Evaluation of an *In Vitro* Cytotoxicity Assay for Specific Groups of Chemicals

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CONTENTS

SUMMARY

The FRAME KB cytotoxicity assay is an *in vitro* test for basal cytotoxicity which measures the sub-lethal inhibition of cell growth by toxic substances. Exponentially growing 3T3-L1 mouse fibroblasts are exposed to a range of concentrations of a test substance for 72 hours, then relative cell number is estimated by the protein/kenacid blue dye-binding method.

The assay was evaluated for its ability to predict parameters of *in vivo* acute lethal potency. *In vivo/in vitro* comparisons were performed for a set of miscellaneous chemicals and for a set of metal compounds. The degree of correlation was closer for the metal compounds than for the unrelated set, in the *in vitro*/mouse i.p. LD_{50} comparison. The cytotoxicity assay was more useful than metal "softness" (a physico-chemical parameter) for predicting metal compound toxicity *in vivo*.

An investigation of the *in vitro* toxicities of a group of commercial chemicals and formulations revealed very poor *in vivo/in vitro* correlations. Some were toxic to the 3T3-L1 cells, yet of very low toxicity to rats. This was partly due to the poor solubility of some of the substances, which probably caused their virtual non-toxicity to rats by oral dosage. Chemical volatility is another methodological problem for *in vitro* assays. A simple modification of the FRAME KB cytotoxicity assay was successfully developed in order to prevent the underestimation of the cytotoxicities of volatile liquids.

The assay also demonstrated potential use for providing data for the safety assessment of surfactants and toiletry formulations. It is emphasised that the FRAME KB cytotoxicity assay should never be used in isolation, but as part of a battery of tests chosen for a particular type of toxicity and/or type of chemical or formulation.

The F9 embryonal carcinoma cell line was evaluated for its potential usefulness in *in vitro* toxicity testing. F9 cells were induced to differentiate morphologically and biochemically, and it was found that cells in different stages of differentiation did not respond in the same way to toxic chemicals.

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GLOSSARY OF ABBREVIATIONS

σp	metal softness parameter
CO_2	carbon dioxide
dbcAMP	dibutyryl cyclic adenosine monophosphate
DD	differentiated
DG	differentiating
DMSO	dimethyl sulphoxide
EC	embryonal carcinoma
EDTA	ethyl diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FDP	fixed dose procedure
FRAME	Fund for the Replacement of Animals in Medical Experiments
FS	film-sealed
GI	gastro-intestinal
ID_{20}, ID_{50}, ID_{80}	the concentrations of test substances which cause the inhibition of growth of cells in the FRAME KB cytotoxicity assay by 20%, 50% and 80% respectively, compared to control cell growth
IgG	immunoglobulin G
i.p.	intra-peritoneal
i.v.	intra-venous
KB	kenacid blue
LC_{50}	concentration of a test substance which is lethal to 50% of the treated animal population
LD_{50}	the amount of test substance which is lethal to 50% of the treated animal population
MEIC	multicentre evaluation of *in vitro* cytotoxicity
NRU	neutral red uptake
OS	oil-sealed
PBS	phosphate buffered saline
QSAR	quantitative structure activity relationship
RA	retinoic acid
RTECS	Registry of Toxic Effects of Chemical Substances
s.e.m.	standard error of the mean
SSEA-1	stage-specific embryonic antigen
UD	undifferentiated
US	unsealed

CHAPTER 1 INTRODUCTION

1.1 ETHICAL, SCIENTIFIC AND ECONOMIC ISSUES

In recent years, *in vitro* toxicology has rapidly developed into a challenging new scientific discipline, through the support and use of ethical, scientific and economic arguments (Gad, 1989; Goldberg and Frazier, 1989).

The growth of *in vitro* toxicology can be measured by the success during the 1980s of international conferences, such as the Practical *In Vitro* Toxicology meetings and the International Workshops on *In Vitro* Toxicology, and international journals, such as *In Vitro* Toxicology, Toxicology *In Vitro* and Alternatives to Laboratory Animals (Rack and Spira, 1989). Furthermore, the establishment of a database of *in vitro* toxicity test protocols, INVITTOX (Warren *et al.*, 1990) has attracted world-wide interest, demonstrating the expansion of research into *in vitro* toxicology from Europe and America to Asia and Australia.

Ethical arguments against the use of animals in biomedical science have been made for well over a century (Rowan and Andrutis, 1990). Toxicologists began to use laboratory animals in large numbers in the 1960s, and public objection to the massive scale of *in vivo* toxicity testing has grown into the widely-publicised campaigns of today (Rack and Spira, 1989). The targets for such campaigns have principally been the Draize eye and skin irritancy tests and the "classical" LD_{50} test (Rowan and Goldberg, 1985; Goldberg and Frazier, 1989), as these are seen as causing the most unnecessary suffering and death. Thus, *in vitro* toxicity tests have gained the support of many animal welfare organisations, and are seen as one of the most promising means whereby the reduction and replacement of animal usage can be achieved (Rack and Spira, 1989; Zbinden, 1989; Balls, 1991a). Toxicologists themselves have been calling for a more humane approach to the use of animals in toxicity testing (e.g. Zbinden, 1985; Fielder *et al.*, 1987; van den Heuvel, 1990).

However, the majority of the population would agree that the safety of the human consumer is paramount, and hence *in vitro* toxicology should be judged not on whether it is simply an alternative to an *in vivo* test, but whether it is a scientifically valid, relevant and reliable alternative (Balls *et al.*, 1987; Flint, 1988).

The scientific merit of *in vitro* toxicology is not in doubt (Goldberg, 1985; Balls and Southee, 1990; Flint, 1990). The use of *in vitro* techniques in all areas of biomedical science is increasing, because they allow detailed studies of well-defined biological events (Grisham and Smith, 1984; Zbinden, 1990). Their application in toxicology has also been widening, as new methods of tissue and cell culture are developed (Goldberg, 1984), which present new and better ways of investigating mechanisms of toxicity (Purchase, 1986). The practical uses of *in vitro* toxicology are numerous, but in the field of toxicity testing, regulatory guidelines and the lack of "adequately validated" alternative tests (Mehlman *et al.*, 1989; Anon, 1990) have hindered the adoption of *in vitro* tests. However, scientists now see *in vitro* methods as the way forward to a more flexible and scientific approach to safety assessment (Goldberg and Frazier, 1989; Zbinden, 1989; Parish, 1990).

Usually, in comparison to the high cost of laboratory animal experimentation, *in vitro* toxicity tests are inexpensive, and hence the economic argument in their favour is compelling (Hertzfeld and Myers, 1987). However, in the politically-sensitive area of human safety, cost factors are not, and should not, be of prime importance (Roberfroid and Goethals, 1990). Regulatory bodies tend to follow the opinion leaders in industry in the acceptance of new technologies (Hertzfeld and Myers, 1987), but it may not be financially attractive to some sectors of the toxicity testing industry to adopt cheaper non-animal alternatives, however valid they may be. Nevertheless, the majority of companies who perform in-house testing now appreciate the economic benefits

of finding alternatives to animals. The benefits include not only cost savings, but also the favourable publicity in our society, where "cruelty-free" product labelling is increasingly seen as important (Balls, 1991b).

1.2 COMPARISON OF *IN VIVO* AND *IN VITRO* TOXICOLOGY

It has been stated that toxicology should be a single discipline, using *in vitro* and *in vivo* models in an integrated manner, rather like modern physiology (Brown, 1991). It would certainly be of benefit if the barriers between *in vitro* and *in vivo* toxicology could be brought down, but there appears to be little possibility of this happening in the near future. This is partly due to the entrenched attitudes of toxicologists in industry, academia and regulatory bodies (Anon, 1990), and the fact that *in vivo* toxicity tests are governed by strict guidelines which, in general, currently do not permit or encourage the use of an integrated approach. This is despite legislation which requires experimenters to consider whether their use of animals is scientifically justified (Balls, 1989). Fairly or not, *in vitro* toxicology has tended to be held up against the *in vivo* science, for direct comparison rather than using it as part of an integrated or complementary approach. This is for two main reasons. Firstly, during drug development, or retrospective studies on drug toxicity, *in vitro* findings are frequently confirmed by, or compared with, *in vivo* experimentation. Secondly, it is necessary that *in vitro* toxicity tests be validated for use (Balls *et al.*, 1990a). Because *in vivo* tests are regarded (by the regulators) as the current "gold standard" in toxicity testing, it has become common to compare the results of *in vitro* tests with existing *in vivo* data, in order to establish whether equivalent information has been provided. It seems that *in vitro* toxicology must somehow mirror *in vivo* toxicology, for the former to be accepted. Certain *in vivo* toxicity tests are highly inflexible and are performed simply to comply with the regulatory requirements of different countries, with no questioning of their relevance or scientific merit (Zbinden,

1981). The use of the classical LD_{50} has been severely criticised in this respect (Zbinden, 1989; Tamborini *et al.*, 1990), as have the Draize irritancy tests (Zbinden, 1987; Gad, 1989). *In vitro* toxicologists have the opportunity to set new standards in toxicity testing, and provide credible data for safety assessment by the use of experimental protocols which have a strong scientific and mechanistic rationale. However, this will not occur if the *in vitro* tests are required to be validated against the results of *in vivo* tests, which were themselves never validated against human experience (Sharpe, 1985; Hellberg *et al.*, 1990).

Although advances in cell and tissue culture reflect great potential for the future of *in vitro* toxicology, widespread use of *in vitro* toxicological techniques has not yet occurred. The three main uses of *in vitro* toxicology today reflect the Three Rs of Russell and Burch, namely the reduction, refinement and replacement of animal use in experiments (Purchase, 1990a). These uses can be termed, for discussion purposes, screening tests, adjunct studies and replacement tests (Frazier, 1990), although the distinction between them is not always great. Opinions vary as to the value of each of these categories, and their future prospects.

1.3 SCREENING TESTS

A screening test is normally a first-order toxicity test which is relatively simple and rapid to perform. It is therefore useful for testing large numbers of substances, to permit the selection of a small number for further development (Balls *et al.*, 1990a; Frazier, 1990). The use of screening tests (or pre-screens) early in product development is thus mainly elective, because there are few mandatory requirements for them to be performed (Purchase, 1990a). Because of the elective aspect, there is little standardisation between laboratories who use them. Screening tests provide information for three main

purposes (Purchase, 1990a):-

(a) to identify substances which lack the particular toxic property which is to be screened out. Any lack of toxicity *in vitro* is normally confirmed later *in vivo*;

(b) to rank the substances which possess the desired biological activities in order of priority for further evaluation, by identifying which are most toxic;

(c) to identify particular toxic properties of a substance, so that *in vivo* testing can be avoided, or the suffering of animals can be reduced.

Examples of *in vitro* screening tests are those for dust fibrogenicity (Purchase, 1990a), the *Salmonella* mutagenicity tests such as the Ames test (Ames *et al.*, 1975), and tests for skin corrosivity using human, rat or rabbit skin slices (Oliver *et al.*, 1986). The latter test is an example of a test which can help reduce animal suffering. If the test detects corrosive properties in a neat substance, then it will be diluted for application in the *in vivo* experiments. The micromass teratogenicity test (Flint, 1987) has been used to distinguish chemicals that should progress to further development. For example, a series of fungicidal chemicals had been developed, and the micromass assay was used to select out those which demonstrated the greatest potential teratogenicity (Flint, 1986).

Other, more specialised screening procedures include rat brain re-aggregate cultures, which are used as models for central nervous system neurotoxicity (Atterwill, 1990), and primary cultures of porcine thyrocytes which provide an *in vitro* system for testing chemicals for potential thyrotoxicity (Atterwill and Fowler, 1990).

There is considerable scope for *in vitro* tests to be employed in many areas of toxicity testing in early product development (Flint, 1988; Rhodes *et al.*, 1989; Frazier, 1990). The adoption of *in vitro* screening tests in industry is a good example of the successful application of *in vitro* techniques in toxicology. Eventually, screening tests may be found to be good enough to be accepted as replacement tests.

1.4 ADJUNCT TESTS

Adjunct *in vitro* toxicology studies are run in conjunction with *in vivo* experiments, in order to improve the specificity and sensitivity of the *in vivo* work (Parish, 1990; Purchase, 1990a). Specificity, e.g. differences in species susceptibilities, cannot be easily investigated *in vivo*, because the whole body approach is not conducive to the elucidation of mechanisms and sites of action of the toxic substance. Substances whose primary site of action is a particular organ (such as the brain, kidney or liver) are tested in cell or tissue cultures of that organ, from the different animals of interest. Areas of target organ toxicity which have benefited from adjunct *in vitro* studies include nephrotoxicity (Gandolfi and Brendel, 1990; Gonzalez *et al.*, 1990), cardiotoxicity (Toraason *et al.*, 1990) and lung toxicity (Ryrfeldt *et al.*, 1990). The study of liver toxicity using cultures of hepatocytes from different species is one of the most advanced areas of *in vitro* toxicology (Frazier, 1990). Hepatocytes have been used successfully to determine mechanisms of toxicity of a variety of xenobiotics (Blaauboer *et al.*, 1990; Guillouzo *et al.*, 1990), for example di(2-ethylhexyl phthalate) and nafenopin (Bieri *et al.*, 1990).

Improving the sensitivity of *in vivo* studies can be achieved through *in vitro* models which are able to contribute information about the pharmacokinetics or toxicokinetics of a substance (Dean and Chenery, 1990; Purchase, 1990a). The competing routes of metabolic activation or inactivation of a substance

can be measured *in vitro* (Garle and Fry, 1988), but the development of reliable techniques for the detection of metabolism-mediated cytotoxicity are still in their early stages (Glatt *et al.*, 1989; Fry *et al.*, 1990a). The rate of penetration of a chemical through human cadaver skin samples *in vitro* can provide toxicokinetic information which is useful for risk assessment purposes (Rhodes *et al.*, 1989).

Phototoxicity is one area of toxicology where animal methods have not been standardised and there are no regulatory guidelines. Although many *in vivo* procedures exist, they are often performed alongside *in vitro* and human tests, because of the acknowledged limitations of the animal methods (Maurer, 1987). Webster *et al.* (1982) combined human and *in vitro* investigations in an attempt to elucidate the mechanism of phototoxicity of benoxaprofen.

Adjunct studies can therefore provide high quality toxicological data, which otherwise may not have been obtainable *in vivo*. Like screening tests, adjunct studies are generally elective, because each individual substance requires a different approach to elucidate its mechanism of action. The future of *in vitro* systems for this application appears to be very promising.

1.5 REPLACEMENT TESTS

Replacement of animal tests, the third R in Russell and Burch's Three Rs, is highly controversial in toxicology. Whilst it is becoming acknowledged that *in vitro* techniques have much to offer as adjuncts, or can be useful as pre-screens, the true replacement of any regulation-defined *in vivo* test has not occurred. Some believe that whole animal studies will never be replaced (Brown, 1991), particularly in systemic toxicity tests. This belief is based on the fact that the latter depend on the interplay of uptake, distribution, metabolism and excretion of a substance in eliciting the toxic response

(Rowan, 1983; Zbinden, 1989). Although current *in vitro* methods cannot currently model the whole of this pharmacokinetic process, it is not unreasonable to believe that this is achievable in the future (Frazier, 1990; Balls, 1991c). The replacement of *in vivo* tests ultimately depends on the decision-makers at regulatory authority and government level, and, although attitudes are shifting (Balls *et al.*, 1990b), progress is inevitably slow. For example, the Fixed Dose Procedure (FDP) is an *in vivo* acute systemic toxicity test which has recently been validated (but still not universally accepted) as an alternative to the classical LD_{50} test (van den Heuvel *et al.*, 1990). Despite the overwhelming scientific evidence in favour of the FDP, and the discrediting of the LD_{50} test, the FDP is still only deemed an *alternative* to the LD_{50}, not a replacement (van den Heuvel, 1990). Regulatory toxicologists naturally have human safety as their prime concern, and therefore appear unwilling to change a system which they perceive as being satisfactory for the purpose of safety assessment.

The diversity and complexity of toxic mechanisms and sites of action of all chemicals within one type of toxicity cannot, and probably will not, satisfactorily be modelled with one *in vitro* test system. Therefore, an *in vivo* test which *can* evaluate many mechanisms within one type of toxicity is unlikely to be replaced with one *in vitro* test (Frazier, 1990). In several areas of *in vitro* toxicology, a battery of test systems has been proposed (Shopsis *et al.*, 1985; Balls *et al.*, 1991a). Each battery would consist of a number of alternative tests which together would provide a span of the toxic mechanisms or target processes of interest (Shopsis *et al.*, 1987; Ekwall *et al.*, 1991). "Alternative tests" includes not only *in vitro* procedures, but also computer molecular and mathematical modelling techniques (Parke *et al.*, 1990). Any test battery based on mechanistic relevance in this way, and found to be highly predictive in validation studies, could offer a feasible and more acceptable means of replacing an *in vivo* test completely (Frazier, 1990).

1.6 VALIDATION

For an *in vitro* toxicity test to be accepted as a replacement to an *in vivo* test, the former must be rigorously and adequately validated (van den Heuvel and Fielder, 1990). Until recently, the validation process has not been strictly defined, and several expensive validation studies have been performed which have achieved very little (Balls and Clothier, 1989). Although international validation workshops have made recommendations as to how studies should be organised (Balls *et al.*, 1990a, Balls *et al.*, 1990b), it is not clear who should have the final decision on whether a test has been adequately validated or not.

Validation may be defined as "the process by which the reliability and relevance of a procedure are established for a specific purpose" (Balls *et al.*, 1990a). Toxicity tests are designed to predict adverse effects in humans, and it is therefore logical that a test should be validated using data from human experience (Hellberg *et al.*, 1990). Human systemic poisoning data is available for some common drugs and poisons (Ekwall, 1980), but is insufficient or unavailable for other types of toxic effect and other classes of substances (Balls and Clothier, 1989). It would therefore be difficult to interpret the *in vitro*/human comparison when the human data was unreliable or anecdotal. The Scandinavian multi-centre evaluation of *in vitro* cytotoxicity (MEIC) involves chemicals selected for inclusion by the virtue of having human poisoning data of sufficient quality to permit a meaningful estimate of human lethal blood concentrations (Ekwall *et al.*, 1989). Values have been calculated for 50 human poisons, using information supplied by the Swedish Poisons Information Centre and from the literature.

Toxicity data from animals, however, constitute a much larger and more available information set with which to compare *in vitro* results. However, if animal data are to be used, their drawbacks must be acknowledged, so that

any comparisons do not regard the *in vivo* results as inviolable, absolute values. These drawbacks include: the reliability of the data source itself (for example, the Registry of Toxic Effects of Chemical Substances (RTECS; Lewis and Tatken, 1982) is known to contain a significant proportion of errors); the underlying requirement to accept the extrapolation of the animal model to man; variability in test protocols; and ambiguous classification of results (Balls *et al.*, 1990a). In order to overcome some of these drawbacks, the establishment of an international reference chemical data bank has been proposed (Purchase, 1990b). The purpose of the data bank would be to provide appropriate selections of chemicals, backed by a reliable source of toxicological data and expert advice for validation studies. The international databank of *in vitro* toxicity test protocols, INVITTOX (Warren *et al.*, 1990), also has a role in validation. By disseminating protocols to laboratories in many countries, more scientists will try out the methods and therefore contribute to the range of chemicals tested, as well as providing further information on the interlaboratory reproducibility and usefulness of the methods.

Numerous *in vitro* alternatives to *in vivo* eye irritancy tests have been proposed (Wilcox and Bruner, 1990). Several of these have been the subject of validation studies designed to measure the degree of correlation between the *in vitro* results and *in vivo* irritancy data. The methods which have received the most attention are the Neutral Red Uptake cytotoxicity test (Borenfreund and Puerner, 1985), the chicken egg chorio-allantoic membrane assay (van Erp and Weterings, 1990), EYTEX™ (Gordon and Bergman, 1987), and the bovine isolated eye assay (van Erp and Weterings, 1990). Interlaboratory trials have been taking place in Europe and America (e.g. Kalweit *et al.*, 1990), but the outcomes are not yet fully known. Nevertheless, certain industrial in-house testing laboratories, such as Avon Products Inc., have felt confident enough in some of the alternative methods that Draize ocular testing of their products

is no longer conducted. The complete replacement of Draize eye testing protocols at regulatory level may seem a long way off to some observers (Gad, 1989; Wilcox and Bruner, 1990), but the belief now exists in many circles that it is an achievable objective.

In contrast, there has been slow progress towards validating suitable alternatives in the field of acute systemic toxicity. Several *in vitro* tests have been developed as alternatives in this field, mainly based on the measurement of basal cytotoxicity in cell cultures (Ekwall, 1983a). A substance which is basally cytotoxic is one which interferes with the basic cellular functions or structures which are present in all cells (Bernson *et al.*, 1986; Fry *et al.*, 1990b). Examples are anti-metabolites, microtubule inhibitors and surfactants. Hence, basal, or "general", cytotoxicity tests tend to use relatively undifferentiated cell lines, which may be continuous or finite-lived, and have no specialised functional characteristics (Ekwall and Ekwall, 1988).

The endpoint most commonly employed to measure toxic interference with these basic attributes is the sublethal inhibition of cell growth (Flint, 1988). Stark *et al.* (1986) described this endpoint (quantified by various assays such as vital dye uptake, total cell protein, cell counting or colony formation) as being ideal, because it is relevant to virtually any mode of insult. However, it is not relevant if the test substance requires metabolic activation to produce a cytotoxic effect and the cells in culture do not possess the metabolic capacity to activate the test substance. In this situation, it must be acknowledged that the cytotoxicity test will measure the effects of the parent compound, and not the metabolites which are responsible for toxicity *in vivo*. It is also not relevant if one wishes to examine substances which cause organ-specific or tissue-specific effects, i.e. they interfere with cell or tissue functions or structures which are specific to a particular tissue or organ. For this, one requires a cell-line or culture which possesses the specialised attributes of that tissue or

organ. In order to establish whether or not the cytotoxicity measured in the specialised cell culture is basal, one must also test the substance in parallel in an undifferentiated cell line (Ekwall and Ekwall, 1988).

In addition to *in vitro* alternatives, there have been several attempts to model acute systemic toxicity by quantitative structure-activity relationships (QSAR) (Enslein, 1988; Phillips *et al.*, 1990). Most have attempted to predict LD_{50} values, but Phillips *et al.* (1990) concluded that the LD_{50} as a toxic endpoint cannot be predicted by QSAR, because of the diversity of toxic mechanisms involved, unless a set of compounds with a common mechanism of action were chosen. Knowledge of such mechanisms must be developed further before QSAR can exert a significant influence in predictive toxicology (Purchase *et al.*, 1990).

The results of some *in vitro* cytotoxicity tests have been compared to animal acute lethal potency parameters, such as LD_{50} values (e.g. Walum and Peterson, 1983; Shopsis and Eng, 1985; Borenfreund and Babich, 1987a; Simmons *et al.*, 1987; Clothier *et al.*, 1989; Fry *et al.*, 1990b) and to human toxicity parameters (Ekwall *et al.*, 1989; Knobeloch *et al.*, 1990). In some cases, too few chemicals were compared for definite conclusions to be drawn, although, in general, the indications were that *in vitro* and *in vivo* parameters correlated reasonably well (Ekwall and Ekwall, 1988). The MEIC study found that for 10 unrelated drugs and poisons, there was a good correlation between *in vitro* cytotoxicity assays and rodent oral LD_{50} or human acute lethal toxicity values (Ekwall *et al.*, 1989). The multivariate cytotoxicity model was able to predict the human values as accurately as the mouse oral LD_{50}. Clothier *et al.* (1989) found that the cytotoxicities of 59 miscellaneous pure chemicals correlated well with rat oral and mouse i.p. LD_{50} values. Rat oral LD_{50} data were considered appropriate because they are widely used as a parameter by which to classify chemicals for hazard labelling, and as a basis for selecting

dosage in other *in vivo* tests. Mouse i.p. values are also reasonably abundant in the literature, and have been proposed as a more logical parameter with which to compare *in vitro* data (Fry *et al.*, 1988a). Clothier *et al.* (1989) and Fry *et al.* (1990b) both concluded that the mouse i.p. LD_{50} values correlate better than rat oral LD_{50} values with *in vitro* cytotoxicity test results.

The choice of chemicals for an *in vitro/in vivo* comparison can influence the significance of the correlation found. For some validation studies, it is considered appropriate to select chemicals with a wide variety of toxic potency and mode of action (Purchase *et al.*, 1987). This serves to demonstrate whether the test under validation can detect the same spread of toxic potency as the "gold standard" test. The selection of chemicals with many modes of action can serve to evaluate whether the test under validation can detect toxicity by all these modes of action. However, while many *in vitro* cytotoxicity tests will be able to demonstrate a correlation with *in vivo* data for a group of unrelated chemicals, it is difficult to interpret how useful the *in vitro* test would be at predicting the *in vivo* toxicity of further, unrelated chemicals.

It is therefore becoming recognised that the *in vitro* testing of chemicals which are related will permit better interpretation of the results (Balls *et al.*, 1990a; Phillips *et al.*, 1990). Several approaches can be taken. Pairs, or small groups of chemical analogues can be tested in order to establish whether one or more has the particular toxic property of interest (Purchase *et al.*, 1987). This is used particularly during the validation of *in vitro* tests for teratogenicity and genotoxicity. A different approach is to select a larger number of chemicals related by their chemical structure and/or mode of toxicity. If a strong *in vitro/in vivo* correlation is noted for this group, then this will allow greater confidence to be placed in the *in vitro* prediction of the *in vivo* toxicities of further chemicals which are known to have the same structure or toxic mechanism. This in turn may lead to the acceptance of the test as valid for

that type of substance, but not for other types of substance (Flint, 1990). Another method is to test substances related by their commercial use, such as shampoos, fungicides or solvents. This may permit the manufacturer to select the least harmful substance from a group which were equally active for their desired purpose. Alternatively, it can provide a database of a range of *in vitro* toxicities for substances in a particular product group (whose safety profiles are known), which may be referred to when testing *(in vitro)* further products from that group. According to whether a new product in the group gives an *in vitro* toxicity result inside or outside the known range, the information can be used in the safety assessment process.

1.7 *IN VITRO* TOXICOLOGY IN INDUSTRY

The question of the use of an *in vitro* toxicity test in industry is very relevant. There is little point in developing an *in vitro* test which cannot be adapted for use within industry, as it is their responsibility for testing novel substances for safety assessment purposes. Toxicity studies are carried out by industrial companies on chemicals and formulations for many reasons. Some studies are for research and evaluation purposes, when elective screening tests are performed and decisions about priority among candidate compounds are made (Purchase, 1990a); each company may perform a different set of tests at this stage. In the final development of a novel substance (pure chemical or formulation), definitive data must be obtained according to official protocols, in order to satisfy regulatory requirements so that it can be legally marketed. These sets of mandatory toxicity tests differ according to the proposed use of the substance (van den Heuvel and Fielder, 1990). The regulatory agencies have a duty to ensure that the new substances are not likely to endanger the health of the industrial worker in the manufacturing company (by applying adequate hazard warning labels) or the final user (by ascertaining that they are not harmful in normal use). At present, the vast majority of these mandatory

tests are performed in animals. Only fully validated alternative tests will be considered as possible replacements for the *in vivo* tests. *In vitro* genotoxicity tests have been accepted by the U.K. Department of Health as a valid part of genetic toxicity testing. A certain amount of scientific freedom is allowed in the choice of these genotoxicity tests, but *in vivo* tests still cannot be avoided altogether (Richold, 1990). The UK Environmental Mutagen Society has provided a useful forum for industrialists and academics, and this has assisted in the standardisation of test protocols (Venitt and Parry, 1984).

An industry/academia collaboration of this sort would potentially speed up the adoption of other types of *in vitro* toxicity tests. In the early 1980s, the Fund for the Replacement of Animals in Medical Experiments (FRAME) initiated a research project on *in vitro* cytotoxicology, the aim of which was to investigate the extent to which "cell cultures could profitably and reliably be used to replace live animal methods used in routine tests on the acute toxicity of chemicals" (Balls and Bridges, 1984). A basal cytotoxicity test was developed through the collaboration of four UK research laboratories and the financial support of several large companies involved in the toxicity testing of chemicals (Balls and Horner, 1985; Knox *et al.*, 1986; Balls *et al.*, 1987). The basis of this test is that a cytotoxic chemical, regardless of the site or mechanism of its action, will interfere with the proliferation of cells which normally grow in culture. The sublethal inhibition of the growth, related to the concentration of the test chemical applied, will provide an indication of cytotoxic potency. The cytotoxicity test underwent inter-laboratory evaluation and was found to be reliable and reproducible (Balls and Horner, 1985; Knox *et al.*, 1986). The test became known as the FRAME Kenacid Blue (KB) method or assay. The KB stain binds to protein, and is used to measure the relative amounts of cellular protein in culture wells, following the exposure of exponentially growing cultures of cells to test materials. The interlaboratory evaluation involved the testing of chemicals whose modes of action and known

toxic potencies were chosen to be as wide-ranging as possible. However, they tended to include a large proportion of chemicals likely to cause toxicity following systemic, rather than topical, exposure. In addition, some chemicals were deliberately included because of the difficulties they would be likely to cause in *in vitro* tests, such as aqueous insoluble or volatile substances, or those which require metabolic activation to exert their toxicity (Knox *et al.*, 1986). It is known that the majority of cell lines possess very little enzymic capacity for the activation of such chemicals (Fry *et al.*, 1988b).

When the human fibroblast-like BCL-D1 cell line used in the initial evaluation, suddenly and unexpectedly ceased to be available, the test was re-evaluated with the murine fibroblast-like 3T3-L1 cell line (Riddell *et al.*, 1986a,b). An extended set of test substances was then chosen to include all the chemicals from the first evaluation (Knox *et al.*, 1986), but attention was also paid to the selection of some sets of related chemicals, such as metal compounds, surfactants and straight chain alcohols (Clothier *et al.*, 1988; Hulme *et al.*, 1989). Hunt *et al.* (1987) evaluated the FRAME KB cytotoxicity assay for testing water toxicity, but the application of the assay in the context of the safety assessment of chemicals and formulations for industrial and regulatory purposes has yet to be ascertained.

In this thesis, the FRAME KB cytotoxicity assay is evaluated by its application to several sets of related substances, to examine whether the *in vivo* predictivity of the assay is improved by selecting related groups. Metal compounds, surfactants, toiletries, industrial biocides and formulations were tested, and the results obtained were considered for their relevance to the type of *in vivo* toxicity data required for these substances. In response to the stated lack of confidence in results for volatile liquids (Knox *et al.* 1986; Clothier *et al.*, 1988), an investigation was made into whether the basic cytotoxicity assay could be adapted to permit volatile liquids to be tested with greater

confidence. A series of preliminary experiments were also undertaken, in order to evaluate the potential usefulness of a differentiating embryonal carcinoma cell line in the development of an *in vitro* test for teratogenicity.

CHAPTER 2 METHODS AND MATERIALS

2.1 Methods

2.1.1 The FRAME KB cytotoxicity assay

2.1.2 Modification of the FRAME KB cytotoxicity assay for tests on volatile chemicals

2.1.3 Experimentsl with F9 embryonal carcinoma cells

2.1.4 Statistical tests

2.2 Reagent recipes

2.2.1 3T3-L1 cell culture reagents

2.2.2 FRAME KB cytotoxicity assay reagents

2.2.3 F9 cell culture, Bradford assay and laminim ELISA reagents

2.3 Suppliers

2.1 METHODS

2.1.1 FRAME Kenacid Blue (KB) Cytotoxicity Assay

The KB assay method was used for testing the 150 chemicals (Chapter 3), the metal compounds (Chapter 4) and the pure surfactants (Chapter 6). The Rhone-Poulenc Ltd. products (Chapter 5) and the cosmetic and toiletry formulations (Chapter 6) were also tested by this method, but in 96-well culture plates only.

2.1.1.1 **Cell culture**

3T3-L1 embryonic mouse fibroblasts were kindly supplied by Professor John Mayer, Department of Biochemistry, University of Nottingham. The cells were routinely cultured in 75cm^2 tissue culture flasks in 3T3-L1 culture medium at 37°C in a 5% CO_2/95% air atmosphere. They were dissociated for subculture twice weekly with 0.05% (w/v) trypsin/0.02% (w/v) EDTA. Cell stocks were maintained under liquid nitrogen (-196°C) or at -70°C in 3T3-L1 cell freezing medium. Fresh cultures were regenerated from frozen stocks once the existing line had undergone 50 subcultures.

2.1.1.2 **Cytotoxicity testing**

The method used was based on that of Knox *et al.* (1986). 3T3-L1 cells were seeded at a density of 1 x 10^4/cm^2 in 23 wells of a 24-well plate (2 x 10^4 cells in 1ml of medium), or 95 wells of a 96-well plate (1.6 x 10^4 cells in 0.2ml of medium). The remaining well received medium only. Control studies showed that different sized culture surfaces produced the same cytotoxicity test results. After overnight incubation, the medium was aspirated and replaced by medium containing a dissolved test substance. The substances were dissolved

directly into culture medium where possible. Substances which were insufficiently soluble in aqueous medium were dissolved initially in methanol, ethanol or dimethyl sulphoxide. The final concentration of these solvents was always 1% (v/v), at which level the rate of control cell growth was not adversely affected. Test substance solutions, and control medium or 1% (v/v) solvent controls were all included in triplicate.

After 72 hours of test substance exposure, the medium was removed and cell monolayers were washed twice with warm PBS. Cells were then fixed *in situ* with KB fixative for 20-30 minutes. The fixative was replaced with KB stain and the plates were agitated for a further 20 minutes. The stain was washed away with several changes of KB wash solution. Each well then received an exact volume of KB desorb solution (24-well plate - 1ml; 96-well plate - 0.2ml). Plates were agitated until the desorbed stain solutions were homogenous. The absorbances of 24-well plate wells were measured at 570nm in a Dynatech Minireader. 96-well plates were read in a Kontron SLT-210 plate reader at 577nm, against a 404nm reference filter. In both cases the absorbance of the well without cells was used as the blank.

2.1.1.3 **Calculation of results**

Each substance was tested on at least three separate occasions. The mean absorbance of the triplicate wells for each test substance concentration on each occasion was expressed as a percentage of the mean absorbance of the control wells. KB absorbance has been shown to have a linear relationship with total cell number and total cell protein (Knox *et al.*, 1986). Hence, the concentrations of substances which reduced the final total cellular protein content of wells by 20%, 50% and 80% (the ID_{20}, ID_{50} and ID_{80} values respectively) could be calculated from the dose-response (concentration - % of control well absorbance) curves. The mean ID_{20}, ID_{50} and ID_{80} values from

the three (or more) experiments were then calculated.

2.1.2 Modification of the FRAME KB Cytotoxicity Assay for tests on volatile chemicals

This assay method for volatile materials was used for testing the chemicals discussed in Chapter 7. 3T3-L1 cells were cultured as described in section 2.1.1.1, except that the ingredients for the 3T3-L1 culture medium were supplied by Northumbria Biologicals Ltd.

2.1.2.1 **Cell culture and cytotoxicity tests**

3T3-L1 cells were seeded at a density of 0.5 x $10^4/cm^2$ in 95 wells of a 96-well plate (0.15 x 10^4 cells in 0.15ml of medium). The remaining well received medium only. After overnight incubation, the medium was aspirated and replaced by medium containing a dissolved test substance (solid or liquid). The test substances were made into solutions as described in section 2.1.1.2, except that organic solvents were used at 0.1% (v/v).

Unsealed (US) culture plates were returned to the incubator as usual. Every well in the oil-sealed (OS) culture plates received 0.1ml of light paraffin oil which, due to its low density, floated on top of the medium. The film-sealed (FS) culture plates had the adhesive film firmly attached directly onto the top of the wells. The lids were replaced and all plates were returned to the incubator.

After 72 hours of test substance exposure, the cultures were examined by phase-contrast microscopy. The medium was then removed and the cell monolayers were washed twice with warm PBS. Cells were then fixed *in situ* with KB fixative for up to 4 hours. The fixative was then replaced with KB

stain. In the absence of a plate agitator or shaker, the stain was left in contact with the cells for 2 hours. The stain was washed away with several changes of KB wash solution. Each well then received 0.15ml of KB desorb solution. The KB stain was desorbed by trituration. The absorbances of each well were measured at 595nm in a Flow Titertek Multiscan plate reader. The absorbance values were read directly into a computer program, Titersoft, which calculated the mean percentage absorbances of test substance treated wells compared with the untreated control wells. The absorbance of the well without cells was used as the blank.

2.1.2.2 **Calculation of results**

Each substance was tested on at least three separate occasions. The mean absorbance of the triplicate wells for each substance concentration on each occasion was expressed as a percentage of the mean absorbance of the control wells. Hence the concentrations of each test substance which reduced the final total cellular protein content of wells by 20%, 50% and 80% (The ID_{20}, ID_{50} and ID_{80} values, respectively) could be calculated from the dose-response (concentration - % of control well absorbance) curves. The mean ID_{20}, ID_{50} and ID_{80} values from the three (or more) experiments were then calculated.

2.1.3 Experiments with F9 embryonal carcinoma cells

These methods were used in the experiments described in Chapter 8.

2.1.3.1 **Cell culture**

F9 murine embryonal carcinoma cells (a gift from Dr A K Daly, Department of Pharmacological Sciences, University of Newcastle-upon-Tyne) were cultured in 75cm^2 tissue culture flasks in F9 culture medium at 37°C in a 5%

CO_2/95% air atmosphere. F9 cells require culture surfaces to be coated with gelatin to aid adherence. Hence, prior to use, the surfaces of flasks and plates were rinsed with a 0.1% (w/v) porcine gelatin solution. Excess solution was aspirated and the vessels were left to dry overnight. Cells were subcultured every 2-3 days following treatment with the trypsinisation reagent. Cell stocks were maintained under liquid nitrogen (-196°C) or at -70°C in F9 freezing medium. Fresh cultures were regenerated from frozen stocks every 8 to 10 weeks (i.e. every 25 to 30 passages).

2.1.3.2 **Cytotoxicity testing**

On undifferentiated cells

The method used was based on that of Knox *et al.* (1986), using 24-well plates, but with the test chemical exposure time reduced from 72 to 48 hours. Undifferentiated F9 cells were seeded at 2.5 x $10^4/cm^2$ (5 x 10^4 cells in 1ml of medium) in 23 wells of a 24-well plate. The remaining well received medium only. After overnight incubation, the medium was aspirated and replaced by 1ml of medium containing a dissolved test chemical. Chemicals which did not dissolve directly into medium at the concentrations required were dissolved initially in either ethanol or DMSO. The final concentration of the ethanol or the DMSO was 1% (v/v) in the medium. Medium only, and 1% solvent control wells were also included in triplicate. This allowed six or seven chemical concentrations to be tested in triplicate per plate, with the highest dose being in duplicate only (to allow for the well without cells).

After 48 hours exposure, the total well protein was determined by the FRAME KB cytotoxicity assay (as described in section 2.1.1.2). Absorbances were measured at 570nm in a Dynatech Mini-reader. The well without cells was used as the blank.

On differentiating cells

Undifferentiated F9 cells were seeded at 2.5 x 10^4/cm^2 (5 x 10^4 cells in 1ml of medium) in culture medium containing 0.1μM retinoic acid (RA) in 23 wells of a 24-well plate. They were then treated with test chemicals as for undifferentiated cells, but the test chemical and control solutions also contained 0.1μM RA. At the end of the 48 hour exposure period, the cells were washed with PBS to remove serum proteins. The protein in each well was solubilised with 1ml of 1M sodium hydroxide for 2 hours with shaking. Protein extracts were assayed for total protein using the Bradford method (Bradford, 1976). The dye reagent was filtered twice before use, and was stable for two weeks. 100μl of the cell protein extract solution was mixed with 1ml of dye solution in a disposable plastic test tube. The absorbance of the solution (placed in a 1ml disposable plastic cuvette) was then read at 595nm in a Cecil spectrophotometer, using 1M sodium hydroxide in the dye solution as the blank. A standard curve was prepared from a range of dilutions (0 to 600μg/ml) of a Sigma protein standard solution diluted in 1M sodium hydroxide. Control well total protein levels (i.e. 100% cell growth) were usually between 90 and 130μg per well.

On differentiated cells

Differentiated F9 cells were obtained by seeding and growing previously undifferentiated cells for 5 or 6 days in the presence of 0.1μM RA in 75cm^2 flasks. Because of continued growth during this period, the cells were subcultured on day 3 into a fresh solution of 0.1μM RA in medium. Cells were seeded for tests at 5 x 10^4/cm^2 (10 x 10^4 cells in 1ml of medium) in culture medium containing 0.1μM RA in 23 wells of a 24-well plate. Chemical treatments and the Bradford protein assay were conducted as for the differentiating cells.

2.1.3.3 **Calculation of results**

Each chemical was tested on at least three separate occasions.
Undifferentiated F9 cells: The mean absorbance of the triplicate wells for each chemical concentration on each occasion was expressed as a percentage of the mean absorbance of the control wells.
Differentiating and differentiated F9 cells: The mean quantity of protein in the triplicate wells for each chemical concentration on each occasion was expressed as a percentage of the mean quantity of protein in the control wells.

The concentrations of the test chemicals which reduced the final total cellular protein content of wells by 20%, 50% and 80% (the ID_{20}, ID_{50} and ID_{80} values respectively) could be calculated from the dose-response (concentration - % of control well absorbance or protein) curves. The mean ID_{20}, ID_{50} and ID_{80} values from the three (or more) experiments were then calculated.

2.1.3.4 **Enzyme-Linked Immunosorbent Assay (ELISA) for Laminin**

Outline of the assay

The ELISA method was based on that of Williams *et al.* (1987), which had been developed to measure retinoid-induced F9 cell differentiation into parietal endoderm-like cells. Briefly, cell culture medium samples were co-incubated with a rabbit IgG anti-mouse laminin antibody. The mixture was then transferred to microtitre wells coated with laminin. Any unbound anti-laminin in the mixture became immobilised on the solid phase. This was then detected using an alkaline phosphatase/goat anti-rabbit IgG antibody conjugate, with p-nitrophenol phosphate as the substrate to generate colour.

Laminin assay procedure

Samples of medium were collected after the 48 hour exposure period from every culture well in the cytotoxicity tests (including both differentiating and differentiated F9 cells) and stored at -20°C until required for the assay. Samples were thawed then centrifuged at 2500g to remove dead cells. Each sample was diluted to 40% (v/v) with PBST. 50μl of the diluted medium was then mixed with an equal volume of a 1 in 12000 dilution of rabbit IgG anti-mouse laminin antibody in PBST, in the wells of a 96-well flexible polyvinyl ELISA plate. The medium and anti-laminin antibody mixture was incubated at 22-24°C overnight, during which time any laminin in the medium became bound to the anti-laminin antibody. At the same time, a laminin-coated 96-well polyvinyl ELISA plate was prepared by incubating 50μl of 1μg/ml mouse laminin in a pH 9.6 coating buffer in each well, overnight at 4°C.

The laminin-coated plate was allowed to come to room temperature and was washed with PBST to remove any unbound laminin. The washing technique consisted of filling each well with PBST and immediately shaking it out. This was repeated twice more. After the final wash, all liquid was removed from wells onto paper towels by flicking the plate. 50μl aliquots of the medium/anti-laminin incubations were transferred to the laminin-coated plate, and incubated for 30 minutes at room temperature. During this time, any excess unbound anti-laminin antibody became immobilised on the laminin-coated solid phase. The plate was washed three times with PBST, as above. The presence of immobilised rabbit anti-laminin was detected by the addition to every well of 50μl of a 1 in 1000 dilution of alkaline phosphatase/goat anti-rabbit IgG antibody conjugate in PBST. After incubation at room temperature for 60 minutes, any unbound conjugate was washed out twice with PBST, then twice with pH 9.8 substrate buffer, to bring the plate to the correct pH. 100μl of freshly-prepared phosphatase substrate (1mg/ml p-nitrophenol phosphate

in pH 9.8 substrate buffer) was added to each well. The production of a yellow colour, due to the conversion by alkaline phosphatase of p-nitrophenol phosphate to p-nitrophenol, was measured at 404nm in a Kontron SLT-210 96-well plate reader. The colour was allowed to develop for between 100 and 200 minutes, until the absorbance had reached approximately 1.0 in the control (no laminin) wells. A standard curve was prepared using a range of dilutions from 5 to 200ng/ml of the purified mouse laminin in 40% culture medium/60% PBST (v/v). The standard dilutions were assayed on four separate occasions and a standard curve was constructed from the mean of the results (Figure 2.1). The full range of standard dilutions was not included in every assay run. Instead, 40ng/ml and 200ng/ml dilutions were assayed in each run to confirm that the assay was performing correctly.

Absorbance results were expressed as a percentage of the maximum absorbance (i.e. the mean absorbance of the 0 ng/ml laminin wells) generated in each assay run. The limits of detection of the ELISA were taken as 5 to 100ng/ml, i.e. between 80% and 30% maximum absorbance. In order to detect greater than 100ng/ml, medium samples were diluted below 40% in PBST in the initial dilution. The final dilution factor of medium in the medium/anti-laminin coincubation was 1 in 5, hence laminin concentrations read from the standard curve were multiplied by five.

2.1.4 Statistical tests

Linear regression analysis was performed using the Techni-Curve graph-plotting software programme. The two-sample median test was performed using the Unistat statistics software programme.

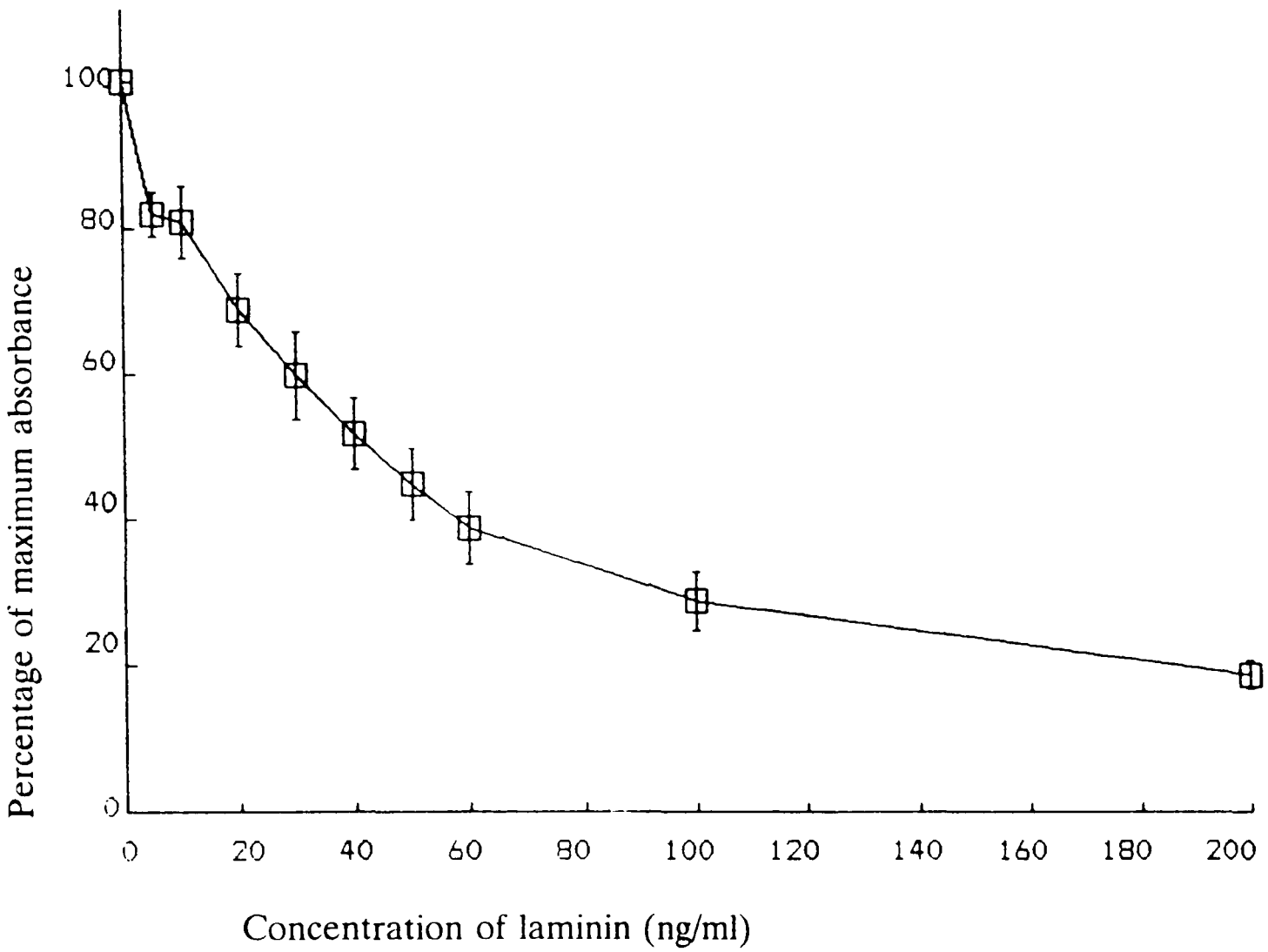

Figure 2.1. Laminin ELISA standard curve

2.2 REAGENT RECIPES

2.2.1 3T3-L1 cell culture reagents

3T3-L1 culture medium

Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with:
10% (v/v) new-born calf serum (heat-inactivated)
2mM L-glutamine
100 IU/ml sodium benzylpenicillin
100μg/ml streptomycin sulphate
2μg/ml Fungizone

3T3-L1 trypsinisation reagent

0.05% (w/v) trypsin
0.02% (w/v) EDTA
in Puck's Saline (Gibco)
or PBS (Northumbria Biologicals)

3T3-L1 freezing medium

90% (v/v) 3T3-L1 culture medium
10% (v/v) dimethyl sulphoxide

2.2.2 FRAME Kenacid Blue (KB) cytotoxicity assay reagents

KB Fixative

1% (v/v) glacial acetic acid
49% (v/v) distilled water
50% (v/v) absolute ethanol

KB stain

0.04% (w/v) Kenacid Blue R
25% (v/v) absolute ethanol
63% (v/v) distilled water

Immediately before use, the stain was filtered and 12% (v/v) glacial acetic acid was added.

KB wash

10% (v/v) absolute ethanol
5% (v/v) glacial acetic acid
85% (v/v) distilled water

KB desorbing solution

9.82% (w/v) potassium acetate
70% (v/v) absolute ethanol
30% (v/v) distilled water

2.2.3 F9 cell culture, Bradford assay and laminin ELISA reagents

F9 culture medium

Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with:
10% fetal calf serum
2mM L-glutamine
100 IU/ml sodium benzylpenicillin
100μg/ml streptomycin sulphate

F9 trypsinisation reagent

0.05% (w/v) trypsin
0.02% (w/v) EDTA in Puck's saline

F9 freezing medium

DMEM, supplemented with:
25% fetal calf serum
10% glycerol

0.1% porcine gelatin solution

0.1% (w/v) porcine gelatin
Double distilled water
Autoclaved

Bradford protein assay dye reagent

0.01% (w/v) Coomassie Brilliant Blue G-250
4.7% (v/v) absolute ethanol
8.5% (v/v) phosphoric acid

PBST

Phosphate buffered saline
0.05% (v/v) Tween 20
0.02% (w/v) sodium azide

Coating buffer, pH 9.6

35mM sodium hydrogen carbonate
0.02% (w/v) sodium azide

Substrate buffer, pH 9.8

9.7% (v/v) diethanolamine
0.02% (w/v) sodium azide
0.098mM magnesium chloride hexahydrate

2.3 SUPPLIERS

Gibco Ltd.

Dulbecco's Modification of Eagle's Minimal Essential Medium (supplied as 10X, without sodium bicarbonate)
Fetal calf serum
New-born calf serum (heat-inactivated)
200mM L-glutamine
0.05% (w/v) trypsin, 0.02% (w/v) EDTA in Puck's Saline
7.5% (w/v) sodium bicarbonate
24 well tissue culture plates (Nunc)
75cm^2 tissue culture flasks (Nunc)
Purified laminin (produced from Engelbreth-Holm-Swarm sarcoma in mice)
Rabbit anti-mouse laminin antibody

Northumbria Biologicals Ltd.

Dulbecco's Modification of Eagle's Minimal Essential Medium (supplied as 1X, with sodium bicarbonate)
New-born calf serum (heat-inactivated)
0.05% (w/v) Trypsin, 0.02% (w/v) EDTA in PBS
Phosphate-buffered saline without Ca^{2+} and Mg^{2+}
200mM L-glutamine
250μg/ml Fungizone
10000 IU/ml sodium benzylpenicillin, 10000μg/ml streptomycin sulphate in PBS
75cm^2 tissue culture flask (Costar)
96-well tissue culture plates (Costar)
Adhesive microwell plate sealing film (Costar C3095)

Aldrich Chemical Co. Ltd.

Cadmium chloride
5-Fluorouracil
6-Aminonicotinamide
Diethylstilboestrol
Acetylsalicylic acid
Phenytoin
Pentachlorophenol
Isoniazid
Saccharin
Trichloroacetic acid
Sodium chloride
Vanadium (V) oxide
Silver (I) nitrate
Mercury (II) chloride
Copper (I) chloride
Thallium (I) sulphate
Thallium (I) acetate
Zinc (II) chloride
Tellurium (IV) chloride
Cobalt (II) chloride
Selenium (IV) oxide
Rhodium (III) chloride trihydrate
Sodium (I) tetrathionate dihydrate
Tin (II) chloride
Nickel (II) chloride hexahydrate
Lead (II) nitrate

Palladium (II) chloride
Yttrium (III) nitrate hydrate
Gadolinium (III) chloride hexahydrate
Lanthanum (III) chloride heptahydrate
Sodium (I) fluoride
Potassium (I) fluoride
Lithium (I) fluoride
Aluminium (III) nitrate nonahydrate
Lithium (I) chloride
Strontium (II) chloride hexahydrate
Lithium (I) sulphate
Magnesium (II) chloride hexahydrate
Lithium (I) iodide
Lithium (I) bromide
Potassium (I) iodide
Potassium (I) sulphate
Sodium (I) sulphate
Potassium (I) bromide
Sodium (I) iodide
Sodium (I) bromide
Ammonium chloride
Sodium dodecyl sulphate
Methanol
Ethanol
Propan-lol
Butan-l-ol
Pentanol
Hexanol
Heptanol
Octanol
Acetaldehyde
Acetonitrile
Acetone
Pyrrole
Allyl alcohol
Ethyl acetate
2-Methoxyethanol
Glycerol
1,2,4-Trichlorobenzene
1,2-Dibromomethane
Aniline
Tri-n-butytinchloride
Brij 35

Sigma Chemical Co. Ltd.

Alkaline phosphatase/goat anti-rabbit IgG antibody conjugate
p-Nitrophenol phosphate
Protein standard solution (80mg/ml)
Diethanolamine
Porcine skin gelatin, type A, approx 175 Bloom
Dibutyryl cyclic adenosine monophosphate
Coomassie Brilliant Blue G-250
Light paraffin oil (M3516)
(all-trans) Retinoic acid
Caffeine
Cyclophosphamide
Tween 20
Sodium azide
Sodium hydrogen carbonate
Colchicine
Dexamethasone
2,4-Dinitrophenol

Chloramphenicol
Lead (IV) tetra-acetate
Chromium (III) chloride hexahydrate
Dimethyl formamide
Cetyl pyridinium bromide
Cetyl dimethyl ethyl ammonium bromide
Hexadecyl trimethyl ammonium bromide
Benzalkonium chloride
Dodecyl trimethyl ammonium bromide
Triton X-100
Benzethonium chloride
Nonidet P40
N-Lauroyl sarcosine, sodium salt
Dodecyl benzene sulphonic acid, sodium salt
Deoxycholic acid, sodium salt
Tween 40
Tween 60
Tween 80
Tween 85
1-Decanesulphonic acid, sodium salt
Caprylic acid, sodium salt
Cholic acid
1-Heptanesulphonic acid, sodium salt
CHAPS
CHAPSO
Taurocholic acid, sodium salt
Zinc pyrithione

BDH Ltd.

Phosphoric acid
Dimethyl sulphoxide
Sodium hydroxide
Potassium (I) chloride
Calcium (II) chloride dihydrate
Barium (II) acetate
Aluminium (III) chloride hexahydrate
Tin (IV) chloride pentahydrate
Copper (II) chloride
Zinc (II) acetate
Manganese (II) sulphate tetrahydrate
Nickel (II) sulphate heptahydrate
Iron (II) sulphate heptahydrate
Iron (III) sulphate
Benyllium sulphate tetrahydrate
Kenacid Blue R
Glacial acetic acid
Sulphuric acid
Hydrochloric acid

Rhone-Poulenc Ltd.

Thalidomide (TOX 16)
Coded chemicals TOX 01 to TOX 46, as described in Chapter 5.

Oxoid

Dulbecco's 'A' Phosphate Buffered Saline tablets (1 tablet dissolved in 100ml of double distilled water)

Avon

6-Methylcoumarin
Nonoxynol-11
Teals
Miranol

Applied Biosystems

Trifluoroacetic acid

James Burroughs

Methanol
Ethanol (absolute)

Boots the Chemists Ltd.

Anti-dandruff shampoo
Silk protein dandruff shampoo

Revlon Inc.

Flex conditioner
Flex shampoo

Hennara

Henna shampoo

Beechams

Vidal Sassoon Wash 'n' Go conditioning shampoo for frequent use

E. R. Squibb Ltd.

Fungizone

Evans Medical Ltd.

Streptomycin sulphate

Glaxo Laboratories Ltd.

Sodium benzylpenicillin

CHAPTER 3 THE *IN VITRO* TOXICITIES OF 150 MISCELLANEOUS CHEMICALS

3.1 INTRODUCTION

During the development of an *in vitro* toxicity test, such as the FRAME KB cytotoxicity assay, it is desirable that a wide variety of chemical types are tested and that the range of toxic concentrations detectable by the test is established. Within the wide variety of chemical types, if possible, there should be a reasonable number of representatives from each chemical type. This permits the recognition of those types of chemicals for which the assay is appropriate or inappropriate.

Hence, 150 chemicals were selected to be tested in the FRAME KB cytotoxicity assay, so that many modes of toxic action were covered and a wide range of toxic potencies would be spanned. Some chemical were chosen to be part of related subgroups, such as metal compounds, alcohols and surfactants. A number of chemicals were included because of their industrial use, and the fact that industrial sponsors of the FRAME Research Programme on Cytotoxicology were interested in their *in vitro* toxicity. Certain types of chemicals were known to cause difficulties in *in vitro* toxicity tests, and therefore representatives of these types (for example, volatile or unstable substances, those with poor aqueous solubility and those which exert toxicity *in vivo* through their metabolites) were tested in order to determine whether the FRAME KB cytotoxicity assay could be applied to them.

Results from a group of miscellaneous chemicals in an *in vitro* toxicity test can be compared to *in vivo* toxicity parameters, or, smaller groups of related substances can be used for comparisons. The results for the 150 chemicals in the FRAME KB cytotoxicity assay are therefore presented, not only as a miscellaneous set for comparison to *in vivo* toxicity parameters, but also as a basis for the studies of related substances (metal compounds, Chapter 4; industrial chemicals and mixtures, Chapter 5; surfactants and toiletry products,

Chapter 6; volatile liquids, Chapter 7; and chemicals recommended for inclusion in *in vitro* teratogenicity test validation, Chapter 8).

Because the FRAME KB cytotoxicity assay involves the continuous exposure of cells for 72 hours, and also because a large proportion of the 150 chemicals are likely to cause toxicity following systemic exposure, the *in vivo* toxicity parameters for which the assay would most likely be predictive are measures of acute lethal potency. Although it is desirable to use indices of toxicity in humans wherever possible (Hellberg *et al.*, 1990), the data are difficult to obtain and are inherently of variable quality (Balls *et al.*, 1990a; Flint, 1991).

Currently-accepted indices of acute lethal potency include rodent LD_{50} values, which are therefore available in relative abundance in the literature. Rat oral LD_{50} values are commonly used to assign chemicals to hazard categories for labelling purposes. However, it has been suggested that *in vitro* cytotoxicity data might correlate better with LD_{50} values obtained from parenteral (i.p. or i.v.) dosage (Fry *et al.*, 1988a). Hence, rat oral and mouse i.p. LD_{50} values were compared with the *in vitro* toxicities of the 150 miscellaneous chemicals.

3.2 RESULTS

3.2.1 *In vitro* toxicities of 150 chemicals

The 150 chemicals gave a very wide range of cytotoxicities to the 3T3-L1 cells. The results are presented in rank order of ID_{50} (μg/ml) (Table 3.1), with values covering seven orders of magnitude, from <0.01 to 134,000μg/ml. There is a fairly even spread of results across this concentration range.

The ID_{50} value is used as the parameter by which to rank the chemicals (and thereby to judge their relative cytotoxicities), because it is taken from the

Table 3.1 *In vitro* toxicities of 150 test chemicals listed in order of ID_{50} (μg/ml)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
001	5-Fluorouracil	D	<0.01	<0.01	<0.01
002	6-Mercaptopurine monohydrate	D	<0.01	<0.01	<0.01
003	Acrolein	E	<0.01	<0.01	<0.01
004	Rotenone	D	<0.1	<0.1	<0.1
005	Vincristine sulphate	W	0.01 ± 0	<0.1	>100
006	Cycloheximide	M	<0.1	<0.1	0.3 ± 0.1
007	Cadmium (II) chloride	W	0.1 ± 0	0.1 ± 0	0.2 ± 0.1
008	Tributyltin chloride	E	0.1 ± 0.01	0.3 ± 0.01	0.4 ± 0
009	Silver (I) nitrate	W	1 ± 0	2 ± 0	2 ± 0
010	Vanadium (V) oxide	W	0.2 ± 0.1	2 ± 0	3 ± 1
011	p-Chloromercuribenzoic acid	D	<0.1	3 ± 1	8 ± 1
012	Copper (I) chloride	W	2 ± 0	3 ± 0	5 ± 1
013	Copper (II) chloride	W	2 ± 1	3 ± 1	4 ± 1
014	Mercury (II) chloride	E	2 ± 1	4 ± 1	6 ± 0
015	Chloropromazine hydrochloride	W	3 ± 0	5 ± 1	7 ± 1
016	Benzalkonium chloride	W	4 ± 1	7 ± 0	9 ± 2
017	Chloroquine sulphate	W	4 ± 0	7 ± 0	>10
018	Colchicine	E	2 ± 1	8 ± 1	>10
019	Hexachlorophene	E	3 ± 1	9 ± 2	14 ± 2
020	Zinc (II) chloride	W	6 ± 1	9 ± 0	12 ± 1

Table 3.1. (contd)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
021	p-Aminophenol	D	4 ± 2	10 ± 1	33 ± 12
022	Selenium (IV) oxide	W	5 ± 1	10 ± 2	15 ± 2
023	Cobalt (II) chloride	W	3 ± 1	11 ± 1	24 ± 6
024	Propanolol hydrochloride	M	8 ± 1	12 ± 0	14 ± 0
025	Diethylstilboestrol	E	4 ± 1	14 ± 3	60 ± 17
026	Triton X-100	W	2 ± 2	14 ± 3	50 ± 12
027	Thallium (I) sulphate	W	7 ± 3	15 ± 3	25 ± 5
028	Manganese (II) sulphate tetrahydrate	W	11 ± 2	15 ± 1	25 ± 3
029	Zinc acetate	W	12 ± 1	15 ± 1	18 ± 1
030	Diethyl maleate	E	12 ± 0	17 ± 2	27 ± 3
031	Thallium (I) acetate	W	9 ± 1	17 ± 2	30 ± 2
032	6-Aminonicotinamide	D	11 ± 1	20 ± 6	27 ± 2
033	Iron (II) sulphate heptahydrate	W	I	20 ± 9	I
034	Tellurium (IV) chloride	D	8 ± 1	22 ± 2	31 ± 2
035	Bendiocarb	D	7 ± 2	30 ± 5	147 ± 3
036	Pentachlorophenol	E	22 ± 1	31 ± 1	38 ± 1
037	Nickel (II) sulphate heptahydrate	W	20 ± 3	33 ± 3	53 ± 3
038	Phenylbutazone	D	29 ± 1	45 ± 1	65 ± 3
039	Versalide	E	30 ± 1	45 ± 1	>50
040	Butylated hydroxyanisole	E	30 ± 1	45 ± 1	>50

Table 3.1. (contd)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
041	Ioxynil	D	32 ± 4	47 ± 1	57 ± 1
042	Fungizone	W	8 ± 2	48 ± 3	85
043	Retinoic acid	D	40 ± 10	49 ± 10	57 ± 9
044	Dieldrin	D	26 ± 2	50 ± 3	123 ± 25
045	Tin (II) chloride	W	21 ± 6	53 ± 10	124 ± 31
046	Sodium (I) fluoride	W	35 ± 6	53 ± 4	73 ± 4
047	Rhodium (III) chloride trihydrate	W	26 ± 3	55 ± 2	100 ± 1
048	Tin (IV) chloride pentahydrate	W	34	64 ± 3	98
049	2,4-Dinitrophenol	D	34 ± 10	72 ± 9	87 ± 10
050	Sodium tetrathionate dihydrate	W	33 ± 4	74 ± 5	128 ± 22
051	Nickel (II) chloride hexahydrate	W	43 ± 3	77 ± 13	98 ± 18
052	Lithium (I) fluoride	W	51 ± 12	77 ± 25	142 ± 24
053	Paracetamol	M	44 ± 4	90 ± 10	136 ± 18
054	Sodium dodecyl sulphate	W	66 ± 11	94 ± 12	162 ± 17
055	Phenacetin	D	84 ± 1	95 ± 1	>100
056	Palladium (II) chloride	W	62 ± 14	103 ± 2	115
057	Methylparaben	E	64 ± 4	106 ± 16	>300
058	Lead (II) nitrate	W	53 ± 21	119 ± 45	345 ± 19
059	Lead (IV) tetra-acetate	D	82 ± 22	119 ± 5	286 ± 34
060	1,2,4-Trichlorobenzene	E	89 ± 11	122 ± 14	>200

Table 3.1. (contd)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
061	Acrylamide	W	>100	123 ± 8	182 ± 8
062	Iron (III) sulphate	W	37 ± 7	123 ± 24	309 ± 69
063	Naphthalene	D	44 ± 13	123 ± 1	189 ± 9
064	Dodecylbenzene sulphonic acid, sodium salt	W	120 ± 6	137 ± 1	150 ± 2
065	Potassium (I) fluoride	W	70 ± 16	150 ± 6	260 ± 25
066	Beryllium (II) sulphate tetrahydrate	W	66 ± 9	157 ± 17	387 ± 48
067	Tween 40	E	53 ± 5	159 ± 49	295 ± 50
068	2,4-Dichlorophenoxyacetic acid	M	70 ± 15	162 ± 19	>500
069	6-Methylcoumarin	E	130 ± 7	169 ± 16	403 ± 140
070	Diethyl phthalate	E	93 ± 22	198 ± 8	281 ± 8
071	Chloramphenicol	M	96 ± 28	226 ± 41	397 ± 42
072	2-Chloroquinoline	E	145 ± 25	233 ± 17	331 ± 42
073	Tween 60	E	146 ± 31	244 ± 27	345 ± 9
074	Dexamethasone	D	74	252 ± 43	>400
075	Tween 20	W	123 ± 5	262 ± 43	396 ± 42
076	Yttrium (III) nitrate hexahydrate	W	227 ± 20	262 ± 7	286 ± 3
077	Phenytoin	D	57 ± 3	265 ± 44	>1000
078	Acrylonitrile	E	147 ± 28	266 ± 40	290 ± 16
079	Thioacetamide	E	167 ± 11	274 ± 8	>500

Table 3.1. (contd)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
080	Warfarin	D	187 ± 39	285 ± 26	398 ± 25
081	1-Octanol	E	236 ± 6	298 ± 4	360 ± 2
082	Indium (III) nitrate hydrate	W	26 ± 3	303 ± 57	546 ± 97
083	3-Chloroaniline	E	97 ± 11	308 ± 68	460 ± 138
084	L-Ascorbic acid	W	295 ± 60	323 ± 58	351 ± 55
085	Ammonium chloride	D	168 ± 47	337 ± 63	>500
086	Allyl alcohol	W	191 ± 23	355 ± 52	521 ± 34
087	Phorone	E	188 ± 16	378 ± 27	600 ± 68
088	Bromobenzene	E	284 ± 23	389 ± 29	486 ± 38
089	Gadolinium (III) chloride hexahydrate	W	360 ± 3	389 ± 6	419 ± 12
090	Iproniazid phosphate	W	117 ± 20	393 ± 37	420 ± 76
091	Lanthanum (III) chloride heptahydrate	W	373	441 ± 34	567 ± 19
092	Acetylsalicylic acid	E	153 ± 28	465 ± 86	821 ± 52
093	Tween 85	E	253 ± 149	507 ± 50	821 ± 7
094	Trichloroacetic acid	W	311 ± 69	524 ± 109	658 ± 126
095	2,4,6-Trinitrobenzene sulphonic acid	W	269 ± 42	536 ± 46	772 ± 11
096	Phenobarbital	M	<500	543 ± 15	917 ± 34
097	Lithium (I) chloride	W	343 ± 67	554 ± 95	810 ± 73

Table 3.1. (contd)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
098	Carbon tetrachloride	E	506 ± 32	600 ± 28	665 ± 32
099	Tween 80	E	135 ± 25	619 ± 69	836 ± 9
100	Isoxepac	D	292 ± 90	644 ± 38	>1000
101	1,2-Dibromoethane	D	589	645 ± 240	924 ± 250
102	Imidazole	D	371 ± 44	688 ± 12	>750
103	Frusemide	D	628 ± 70	756 ± 52	873 ± 45
104	Aluminium (III) chloride hexahydrate	W	738 ± 120	830 ± 60	1050 ± 82
105	Acetonylacetone	W	798 ± 34	937 ± 23	>1000
106	Barium (II) acetate	W	566 ± 280	982 ± 62	2470
107	1-Heptanol	E	887 ± 26	1010 ± 86	1150 ± 74
108	Caffeine	W	330 ± 30	1110 ± 57	3240 ± 310
109	Aluminium (III) nitrate nonahydrate	W	953 ± 75	1190 ± 37	1280
110	L-Cysteine hydrochloride monohydrate	W	498 ± 120	1190 ± 160	1650
111	Aniline	E	1320 ± 170	1480 ± 330	2540 ± 190
112	Chromium (III) chloride hexahydrate	W	857 ± 160	1500 ± 24	2170 ± 84
113	Thiophene	E	1240 ± 31	1680 ± 130	>2000
114	Cyclophosphamide	W	1320 ± 150	1800 ± 490	2470 ± 100

Table 3.1. (contd)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
115	Isoniazid	W	1320 ± 78	2170 ± 330	3970 ± 730
116	Lithium (I) sulphate	W	953 ± 210	2460 ± 600	6460 ± 2000
117	Lithium (I) bromide	W	1790 ± 390	3290 ± 130	7050 ± 630
118	1-Pentanol	W	2880 ± 490	3540 ± 550	4160 ± 610
119	1-Hexanol	W	2580 ± 530	3600 ± 540	4320 ± 960
120	Calcium (II) chloride dihydrate	W	2230	3860	8900
121	Sodium benzylpenicillin	W	1730	3970 ± 78	6280 ± 48
122	Strontium (II) chloride hexahydrate	W	1940 ± 290	4060 ± 220	10900 ± 940
123	Streptomycin sulphate	W	1450	4160 ± 430	6990 ± 240
124	Potassium (I) chloride	W	3060 ± 300	4360 ± 200	5560 ± 75
125	Lithium (I) iodide	W	2470 ± 810	4690 ± 460	7710 ± 1100
126	1-Butanol	W	3320 ± 970	4780 ± 490	6320 ± 570
127	Magnesium (II) chloride hexahydrate	W	3260 ± 420	5600 ± 750	7910 ± 960
128	Sodium chloride	W	1790 ± 700	6230 ± 720	8390 ± 660
129	Pyrrole	W	5740 ± 230	6570 ± 310	7430 ± 380
130	Potassium iodide	W	3280 ± 860	7260 ± 440	8790 ± 440
131	Potassium (I) bromide	W	5900 ± 140	7780 ± 390	9540 ± 360
132	Potassium (I) sulphate	W	6270 ± 990	7860 ± 690	9230 ± 330
133	Sodium (I) sulphate	W	6090 ± 890	8930 ± 560	10000 ± 390

Table 3.1. (contd)

No.	Test Chemical	Solvent	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
134	Sodium (I) bromide	W	7250 ± 450	9840 ± 910	11300 ± 1000
135	Kanamycin sulphate	W	2960 ± 970	11000 ± 1200	19200
136	1-Propanol	W	7580 ± 480	11200 ± 480	17000
137	Sodium (I) iodide	W	8730 ± 1700	11800 ± 1500	16400 ± 1300
138	1,2-Dichloroethane	W	8020 ± 1600	13600 ± 2800	15900 ± 4000
139	2-Propanol	W	7750 ± 1000	14200 ± 1400	25200
140	Dimethyl sulphoxide	W	11800 ± 2100	22400 ± 1700	38300 ± 5000
141	Acetonitrile	W	14300 ± 710	23100 ± 2200	31400 ± 4000
142	1,2-Propanediol	W	19100 ± 1900	26000 ± 2600	46200 ± 8900
143	Acetone	W	26900 ± 7400	35900 ± 8500	43700 ± 6700
144	Ethanol	W	23200 ± 1700	36100 ± 870	38700 ± 1300
145	D-Fructose	W	15500 ± 2100	39200 ± 1200	55900 ± 340
146	Ethyl acetate	W	24600 ± 4900	43300 ± 5800	60000 ± 7100
147	Methanol	W	36500 ± 9800	46100 ± 10000	47500 ± 5300
148	D-Glucose	W	36400 ± 7600	48500 ± 3900	57900 ± 5200
149	PEG 1900	W	21000 ± 6300	77100 ± 5500	115000 ± 2200
150	PEG 5000	W	22500 ± 5100	134000 ± 6400	>160000

I = indeterminate; the points around the ID_{20} and ID_{80} points of the dose-response curve were too variable for values to be recorded with confidence. Solvent code: D = dimethyl sulphoxide; E = ethanol; M = methanol; W = water.

middle of the dose-response curve and is therefore subject to less variation (Knox *et al* 1986). The ID_{20} and ID_{80} values are, however, useful for providing information about the slope and shape of the dose-response curve (Knox *et al.*, 1986; Riddell *et al.*, 1986a). For example, bendiocarb (position 35) and pentachlorophenol (position 36) have very similar ID_{50} (μg/ml) values, but the dose-response curve for pentachlorophenol was much steeper (Table 3.1). The dose-response curve shapes were generally of the S-shape type and symmetrical about the ID_{50}. Some were exponential, i.e. the toxicity increased slowly between the ID_{20} and ID_{50} but rapidly from the ID_{50} to the ID_{80} (or vice versa). For example, acrylonitrile, iproniazid phosphate and methanol (positions 78, 90 and 147, Table 3.1) gave exponential dose-response curves, while diethylstilboestrol (position 25, Table 3.1) demonstrated an inverse exponential curve.

The s.e.m.s for most of the results represented approximately 10% of the mean ID values, although in some cases they were larger than this. However, only in the case of iron (II) sulphate heptahydrate (position 33, Table 3.1) was the variation in individual ID_{20} and ID_{80} results so great that the mean values had to be recorded as indeterminate. The variation in its ID_{50} values were also large ($20 \pm 9\mu$g/ml).

3.2.2 Expression of results

In vivo LD_{50} toxicity values are normally expressed in units of weight per kg of body weight, and the expression of *in vitro* toxicities in terms of μg/ml follows from this. However, it would seem logical that toxicities are due to the number of toxic molecules rather than the total weight of molecules

Table 3.2. *In vitro* toxicities of the 150 chemicals listed in order of ID_{50} (mM)

Rank	No.	Test Chemical	MW*	ID_{50} (μg/ml)	ID_{50} (mM)
2.5	001	5-Fluorouracil	130	<0.01	<0.0001
2.5	002	6-Mercaptopurine monohydrate	170	<0.01	<0.0001
2.5	004	Rotenone	394	<0.1	<0.0001
2.5	005	Vincristine sulphate	923	<0.1	<0.0001
5	003	Acrolein	56	<0.01	<0.0002
6	006	Cycloheximide	281	<0.1	<0.0004
7.5	007	Cadmium (II) chloride	183	0.1	0.001
7.5	008	Tributyltin chloride	326	0.3	0.001
10.5	011	p-Chloromercuribenzoic acid	357	3	0.01
10.5	010	Vanadium (IV) oxide	182	2	0.01
10.5	009	Silver (I) nitrate	170	2	0.01
10.5	015	Chlorpromazine hydrochloride	355	5	0.01
16	014	Mercury (II) chloride	272	4	0.02
16	017	Chloroquine sulphate	418	7	0.02
16	018	Colchicine	399	8	0.02
16	016	Benzalkonium chloride	353ave	7	0.02
16	013	Copper (II) chloride	134	3	0.02
16	019	Hexachlorophene	407	9	0.02
16	026	Triton X-100	647ave	14	0.02
20.5	012	Copper (I) chloride	99	3	0.03
20.5	027	Thallium (I) sulphate	505	15	0.03
22	024	Propranolol hydrochloride	296	12	0.04

Table 3.2. (contd.)

Rank	No.	Test Chemical	MW*	ID_{50} (μg/ml)	ID_{50} (mM)
23.5	025	Diethylstilboestrol	268	14	0.05
23.5	042	Fungizone	924	48	0.05
26.5	031	Thallium (I) acetate	263	17	0.07
26.5	020	Zinc (II) chloride	136	9	0.07
26.5	028	Manganese (II) sulphate tetrahydrate	223	15	0.07
26.5	033	Iron (II) sulphate heptahydrate	278	20	0.07
29.5	029	Zinc (II) acetate	184	15	0.08
29.5	034	Tellurium (IV) chloride	269	22	0.08
32	023	Cobalt (II) chloride	130	11	0.09
32	022	Selenium (IV) oxide	111	10	0.09
32	021	p-Aminophenol	109	10	0.09
34	030	Diethyl maleate	172	17	0.10
36	036	Pentachlorophenol	266	31	0.12
36	037	Nickel (II) sulphate heptahydrate	281	33	0.12
36	067	Tween 40	1284	159	0.12
39	041	Ioxynil	371	47	0.13
39	044	Dieldrin	381	50	0.13
39	035	Bendiocarb	223	30	0.13
42	032	6-Aminonicotinamide	137	20	0.15
42	038	Phenylbutazone	308	45	0.15
42	043	Retinoic acid	300	49	0.16
44	039	Versalide	258	45	0.17

Table 3.2. (contd.)

Rank	No.	Test Chemical	MW*	ID_{50} (μg/ml)	ID_{50} (mM)
45	048	Tin (IV) chloride pentahydrate	351	64	0.18
46	073	Tween 60	≈1300	244	0.19
47.5	047	Rhodium (III) chloride trihydrate	263	55	0.21
47.5	075	Tween 20	1228	262	0.21
49	050	Sodium tetrathionate dihydrate	306	74	0.24
50	040	Butylated hydroxyanisole	180	45	0.25
51	059	Lead (IV) tetraacetate	443	119	0.27
52	045	Tin (II) chloride	190	53	0.28
53	062	Iron (III) sulphate	400	123	0.31
54	051	Nickel (II) chloride hexahydrate	238	77	0.32
55	054	Sodium dodecyl sulphate	288	94	0.33
56	058	Lead (II) nitrate	331	119	0.36
58	093	Tween 85	≈1300	507	0.39
58	049	2,4-Dinitrophenol	184	72	0.39
58	064	Dodecylbenzene sulphonic acid, sodium salt	349	137	0.39
60	099	Tween 80	1310	619	0.47
61	055	Phenacetin	179	95	0.53
62	056	Palladium (II) chloride	177	103	0.58
63	053	Paracetamol	151	90	0.60
64	074	Dexamethasone	393	252	0.64
65	060	1,2,4-Trichlorobenzene	182	122	0.67
66	076	Yttrium (III) nitrate hexahydrate	383	262	0.68

Table 3.2. (contd.)

Rank	No.	Test Chemical	MW*	ID_{50} (μg/ml)	ID_{50} (mM)
67	057	Methylparaben	152	105	0.69
68	071	Chloramphenicol	323	226	0.70
69	068	2,4-Dichlorophenoxyacetic acid	221	162	0.73
70.5	066	Beryllium (II) sulphate tetrahydrate	177	157	0.89
70.5	070	Diethyl phthalate	222	198	0.89
72	080	Warfarin	308	285	0.92
73	063	Naphthalene	128	123	0.96
74.5	082	Indium (III) nitrate hydrate	301	303	1.0
74.5	089	Gadolinium (III) chloride hexahydrate	372	389	1.0
76.5	077	Phenytoin	252	265	1.1
76.5	069	6-Methylcoumarin	160	169	1.1
78	091	Lanthanum (III) chloride heptahydrate	371	441	1.2
79	046	Sodium (I) fluoride	42	53	1.3
80.5	090	Iproniazid phosphate	277	393	1.4
80.5	072	2-Chloroquinoline	164	233	1.4
82	061	Acrylamide	71	123	1.7
83.5	095	2,4,6-Trinitrobenzenesulphonic acid	293	536	1.8
83.5	084	L-Ascorbic acid	176	323	1.8
86	103	Frusemide	331	756	2.3
86	081	1-Octanol	130	298	2.3
86	096	Phenobarbital	232	543	2.3
88.5	100	Isoxepac	268	644	2.4

Table 3.2. (contd.)

Rank	No.	Test Chemical	MW*	ID_{50} (μg/ml)	ID_{50} (mM)
88.5	083	3-Chloroaniline	128	308	2.4
90	088	Bromobenzene	157	389	2.5
91.5	092	Acetylsalicylic acid	180	465	2.6
91.5	065	Potassium (I) fluoride	58	150	2.6
93	087	Phorone	138	378	2.7
94	123	Streptomycin sulphate	1458	4160	2.9
95	052	Lithium (I) fluoride	26	77	3.0
96.5	109	Aluminium (III) nitrate nonahydrate	375	1190	3.2
96.5	094	Trichloroacetic acid	163	524	3.2
98.5	101	1,2-Dibromoethane	188	645	3.4
98.5	104	Aluminium (III) chloride hexahydrate	242	830	3.4
100	079	Thioacetamide	75	274	3.6
101	106	Barium (II) acetate	255	982	3.8
102	098	Carbon tetrachloride	154	600	3.9
103	078	Acrylonitrile	53	266	5.0
104	112	Chromium (III) chloride hexahydrate	266	1500	5.6
105	108	Caffeine	194	1110	5.7
106	086	Allyl alcohol	58	355	6.1
107	085	Ammonium chloride	54	337	6.3
108	114	Cyclophosphamide	279	1800	6.5
109	110	L-Cysteine hydrochloride monohydrate	176	1190	6.8
110	105	Acetonyl acetone	114	937	8.2

Table 3.2. (contd.)

Rank	No.	Test Chemical	MW*	ID_{50} (μg/ml)	ID_{50} (mM)
111	107	1-Heptanol	116	1010	8.7
112	102	Imidazole	68	688	10
113	121	Sodium benzylpenicillin	356	3970	11
114	097	Lithium (I) chloride	42	554	13
115	122	Strontium (II) chloride hexahydrate	267	4060	15
116.5	115	Isoiazid	137	2170	16
116.5	111	Aniline	93	1480	16
118	135	Kanamycin sulphate	581	11000	19
119	113	Thiophene	84	1680	20
121	120	Calcium (II) chloride dihydrate	147	3860	26
122	150	PEG 5000	≈5000	134000	27
123	127	Magnesium (II) chloride hexahydrate	203	5600	28
124.5	125	Lithium (I) iodide	134	4690	35
124.5	119	1-Hexanol	102	3600	35
126	117	Lithium (I) bromide	87	3290	38
127	118	1-Pentanol	88	3540	40
128	149	PEG 1900	≈1900	77100	41
129	130	Potassium (I) iodide	166	7260	44
130	132	Potassium (I) sulphate	174	7860	45
131	124	Potassium (I) chloride	75	4360	58
132	133	Sodium (I) sulphate	142	8930	63
133	126	1-Butanol	74	4780	64

Table 3.2. (contd.)

Rank	No.	Test Chemical	MW*	ID_{50} (μg/ml)	ID_{50} (mM)
134	131	Potassium (I) bromide	119	7780	65
135	137	Sodium (I) iodide	150	11800	79
136	134	Sodium (I) bromide	103	9840	96
137	129	Pyrrole	67	6570	98
138	128	Sodium (I) chloride	58	6230	107
139	138	1,2-Dichloroethane	99	13600	138
140	36	1-Propanol	60	11200	186
141	145	D-Fructose	180	39200	218
142	139	2-Propanol	60	14200	236
143	148	D-Glucose	180	48500	269
144	140	Dimethyl sulphoxide	78	22400	287
145	142	1,2-Propanediol	76	26000	342
146	146	Ethyl acetate	88	43300	491
147	141	Acetonitrile	41	23100	562
148	143	Acetone	58	35900	617
149	144	Ethanol	46	36100	783
150	147	Methanol	32	46100	1441

* MW = molecular weight

administered (Stark *et al.*, 1986; Clothier *et al.*, 1988). Hence the ID_{50} values were converted to millimolar concentrations and ranked in terms of ID_{50} (mM) (Table 3.2). This altered the ranking of a few chemicals which have low or high molecular weights. The position of sodium fluoride was changed from position 46 (Table 3.1) to 79 (Table 3.2), i.e. ranking by mM values makes this appear less toxic among the 150 tested. Similarly, PEG 5000 was made to look more toxic in the mM rank. Its position changed from 150 to 122 (Table 3.2).

3.2.3 "Difficult" chemicals

Chemicals with poor aqueous solubility were usually dissolved initially in an organic solvent (see section 2.1.1.2) before dilution into the culture medium. If, at the range-finding stage, it was not possible to reach an ID_{50} concentration for a chemical before its maximum solubility in aqueous medium, then it was discarded and the results are not presented in this chapter. One chemical reacted exothermically with the aqueous medium, so it, too, could not be fully tested.

Some substances are unstable, and degrade chemically with exposure to light, heat or moisture. The results for a moisture-unstable substance, iron (II) sulphate heptahydrate, were very variable (Table 3.1). Other substances known to be chemically unstable in aqueous solution (e.g. L-ascorbic acid and retinoic acid) did not give a level of variation in the results any different from those for the majority of the 150 chemicals.

When high doses of volatile liquids, such as acetonitrile, thiophene and methanol, were tested, it was sometimes noticeable that control cell growth was decreased. Some volatile chemicals, for example, methanol, gave larger s.e.m.s than other volatile chemicals.

3.2.4 Comparison of *in vivo* and *in vitro* toxicity data for 59 chemicals

Rat oral and mouse i.p. LD_{50} data was gathered from the ICI toxicology profiles (Purchase *et al.*, 1986; Clothier *et al.*, 1989), or, if not available from ICI, values were taken from RTECS (Lewis and Tatken, 1982). Although rat oral LD_{50} values were found for 94 of the 150 chemicals tested in the FRAME KB cytotoxicity assay, and mouse i.p. values for 86 of them, both rat and mouse data were available for only 59 chemicals. In order to be comparable with the ID_{50} (mM) values, the LD_{50} values were converted to mmol/kg. The ID_{50} and LD_{50} data for the 59 chemicals are shown in Table 3.3.

Linear regression analysis was used to compare the *in vitro* (log ID_{50}) and *in vivo* rat or mouse (log LD_{50}) toxicities for the 59 chemicals. The correlation coefficients were very similar:

KB ID_{50}/rat oral LD_{50}	r = 0.76
KB ID_{50}/mouse i.p. LD_{50}	r = 0.80

On both linear regression plots (Figures 3.1 and 3.2), there is a wider scattering of points towards the lefthand (more toxic) ends of the graphs. The points which are most distant from the regression line are those where the *in vitro* toxicities would predict the *in vivo* toxicities to be greater than they are.

3.2.5 Conclusions

The FRAME KB cytotoxicity assay is able to detect toxicity to 3T3-L1 cells of a wide range of chemical types with different modes of toxicity, and which are toxic at a very wide range of concentrations. The assay gives reproducible results for the vast majority of chemicals tested. It was found that a small number of chemicals caused problems methodologically and/or in the

Table 3.3. *In vitro* and *in vivo* toxicities of 59 miscellaneous chemicals

Test Chemical	ID_{50} (mM)	Rat oral LD_{50} (mmol/kg)	Mouse i.p. LD_{50} (mmol/kg)
6-Mercaptopurine monohydrate	0.0001	2.5	1.7
Rotenone	0.0001	0.34	0.01
Acrolein	0.0002	0.64	0.13
Cycloheximide	0.0004	0.01	0.49
Cadmium (II) chloride	0.001	0.48	0.05
Chloropromazine hydrochloride	0.01	0.93	0.32
Mercury (II) chloride	0.02	0.004	0.02
Chloroquine sulphate	0.02	2.6	0.23
Benzalkonium chloride	0.02	1.1	0.03
Copper (II) chloride	0.02	1.0	0.06
Zinc (II) chloride	0.07	2.6	0.23
Iron (II) sulphate heptahydrate	0.07	2.1	0.42
Zinc (II) acetate	0.08	14	0.31
Cobalt (II) chloride	0.09	0.62	0.38
Phenylbutazone	0.15	2.4	0.84
Retinoic acid	0.16	6.7	1.2
Tin (II) chloride	0.28	3.7	0.35
Nickel (II) chloride hexahydrate	0.32	0.81	0.20
Sodium dodecyl sulphate	0.33	6.5	0.87
2,4-Dinitrophenol	0.39	0.20	0.14
Phenacetin	0.53	14	5.8
Palladium (II) chloride	0.58	1.1	0.59
Paracetamol	0.60	16	2.4
Chloramphenicol	0.70	7.7	3.9
2,4-Dichlorophenoxyacetic acid	0.73	6.7	1.2
Naphthalene	0.96	14	1.2
Phenytoin	1.1	8.5	0.87
Lanthanum (II) chloride heptahydrate	1.2	17	0.49
Sodium (I) fluoride	1.3	4.3	1.2
Iproniazid	1.4	1.7	3.3
Acrylamide	1.7	2.8	2.4
Phenobarbital	2.3	1.5	1.0
Bromobenzene	2.5	16	6.4
Acetylsalicylic acid	2.6	9.9	1.9
1,2-Dibromoethane	3.4	0.57	1.2
Carbon tetrachloride	3.9	18	30
Acrylonitrile	5.0	2.1	0.74

Table 3.3. (contd.)

Test Chemical	ID_{50} (mM)	Rat oral LD_{50} (mmol/kg)	Mouse i.p. LD_{50} (mmol/kg)
Chromium (II) chloride hexahydrate	5.6	6.7	2.0
Caffeine	5.7	1.8	1.4
Allyl alcohol	6.1	1.1	0.72
Ammonium chloride	6.6	31	9.0
Cyclophosphamide	6.5	0.79	1.7
Acetonyl acetone	8.2	19	14
Lithium (I) chloride	13	18	14
Strontium (II) chloride hexahydrate	15	14	5.7
Isoniazid	16	17	1.0
Aniline	16	5.2	5.3
Calcium (II) chloride dihydrate	26	9.0	2.5
Magnesium (II) chloride hexahydrate	28	40	1.0
Sodium (I) iodide	79	29	5.8
Sodium (I) chloride	107	74	53
2-Propanol	236	97	16
Dimethyl sulphoxide	287	217	168
1,2-Propanediol	342	263	150
Ethyl acetate	491	125	8.1
Acetonitrile	562	91	8.2
Acetone	617	168	22
Ethanol	783	287	130
Methanol	1441	313	263

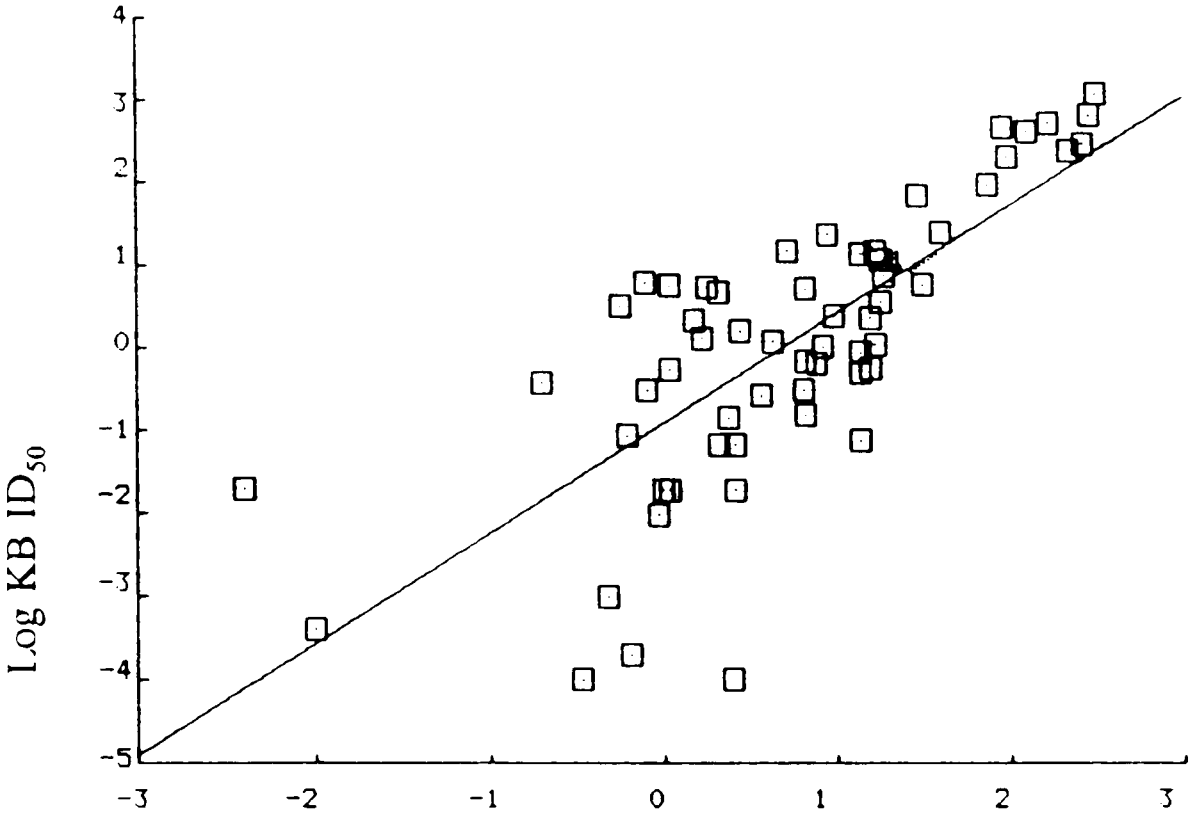

Figure 3.1 Linear regression plot for the comparison of FRAME KB cytotoxicity assay ID_{50} with rat oral LD_{50} for 59 miscellaneous chemicals

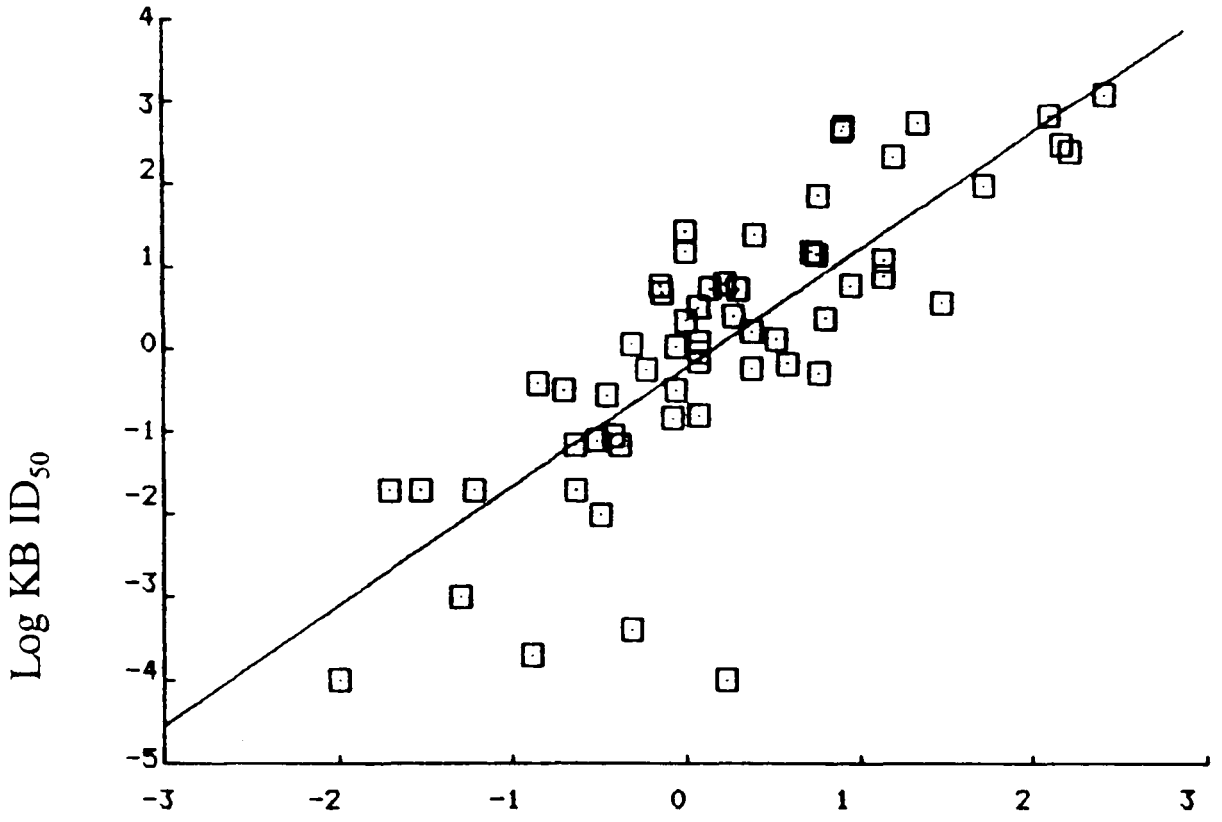

Figure 3.2 Linear regression plot for the comparison of FRAME KB cytotoxicity assay ID_{50} with mouse i.p. LD_{50} for 59 miscellaneous chemicals

interpretation of results. Methodological problems include difficulties in dissolving a substance in aqueous medium, and with volatile liquids, interfering with control cell growth.

For certain types of "difficult" chemicals, the FRAME KB cytotoxicity assay will have to be adapted in order to provide more-meaningful results. This may mean providing alternative methods of solubilising the insoluble chemicals or reducing the evaporation of volatile chemicals.

Most of the alterations in toxicity ranking when converting ID_{50} results from μg/ml to mM were minor. Even where chemicals of low or high molecular weight had their positions altered more considerably, this would not affect the overall assessment of their *in vitro* toxic potency.

The FRAME KB cytotoxicity assay data correlated reasonably well with both the rat oral and mouse i.p. LD_{50} values. Across the toxicity range, the FRAME KB cytotoxicity assay was least accurate in predicting the *in vivo* toxicities of very toxic compounds.

3.3 DISCUSSION

An important aspect of *in vitro* toxicity test development is how the data should be interpreted, how they should be presented, and what they should be compared with. The results for a single test substance in the FRAME KB cytotoxicity assay (ID_{20}, ID_{50} and ID_{80} figures) have no value on their own. However, when compared to the results for all the other chemicals, a rank order of toxicity can be established. This rank order may be compared to the rank order obtained with the same set of chemicals in a different toxicity test. A standard set of ten chemicals has been suggested for this purpose (Clothier *et al.*, 1988). This set was chosen deliberately to span a wide range of toxic

potencies and modes of action, and is also useful during interlaboratory comparisons of absolute values obtained with the standard FRAME KB cytotoxicity assay protocol. Rank ordering has the disadvantage that it implies that the differences from one substance to the next in the ranking are equivalent. This is not the case with the 150 chemicals, because the spread of results across the 7 orders of magnitude is not completely even; there are few chemicals with very low or very high ID_{50} values.

The ID_{50} results for the 150 chemicals could be used to place chemicals in categories of toxic potency, for example, ID_{50} < 0.05mM = very toxic, ID_{50} 0.06 to 1.0mM = toxic, and so on. However, if chemicals are simply categorised as "toxic" or "non toxic", it would not be possible to indicate whether one "toxic" chemical was, in fact, far more toxic than another. ID_{50} values, at one end of a category would tend to be closer to values at the beginning of the next category, than to values at the beginning of the former category. Hence, categorisation is not a very useful way of presenting *in vitro* toxicity results.

Although the mM rank order (Table 3.2) was not very different from the μg/ml rank (Table 3.1), it is still useful to look at both sets of figures. The μg/ml values are often easier to understand, because the experimenter will weigh out a chemical and prepare test solutions in terms of μg/ml or mg/ml. At the very toxic end of the scale, the FRAME KB cytotoxicity assay protocol does not recommend that values below 0.01μg/ml are recorded as definitive, because of the greater risk of inaccuracy in making test chemical dilutions at this level. With very low toxicities (around 100mg/ml), the possibility of the amount of dissolved test compound affecting the volume of the solution must be taken into account. Hence, using μg/ml values makes one more aware of these factors. The mM values offer a more physiological approach, whereby the number, not weight, of test chemical molecules is considered. This has a

scientific basis, and should therefore be the preferred units of expression.

Chemicals such as bromobenzene, cyclophosphamide and paracetamol are metabolised to toxic products *in vivo*. 3T3-L1 cells have little or no enzymes for the metabolic activation of such chemicals, and therefore the FRAME KB cytotoxicity assay has measured the toxicities of the parent compounds, not of the metabolites which are responsible for their toxic effects *in vivo*. Conversely, some chemicals are rapidly detoxified by metabolic processes *in vivo*. These are issues which can lead to difficulties of interpretation, if the *in vitro* results are to be compared to the situation *in vivo*. Horner (1986) and Reader (1988) have examined the problems of metabolism-mediated cytotoxicity. This thesis is therefore complementary to these earlier theses, as it intends to address some of the methodological problems in the FRAME KB cytotoxicity assay, such as those connected with low solubility, and high volatility.

The 59 chemicals used in the KB ID_{50}/LD_{50} comparisons were as varied in their toxic potencies and modes of action as the set of 150. The correlation coefficients were therefore unexpectedly high, if one considers recent discussions (Clothier *et al.*, 1989, Fry *et al.*, 1990b; Phillips *et al.*, 1990) which suggest that only related groups of chemicals would give good *in vitro/in vivo* correlations.

There are several possible reasons why the toxicities of some of the most toxic chemicals *in vivo* were among the most poorly predicted by the FRAME KB cytotoxicity assay. Firstly, the chemical could be detoxified *in vivo* (so that its toxic effects are diminished), whilst *in vitro*, detoxification cannot occur at the same level as *in vivo*, and hence the toxic chemical can exert its effects on the 3T3-L1 cells for longer. Secondly, at the very low concentrations involved, there is a greater possibility of experimental error in both the *in vivo* and *in*

vitro tests. A third reason may be that some of the chemicals may not be well absorbed from the G.I. tract during the rat oral LD_{50}, and hence their *in vivo* toxicities may be 'underestimated', leading to the disparity between oral LD_{50} values and KB ID_{50} results.

The MEIC study has shown close correlations between *in vitro* cytotoxicity and human or rodent lethal blood concentrations for a group of ten unrelated human poisons (Ekwall *et al.*, 1990). Structurally and/or mechanistically related chemicals may give still closer correlations. Hence, a group of structurally and mechanistically related substances (metal compounds) were selected to examine whether better *in vitro/in vivo* correlations could be obtained than with the 59 chemicals.

CHAPTER 4 AN ASSESSMENT OF TWO ALTERNATIVE METHODS FOR PREDICTING THE *IN VIVO* ACUTE TOXICITIES OF METAL COMPOUNDS

4.1 INTRODUCTION

Metal compounds number among the estimated 50 to 70,000 chemical substances in production and use, for which inadequate hazard data or no hazard data exist (Peters and Piersma, 1990). The exposure of humans to metal compounds is increasing, mainly through industrial exposure and via contaminated water or foodstuffs (Friberg and Nordberg, 1986). In addition, the whole ecosystem is suffering increasing exposure through the growing impact of metal ions in the environment, due to pollution and acid rain (Friberg and Nordberg, 1986). The toxicology of metals is important, because of the indestructibility of metal ions, and their tendency to accumulate in body tissues over many years (Clarkson, 1986). Metals and their compounds may act through contact with the skin, pulmonary membranes or the gastro-intestinal mucosa, or systemically in any tissue, and can act as allergens, mutagens, teratogens and carcinogens (Heck and Costa, 1982; Kazantzis, 1986). Certain metals, such as cobalt, zinc and iron, are required in the body as essential trace elements, but they may still exert toxic effects if present in excess (Clarkson, 1986; Bryce-Smith, 1989).

Metal toxicity occurs by many diverse mechanisms, but is explainable, at the most basic level, as interference with cellular biochemical systems (Friberg and Nordberg, 1986). Metals often interact with important sites on cellular enzymes or structural proteins, but may also bind to nucleic acids. The immediate effects are breaking of hydrogen bonds, displacement of other metals (for example, those present as enzyme cofactors) or alteration in the tertiary structure of the target molecule. Binding occurs via co-ordinate-covalent links between the metal cation and electron-donating ligands. The consequences include inhibition of active transport, alteration in the passive permeability characteristics of the cell membrane, alteration of transcriptional and translational processes, and a decrease or increase in the activities of

specific enzymes (Clarkson, 1986).

Protein binding in the plasma varies from metal to metal and may help or hinder the diffusion of the metal through the body (Camner *et al.*, 1986). Cadmium and mercury ions bind strongly to plasma protein, whilst germanium does not bind significantly.

Although metal ions react with a broad range of ligands, they often have very specific target organs or tissues. These are called the critical organs, and the concentration of the metal ion which causes adverse functional changes (reversible or irreversible) in the most sensitive cells of those organs is called the critical concentration (Friberg and Nordberg, 1986). Knowledge of these concentrations is required in order to establish safe overall bodily exposure limits. Little is known, however, about the toxicology of many metals, particularly at the molecular level. Here, *in vitro* models may be useful (Cox and Harrison, 1983; Clarkson, 1986; Elias *et al.*, 1986; Klug *et al.*, 1988; Jenssen and Syversen, 1991).

Metal compound risk assessment is complicated by the many factors which influence the effects of metals on animals, such as the nutritional state, age, or route of exposure of the animal, and the chemical or physical state of the compound (Nordberg *et al.*, 1986). The identification and estimation of risk requires toxicological, medical and scientific judgements, whilst risk evaluation and control must also consider technical and economic factors (Friberg, 1986). The risk analysis process relies on the use of animal models, but difficulties in extrapolating results to man are always present. In addition, the use of animals can be questioned on economic and ethical grounds. Current resources could never realistically provide data on the full backlog of chemicals, so prioritisation is necessary (Purchase, 1990a). Cheaper, non-animal alternative methods of assessing the toxicity of a compound may be

useful in this context. Two such alternatives are the Quantitative Structure Activity Relationship (QSAR) approach, and *in vitro* cytotoxicity testing, e.g. with the FRAME KB cytotoxicity assay. These alternatives could be used at the initial phase of toxicity assessment (Balls, 1986) and could be used as preliminary screens, or as adjuncts to animal tests, or could possibly replace the need for animal tests altogether (Balls and Horner, 1985; Balls *et al.*, 1990a, b).

A QSAR is a mathematical relationship between the quantifiable toxic effects of a chemical and its structure, and aims to predict the toxic effects of similar, as yet untested chemicals (Veith *et al.*, 1985). QSAR studies have attempted to provide predictive models for various different types of toxicity, such as carcinogenicity, eye irritation and acute lethal toxicity (Enslein, 1988; Phillips *et al.*, 1990). Numerous QSAR parameters can be applied in attempts to predict the toxicities of metal compounds in animals (Kaiser, 1985). One parameter, metal softness (σp), has received attention from toxicologists (Jones and Vaughn, 1978; Williams *et al.*, 1982). In addition, Babich and Borenfreund (1987) attempted to demonstrate that *in vitro* toxicity assay data, by correlating well with the softness parameter, can be, by extrapolation, useful both in the prediction of *in vivo* toxicity and in the identification of new and useful QSARS.

The "softness" of a metal ion governs its metal-ligand interactions according to the theory of Hard and Soft Acids and Bases (HSAB; Jones and Vaughn, 1978). σp is defined in terms of the coordinate bond energies (CBE) of the metal fluoride (F) and metal iodide (I), thus:

$$\frac{CBE(F) - CBE(I)}{CBE(F)}$$

QSAR parameters are most likely to be applicable when used to predict toxic endpoints which are caused by the same mechanism (Phillips *et al.*, 1990). For example, correlations of softness to LD_{50} values for metals should only hold where the basis of the lethal toxicity involves metal-ligand binding (Jones and Vaughn, 1978). However, it is questionable whether all metals exert their toxic effects by binding to target molecules (Cox and Harrison, 1983; Hulme *et al.*, 1989).

The FRAME KB cytotoxicity assay results and the softness parameter were therefore assessed for their ability to predict indices of acute toxicity in mammals. As rat oral and mouse i.p. LD_{50} values had been used in the comparison involving unrelated chemicals (chapter 3), the two alternative methods were assessed for their ability to predict these two *in vivo* parameters.

Metal compounds already present in the culture medium were:- calcium (II) chloride (2mM); iron (III) nitrate (0.01mM); magnesium (II) sulphate (1mM) sodium (I) chloride (160mM); sodium (I) hydrogen carbonate (94mM); sodium (I) dihydrogen phosphate.$2H_2O$ (0.5mM) and sodium (I) pyruvate (0.5mM).

4.2 RESULTS

4.2.1 *In vitro* toxicities of 52 metal compounds

4.2.1.1 ***In vitro* toxicities of 52 metal compounds**

The 52 metal compounds tested revealed a wide range of cytotoxicities, with ID_{50} values from 0.1μg/ml to 11800μg/ml (Table 4.1). Heavy metal compounds appear toward the top of the toxicity ranking. By contrast, compounds of the alkali and alkaline earth metals were toward the lower end, giving values considered to be of low toxicity.

Table 4.1. *In vitro* toxicities of 52 metal compounds

			ID_{20} (μg/ml $\pm$ s.e.m.)	ID_{50} (μg/ml $\pm$ s.e.m.)	ID_{80} (μg/ml $\pm$ s.e.m.)
Cadium	II	chloride	0.1 $\pm$ 0	0.1 $\pm$ 0	0.2 $\pm$ 0.1
Silver	I	nitrate	1 $\pm$ 0	2 $\pm$ 0	2 $\pm$ 0
Vanadium	V	oxide	0.2 $\pm$ 0.1	2 $\pm$ 0	3 $\pm$ 1
Copper	I	chloride	2 $\pm$ 0	3 $\pm$ 0	5 $\pm$ 1
Copper	II	chloride	2 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 1
Mercury	II	chloride	2 $\pm$ 1	4 $\pm$ 1	6 $\pm$ 0
Zinc	II	chloride	6 $\pm$ 1	9 $\pm$ 0	12 $\pm$ 1
Selenium	IV	oxide	5 $\pm$ 1	10 $\pm$ 2	15 $\pm$ 2
Cobalt	II	chloride	3 $\pm$ 1	11 $\pm$ 1	24 $\pm$ 6
Thallium	I	sulphate	7 $\pm$ 3	15 $\pm$ 3	25 $\pm$ 5
Manganese	II	sulphate.$4H_2O$	11 $\pm$ 2	15 $\pm$ 1	25 $\pm$ 3
Zinc	II	acetate	12 $\pm$ 1	15 $\pm$ 1	18 $\pm$ 1
Thallium	I	acetate	9 $\pm$ 1	17 $\pm$ 2	30 $\pm$ 2
Iron	II	sulphate.$7H_2O$	I	20 $\pm$ 9	I
Tellurium	IV	chloride	8 $\pm$ 1	22 $\pm$ 2	31 $\pm$ 2
Nickel	II	sulphate.$7H_2O$	20 $\pm$ 3	33 $\pm$ 3	53 $\pm$ 3
Tin	II	chloride	21 $\pm$ 6	53 $\pm$ 10	124 $\pm$ 31
Sodium	I	fluoride	35 $\pm$ 6	53 $\pm$ 4	73 $\pm$ 4
Rhodium	III	chloride.$3H_2O$	26 $\pm$ 3	55 $\pm$ 2	100 $\pm$ 1
Tin	IV	chloride.$5H_2O$	34	64 $\pm$ 3	98
Sodium	I	tetrathionate.$2H_2O$	33 $\pm$ 4	74 $\pm$ 5	128 $\pm$ 22
Nickel	II	chloride.$6H_2O$	43 $\pm$ 3	77 $\pm$ 13	98 $\pm$ 18
Lithium	I	fluoride	51 $\pm$ 12	77 $\pm$ 25	142 $\pm$ 24

Palladium	II	chloride	62 ± 14	103 ± 2	115
Lead	II	nitrate	53 ± 21	119 ± 45	345 ± 19
Lead	IV	tetra-acetate	82 ± 22	119 ± 5	286 ± 34
Iron	III	sulphate	37 ± 7	123 ± 24	309 ± 69
Potassium	I	fluoride	70 ± 16	150 ± 6	260 ± 25
Beryllium	II	sulphate.$4H_2O$	66 ± 9	157 ± 17	387 ± 48
Yttrium	III	nitrate.$6H_2O$	227 ± 20	262 ± 7	286 ± 3
Indium	III	nitrate.xH_2O	26 ± 3	303 ± 57	546 ± 9
Gadolinium	III	chloride.$6H_2O$	360 ± 3	389 ± 6	419 ± 12
Lanthanum	III	chloride.$7H_2O$	373	441 ± 34	567 ± 19
Lithium	I	chloride	343 ± 67	554 ± 95	810 ± 73
Aluminium	III	chloride.$6H_2O$	738 ± 120	830 ± 60	1050 ± 82
Barium	II	acetate	566 ± 280	982 ± 62	2470
Aluminium	III	nitrate.$9H_2O$	953 ± 75	1190 ± 37	1280
Chromium	III	chloride.$6H_2O$	857 ± 160	1500 ± 24	2170 ± 84
Lithium	I	sulphate	953 ± 210	2460 ± 600	6460 ± 200
Lithium	II	bromide	1790 ± 390	3290 ± 130	7050 ± 630
Calcium	II	chloride.$2H_2O$	2230	3860	8900
Strontium	II	chloride.$6H_2O$	1940 ± 290	4060 ± 220	10900 ± 940
Potassium	I	chloride	3060 ± 300	4360 ± 200	5560 ± 75
Lithium	I	iodide	2470 ± 810	4690 ± 460	7710 ± 110
Magnesium	II	chloride.$6H_2O$	3260 ± 420	5600 ± 750	7910 ± 90
Sodium	I	chloride	1790 ± 700	6230 ± 720	8390 ± 660
Potassium	I	iodide	3280 ± 860	7260 ± 440	8790 ± 44
Potassium	I	bromide	5900 ± 140	7780 ± 390	9540 ± 360
Sodium	I	sulphate	6090 ± 890	8930 ± 560	10000 ± 390
Sodium	I	bromide	7250 ± 450	9840 ± 910	11300 ± 1000
Sodium	I	iodide	8730 ± 1700	11800 ± 1500	16400 ± 1300

I = Indeterminate.

Most metal salts ionise in aqueous solutions, hence the relative number of anions and cations in each molecule must be considered. For example, one mole of thallium sulphate (Tl_2SO_4) yields twice as many thallium ions per mole as thallium acetate ($TlCH_3COO$). The toxic effects of thallium would be expected to be related to the number of toxic thallium ions in solution, rather than the weight of compound dissolved in the solution. Hence, although μg/ml ID_{50} values are usually taken as the parameters for comparison of test substances by rank order (Balls & Horner, 1985; Knox *et al.*, 1986), in this case they were converted to millimolar (mM) concentrations to permit the comparison of cytotoxicities in terms of molar concentration (Table 4.2). Thallium sulphate and thallium acetate have ID_{50} values of 15 and 17μg/ml respectively, which, when converted, become 0.03 and 0.07mM. This demonstrates how thallium sulphate, by yielding two Tl^+ ions per mole, is twice as toxic as thallium acetate. Similarly, the ID_{50} (mM) values of the two aluminium compounds (aluminium chloride, 3.4mM, and aluminium nitrate, 3.2mM) are much closer than the ID_{50} (μg/ml) values, 830 and 1190, respectively. However, potassium sulphate (K_2SO_4, ID_{50} 45mM) is not twice as toxic as potassium chloride (KCl), and this indicates that it is not only the potassium ion which is causing toxicity.

By converting ID_{50} values from μg/ml to mM, the compounds whose rankings are altered most are those with either a relatively low or a relatively high molecular weight (Table 4.2). Nickel (II) chloride hexahydrate and lithium fluoride appear to be equally toxic in the μg/ml ranking. However, the mM values reveal that lithium fluoride is almost ten times less toxic than the nickel compound.

4.2.1.2 **Effects of the anion on metal compound toxicity *in vitro***

Since it cannot be assumed that the anions make no contribution to the

Table 4.2. Comparison of rank order of ID_{50}s of 52 metal compounds by millimolar and μg/ml values.

			ID_{50} (mM)	Rank order by ID_{50} (mM)	Rank order by ID_{50} (μg/ml)
Cadmium	II	chloride	0.001	1	1
Vanadium	V	oxide	0.01	2.5	2.5
Silver	I	nitrate	0.01	2.5	2.5
Mercury	II	chloride	0.02	4.5	6
Copper	II	chloride	0.02	4.5	4.5
Copper	I	chloride	0.03	6.5	4.5
Thallium	I	sulphate	0.03	6.5	11
Thallium	I	acetate	0.07	9.5	13
Zinc	II	chloride	0.07	9.5	7
Manganese	II	sulphate.$4H_2O$	0.07	9.5	11
Iron	II	sulphate.$7H_2O$	0.07	9.5	14
Zinc	II	acetate	0.08	12.5	11
Tellurium	IV	chloride	0.08	12.5	15
Cobalt	II	chloride	0.09	14.5	9
Selenium	IV	oxide	0.09	14.5	8
Nickel	II	sulphate.$7H_2O$	0.12	16	16
Tin	IV	chloride.$5H_2O$	0.18	17	20
Rhodium	III	chloride.$3H_2O$	0.21	18	19
Sodium	I	tetrathionate.$2H_2O$	0.24	19	21
Lead	IV	tetraacetate	0.27	20	25.5
Tin	II	chloride	0.28	21	17.5
Iron	III	sulphate	0.31	22	27
Nickel	II	chloride.$6H_2O$	0.32	23	22.5

Lead	II	nitrate	0.36	24	25.5
Palladium	II	chloride	0.58	25	24
Yttrium	III	nitrate.$6H_2O$	0.68	26	30
Beryllium	II	sulphate.$4H_2O$	0.89	27	29
Indium	III	nitrate.xH_2O	1.0	28.5	31
Gadolinium	III	chloride.$6H_2O$	1.0	28.5	32
Lanthanum	III	chloride.$7H_2O$	1.2	30	33
Sodium	I	fluoride	1.3	31	17.5
Potassium	I	fluoride	2.6	32	28
Lithium	I	fluoride	3.0	33	22.5
Aluminium	III	nitrate.$9H_2O$	3.2	34	37
Aluminium	III	chloride.$6H_2O$	3.4	35	35
Barium	II	acetate	3.8	36	36
Chromium	III	chloride.$6H_2O$	5.6	37	38
Lithium	I	chloride	13	38	34
Strontium	II	chloride.$6H_2O$	15	39	42
Lithium	I	sulphate	22	40	39
Calcium	II	chloride.$2H_2O$	26	41	41
Magnesium	II	chloride.$6H_2O$	42	42	45
Lithium	I	iodide	35	43	44
Lithium	I	bromide	38	44	40
Potassium	I	iodide	44	45	47
Potassium	I	sulphate	45	46	49
Potassium	I	chloride	58	47	43
Sodium	I	sulphate	63	48	50
Potassium	I	bromide	65	49	48
Sodium	I	iodide	79	50	52
Sodium	I	bromide	96	51	51
Sodium	I	chloride	107	52	46

toxicity of metal compounds, the toxicities of metal compounds with a common anion were considered. Chloride is the most abundant of those anion already present in the culture medium. Even with a common anion, however, direct comparison between the metal compounds would have to take into account the fact that metals of different valencies and different toxicities will contribute different amounts of chloride to the test chemical solution. However, the culture medium contains over 100mM chloride, so the addition of further chloride would be unlikely to greatly alter the chloride concentration. Therefore, the cytotoxicities of the 22 chlorides were examined (Table 4.3).

Only potassium, sodium, calcium and magnesium chlorides produced ID_{50} (mM) concentrations which could increase the initial medium chloride concentration by more than about 20%. Hence, the anion (in this case, chloride) is potentially important, particularly when applied as a salt of a cation with low cytotoxicity.

4.2.1.3 **Effects of the anion on the *in vitro* toxicities of salts of alkali metals**

The cytotoxicities of five different salts of lithium, sodium and potassium were compared in order to investigate the influence of the anion on metal compound cytotoxicity. Compounds of sodium and potassium in general have very low cytotoxicity to 3T3-L1 cells, thus reducing the risk that cation toxicity would mask any effect caused by the anion.

The results (Table 4.4) show that, of the halides, fluorides are clearly the most toxic *in vitro*. Sulphates also appear to be slightly more toxic than the halides. The rank order of toxicity for the three metals, for all the salts except fluoride, is lithium > potassium > sodium, although the differences are not great (Table 4.4). This ranking was found, despite the high concentration of sodium and chloride ions already present in the culture medium.

Table 4.3 *In vitro* toxicities of the chloride salts of 22 metals

	Metal Valency	ID_{50} (mM)
Cadmium	II	0.001
Mercury	II	0.02
Copper	II	0.02
Copper	I	0.03
Zinc	II	0.07
Tellurium	IV	0.08
Cobalt	II	0.09
Tin	IV	0.18
Rhodium	III	0.21
Tin	II	0.28
Nickel	II	0.32
Palladium	II	0.58
Gadolinium	III	1.0
Lanthanum	III	1.2
Aluminium	III	3.4
Chromium	III	5.6
Lithium	I	13
Strontium	II	15
Calcium	II	26
Magnesium	II	28
Potassium	I	58
Sodium	I	107

Table 4.4. *In vitro* toxicities of salts of three alkali metals

	ID_{50} (mM)		
	Lithium	Sodium	Potassium
Fluoride	3.0	1.3	2.6
Chloride	13	107	58
Bromide	38	96	65
Iodide	35	79	44
Sulphate	22	63	45

4.2.1.4 **Conclusions**

The results for the 52 metal compounds tested in the FRAME KB cytotoxicity assay spanned six orders of magnitude and hence covered a similar range of toxic potencies as the 150 chemicals (Chapter 3).

The conversion of the ID_{50} results from μg/ml to mM removed several discrepancies concerning the toxicity of a metal ion (e.g. with thallium and aluminium). The expression of metal compound toxicity results in mM is therefore necessary, because it takes into account the number of toxic metal ions in solution.

The anion of the metal compound is of little importance in the toxicity of the whole compound, unless the anion is intrinsically toxic and/or the metal is of low toxicity. The most common anions are of low toxicity and do not affect the toxicity ranking of the metals.

4.2.2 Comparison of *in vitro* toxicity data and the metal softness parameter with published *in vivo* toxicity data, by linear regression analysis

In order to test whether the FRAME KB cytotoxicity assay and/or the softness parameter could be useful predictors of mammalian toxicity, both the assay results and the σp values were compared with the parameters of acute *in vivo* toxicity.

Table 4.5 includes the cytotoxicity ID_{50} (mM) values for all 52 metal compounds along with rat oral and mouse i.p. LD_{50} data and softness (σp) values, where available. Rat oral LD_{50} values were available for 27 of the compounds, and mouse i.p. values for 28. Softness (σp) values were available for 31 out of 35 different metal ions present in the compounds, and were taken from Williams *et al.* (1982), except for those for Li^+, Na^+, K^+, Cu^+, Ca^{2+}, Fe^{2+}, Sn^{2+}, Al^{3+}, Y^{3+} and La^{3+}, which were taken from Pearson and Mawby (1967). Both rat and mouse *in vivo* values, as well as softness values, were available for 19 of the compounds.

4.2.2.1 **Selection of data sets for analysis**

In order that correlations of the two alternatives (the FRAME KB cytotoxicity assay and the softness parameter) with acute *in vivo* toxicity data would be comparable, only the 19 compounds with both rat and mouse LD_{50} values were used. Four data sets within this 19 were selected, so that any correlation using a particular data set involved the same set of chemicals.

The first two data sets were used for correlations between the FRAME KB cytotoxicity assay results (ID_{50} in mM) and the rat oral or mouse i.p. LD_{50} values (in mmol/kg). These were:-

Table 4.5. *In vitro* and *in vivo* toxicities and softness values of 52 metal compounds

			ID_{50} (mM)	Rat Oral LD_{50} (mmol/kg)	Mouse i.p. LD_{50} (mmol/kg)	Softness (σp)
Cadmium	II	chloride	0.001	0.48	0.05	0.081
Vanadium	V	oxide	0.01	0.05	-	-
Silver	I	nitrate	0.01	-	0.13	0.073
Mercury	II	chloride	0.02	0.004	0.02	0.064
Copper	II	chloride	0.02	1.0	0.06	0.104
Copper	I	chloride	0.03	2.7	-	0.112
Thallium	I	sulphate	0.03	-	-	0.215
Thallium	I	acetate	0.07	-	0.14	0.215
Zinc	II	chloride	0.07	2.6	0.23	0.115
Manganese	II	sulphate.$4H_2O$	0.07	-	2.4	0.124
Iron	II	sulphate.$7H_2O$	0.07	2.1	0.42	0.129
Zinc	II	acetate	0.08	14	0.31	0.115
Tellurium	IV	chloride	0.08	-	-	-
Cobalt	II	chloride	0.09	0.62	0.38	0.130
Selenium	IV	oxide	0.09	-	-	-
Nickel	II	sulphate.$7H_2O$	0.12	-	0.14	0.126
Tin	IV	chloride.$5H_2O$	0.18	-	0.18	0.075
Rhodium	III	chloride.$3H_2O$	0.21	-	-	0.055
Sodium	I	tetrathionate.$2H_2O$	0.24	-	-	0.211
Lead	IV	tetraacetate	0.27	-	-	-
Tin	II	chloride	0.28	3.7	0.35	0.148
Iron	III	sulphate	0.31	-	-	0.097
Nickel	II	chloride.$6H_2O$	0.32	0.81	0.20	0.126

Lead	II	nitrate	0.36	-	-	0.131
Palladium	II	chloride	0.58	1.1	0.59	0.069
Yttrium	III	nitrate.6H_2O	0.68	-	6.2	0.147
Beryllium	II	sulphate.4H_2O	0.89	0.78	-	0.172
Indium	III	nitrate.xH_2O	1.0	-	-	0.100
Gadolinium	III	chloride.6H_2O	1.0	-	1.4	0.157
Lanthanum	III	chloride.7H_2O	1.2	17	0.49	0.171
Sodium	I	fluoride	1.3	4.3	1.2	0.211
Potassium	I	fluoride	2.6	4.2	-	0.232
Lithium	I	fluoride	3.0	-	-	0.247
Aluminium	III	nitrate.9H_2O	3.2	0.7	-	0.136
Aluminium	III	chloride.6H_2O	3.4	28	-	0.136
Barium	II	acetate	3.8	3.6	-	0.184
Chromium	III	chloride.6H_2O	5.6	6.7	2.0	0.107
Lithium	I	chloride	13	18	14	0.247
Strontium	II	chloride.6H_2O	15	14	5.7	0.172
Lithium	I	sulphate	22	-	-	0.247
Calcium	II	chloride.2H_2O	26	9.0	2.5	0.181
Magnesium	II	chloride.6H_2O	28	40	1.0	0.167
Lithium	I	iodide	35	-	-	0.247
Lithium	I	bromide	38	-	-	0.247
Potassium	I	iodide	44	-	6.7	0.232
Potassium	I	sulphate	45	-	-	0.232
Potassium	I	chloride	58	-	7.4	0.232
Sodium	I	sulphate	63	-	-	0.211
Potassium	I	bromide	65	-	-	0.232
Sodium	I	iodide	79	29	5.8	0.211
Sodium	I	bromide	96	34	-	0.211
Sodium	I	chloride	107	74	53	0.211

Set 1: any compound (n = 19), and
Set 2: chlorides only (n = 15)

The chlorides were chosen as a data set in order to investigate whether the omission of other, potentially toxic, anions from the analysis would affect the correlations.

Softness values can only be used on a comparative scale amongst ions of the same valency (Williams *et al.*, 1982). The compounds therefore had to be divided into the metal valency groupings before correlations involving the softness parameter could be made. There were inadequate (i.e. less than ten) numbers of monovalent, trivalent and tetravalent metals in the available set of 19 compounds for valid correlations to be made. The third and fourth data sets used in comparisons were:

Set 3: divalent metal compounds only (n = 13), and
Set 4: divalent metal chlorides only (n = 11)

Data sets 3 and 4 were used for comparisons between the FRAME KB cytotoxicity assay results or softness values and the *in vivo* data, as well as for comparisons between the FRAME KB cytotoxicity assay results and softness values.

4.2.2.2 **Influence of size of data set on correlation coefficients**

The majority of the correlation coefficients did not alter dramatically when the data sets were reduced from 19 to 15 to 13 to 11 compounds (Table 4.6), or from 13 to 11 (Table 4.7), indicating that the basic correlations are sound, even though a relatively small number of data points were involved. However, it is noteworthy that, in the KB ID_{50}/rat oral LD_{50} comparisons (column A,

Table 4.6. Comparison of the FRAME KB cytotoxicity assay ID_{50} values with rat oral and mouse i.p. LD_{50} values, for four groups of metal compounds

DATA SET			COMPARISON A: Log KB ID_{50} to Log Rat oral LD_{50}	COMPARISON B: Log KB ID_{50} to Log Mouse i.p. LD_{50}
	1	All compounds with both rat and mouse data (n* = 19)	r = 0.74 r(Hg) = 0.82	r = 0.89 r(Hg) = 0.90
	2	All chlorides with both rat and mouse data (n = 15)	r = 0.77 r(Hg) = 0.87	r = 0.90 r(Hg) = 0.89
	3	All divalents with both rat and mouse data (n = 13)	r = 0.63 r(Hg) = 0.74	r = 0.86 r(Hg) = 0.90
	4	All divalent chlorides with both rat and mouse data (n = 11)	r = 0.70 r(Hg) = 0.85	r = 0.88 r(Hg) = 0.91

n = number of metal compounds in data groups
r = correlation coefficient obtained from linear regression analysis
r(Hg) = correlation coefficient obtained when analysis was carried out omitting mercury (II) chloride

Table 4.7 Comparison of the softness (σp) values with rat oral and mouse i.p. LD_{50} values and FRAME KB cytotoxicity assay ID_{50} values for two groups of metal compounds

DATA SET			COMPARISON A Log σp to Log Rat oral LD_{50}	COMPARISON B Log σp to Log Mouse i.p. LD_{50}	COMPARISON C Log σp to Log KB ID_{50}
	3	All divalents with both rat and mouse data (n = 13)	r = 0.77 r(Hg) = 0.67	r = 0.80 r(Hg) = 0.70	r = 0.70 r(Hg) = 0.70
	4	All divalent chlorides with both rat and mouse data (n = 11)	r = 0.81 r(Hg) = 0.74	r = 0.76 r(Hg) = 0.65	r = 0.73 r(Hg) = 0.72

n = number of metal compounds in data groups
r = correlation coefficient obtained from linear regression analysis
r(Hg) = correlation coefficient obtained when analysis was carried out omitting mercury (II) chloride

Table 4.6), there is an increasing difference between the correlation coefficient for each complete data set (r) and the correlation coefficient for the same data set without mercury (II) chloride (r(Hg)), as the data set size decreases.

4.2.2.3 **Comparison of *in vivo* and *in vitro* toxicity data for the metal compounds**

For all four data sets, the FRAME KB cytotoxicity assay gave higher correlation coefficients when compared to mouse i.p. LD_{50} data (Table 4.6, column B) than when compared to rat oral LD_{50} data (Table 4.6, column A). The KB ID_{50}/rat oral LD_{50} linear regression lines are skewed toward the horizontal by an outlying point (Figures 4.1(a), 4.2(a), 4.3(a) and 4.4(a)). This point represents mercury (II) chloride, the most toxic compound in the rat oral LD_{50} test among the metal compounds shown in Table 4.5. By omitting the mercury (II) chloride point from the KB ID_{50}/rat oral LD_{50} comparisons, the regression lines become steeper and the correlation coefficients increase. However, the correlation coefficients are still below the values for the corresponding KB ID_{50}/mouse i.p. LD_{50} comparisons. The slopes of the KB ID_{50}/rat oral LD_{50} regression lines without mercury (II) chloride, move closer to the slopes of the lines for the KB ID_{50}/mouse i.p. LD_{50} comparisons (Figures 4.1(b), 4.2(b), 4.3(b) and 4.4(b).

The KB ID_{50}/rat oral LD_{50} correlation coefficients (Table 4.6, column A) are fairly similar for data sets 1, 2 and 4, although for set 3 (the divalents) it is somewhat lower ($r = 0.63$). Among the KB ID_{50}/mouse i.p. LD_{50} comparisons (Table 4.6, column B), the coefficients are also similar, whichever data are used. Mercury (II) chloride is not distant from the regression line on the KB ID_{50}/mouse i.p. LD_{50} plots (Figures 4.1(b), 4.2(b), 4.3(b) and 4.4(b)), hence, when it was omitted from the KB ID_{50} mouse i.p. LD_{50} comparisons, the coefficients remained virtually unchanged.

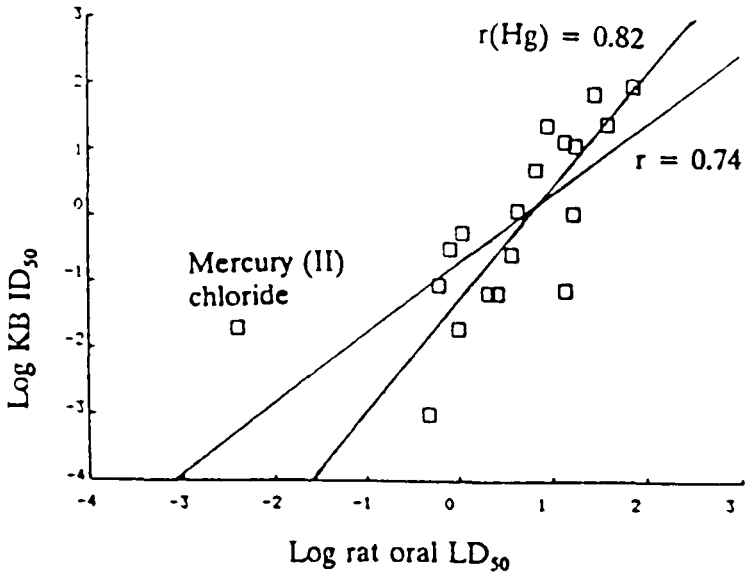

Figure 4.1(a)

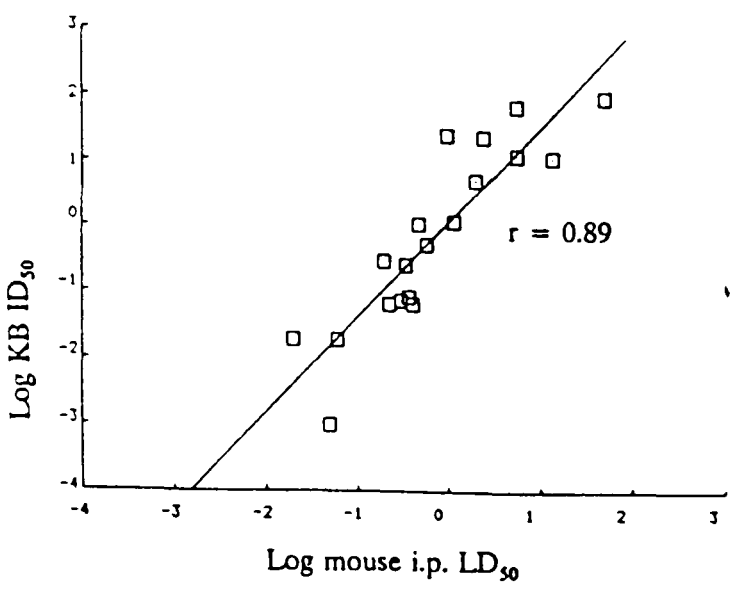

Figure 4.1(b)

Figure 4.1 Linear Regression plots for comparisons of

(a) FRAME KB cytotoxicity assay ID_{50} with rat oral LD_{50}; and
(b) FRAME KB cytotoxicity assay ID_{50} with mouse i.p. LD_{50},

for 19 metal compounds.

r = the correlation coefficient; r(Hg) = the correlation coefficient when mercury (II) chloride is omitted from the comparison

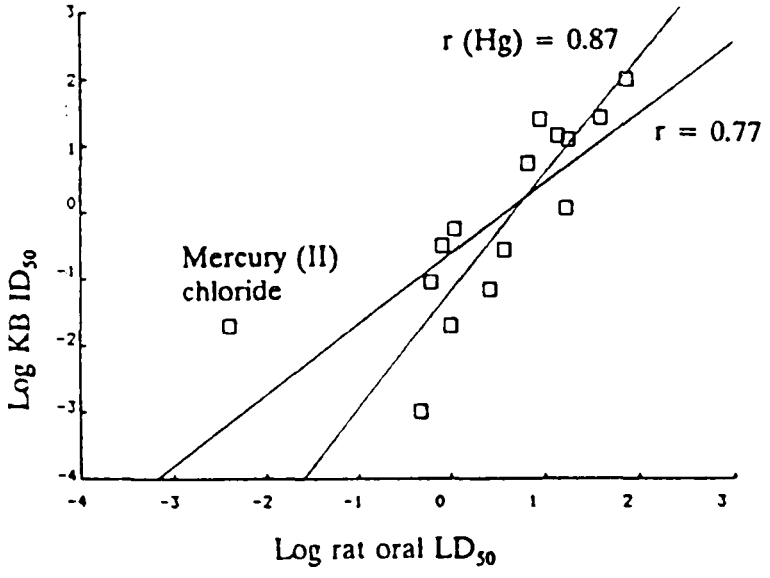

Figure 4.2(a)

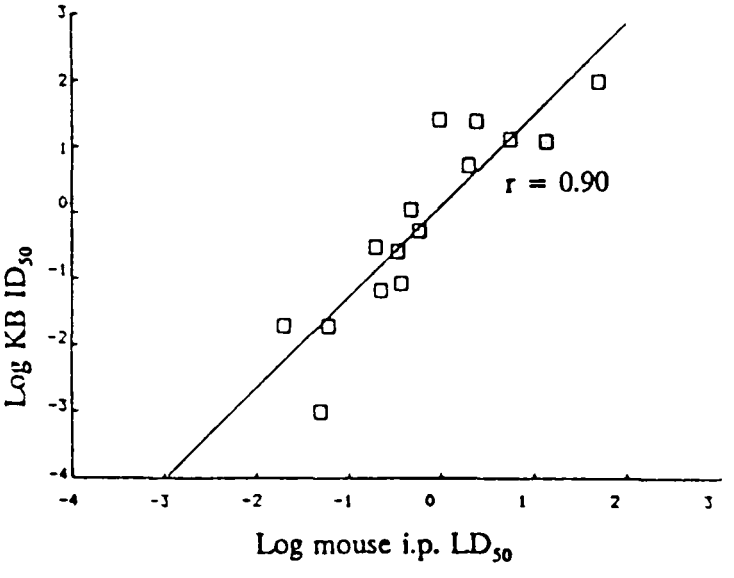

Figure 4.2(b)

Figure 4.2 Linear Regression plots for comparisons of

(a) FRAME KB cytotoxicity assay ID_{50} with rat oral LD_{50}; and
(b) FRAME KB cytotoxicity assay ID_{50} with mouse i.p. LD_{50},

for 15 metal chlorides.

r = the correlation coefficient; r(Hg) = the correlation coefficient when mercury (II) chloride is omitted from the comparison

Figure 4.3 Linear Regression plots for comparison of

(a) FRAME KB cytotoxicity assay ID_{50} with rat oral LD_{50};
(b) FRAME KB cytotoxicity assay ID_{50} with mouse i.p. LD_{50};
(c) Softness parameter (σp) with rat oral LD_{50};
(d) Softness parameter (σp) with mouse i.p. LD_{50}; and
(e) Softness parameter (σp) with FRAME KB cytotoxicity assay ID_{50}

for 13 divalent metal compounds.

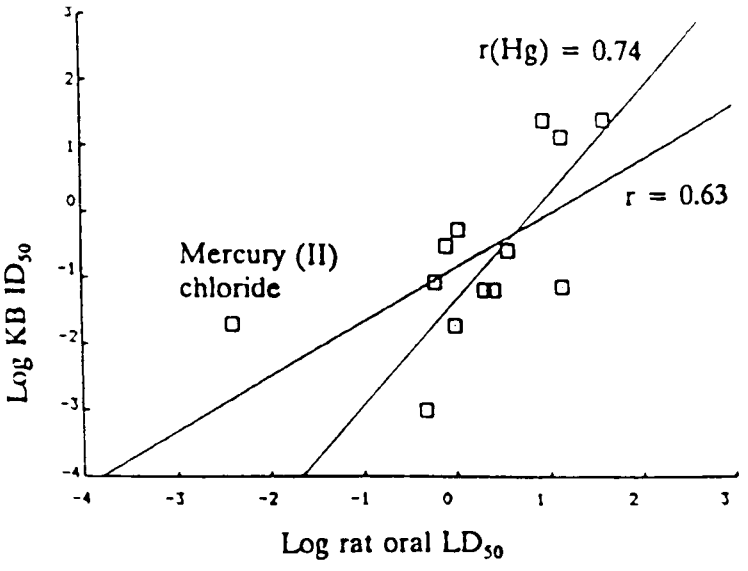

Figure 4.3(a)

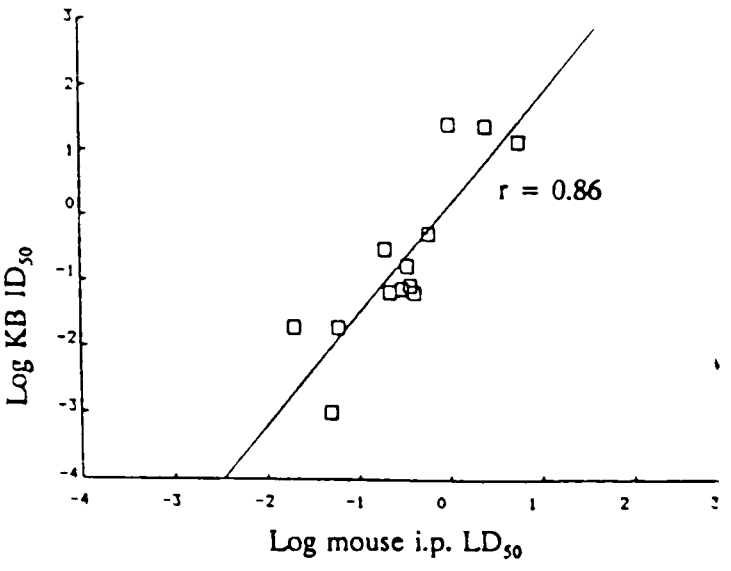

Figure 4.3(b)

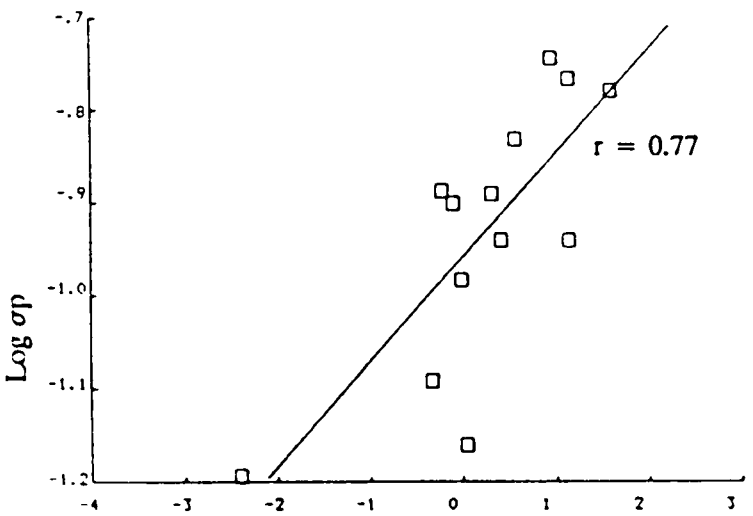

Figure 4.3(c)

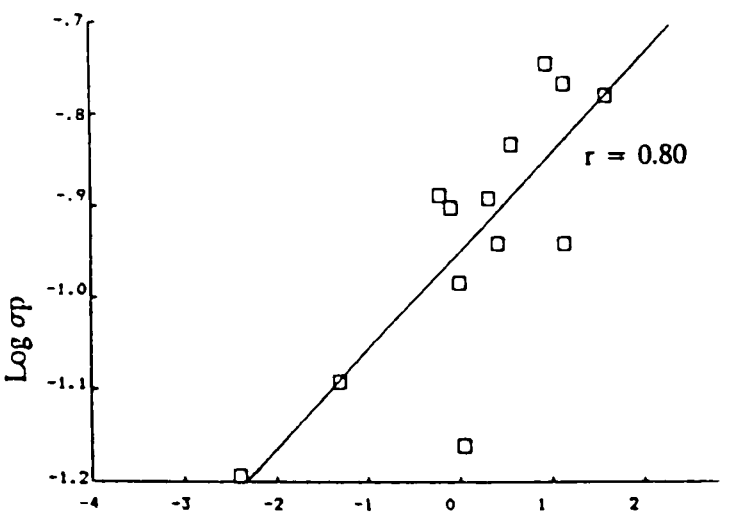

Figure 4.3(d)

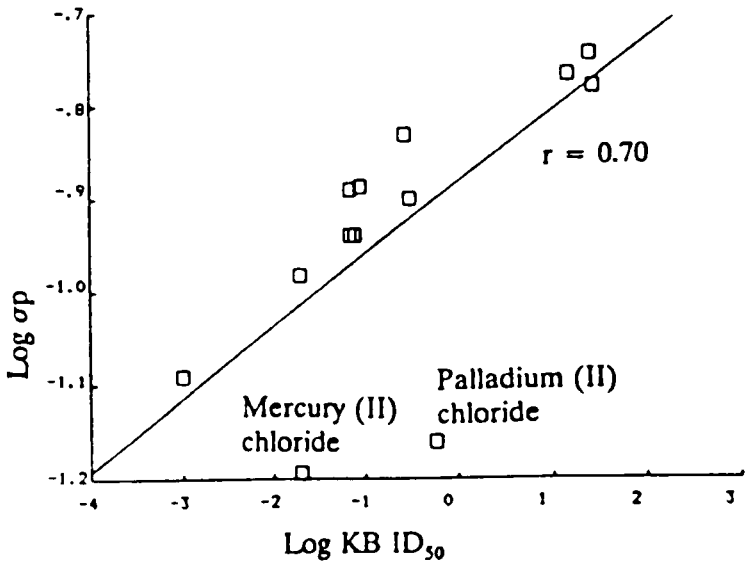

Figure 4.3(e)

r = the correlation coefficient; r(Hg) = the correlation coefficient when mercury (II) chloride is omitted from the comparison

Figure 4.4 Linear Regression plots for comparisons of

(a) FRAME KB cytotoxicity assay ID_{50} with rat oral LD_{50};
(b) FRAME KB cytotoxicity assay ID_{50} with mouse i.p. LD_{50};
(c) Softness parameter (σp) with rat oral LD_{50};
(d) Softness parameter (σp) with mouse i.p. LD_{50}; and
(e) Softness parameter (σp) with FRAME KB cytotoxicity assay ID_{50},

for 11 divalent metal chlorides.

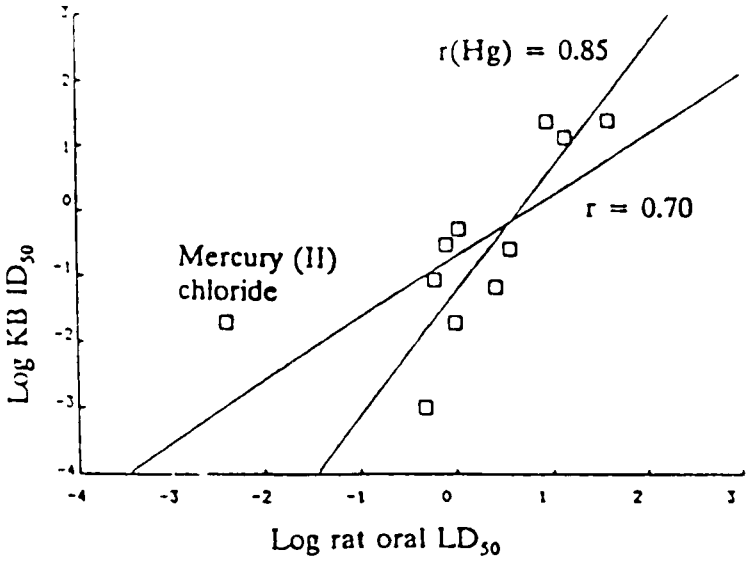

Figure 4.4(a)

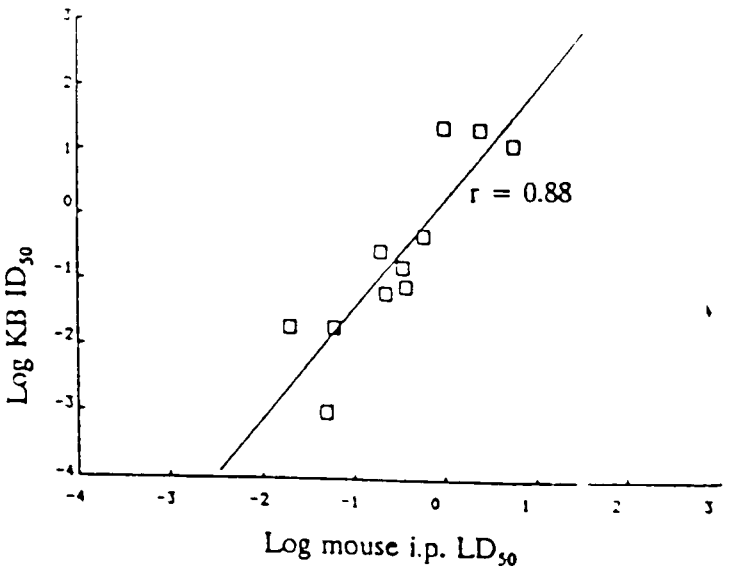

Figure 4.4(b)

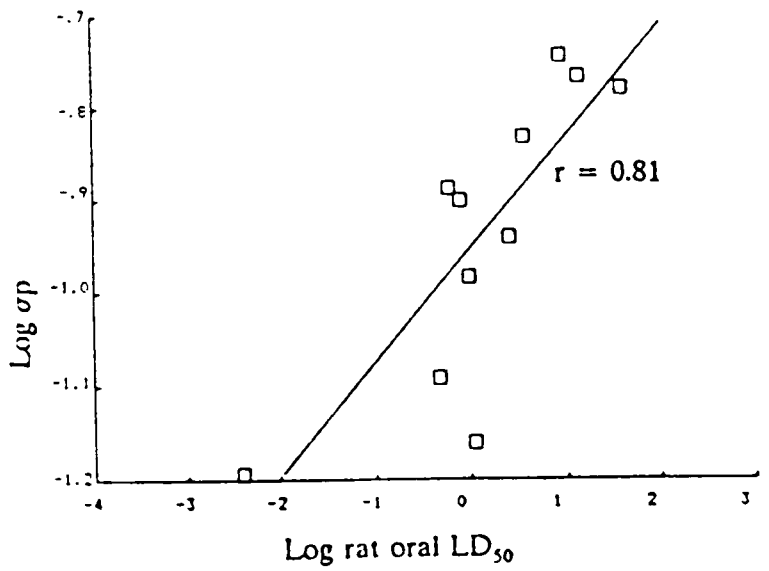

Figure 4.4(c)

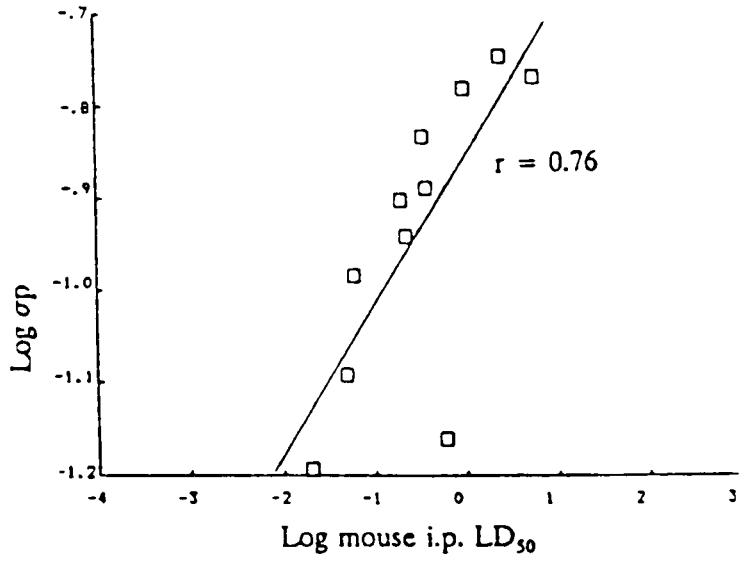

Figure 4.4(d)

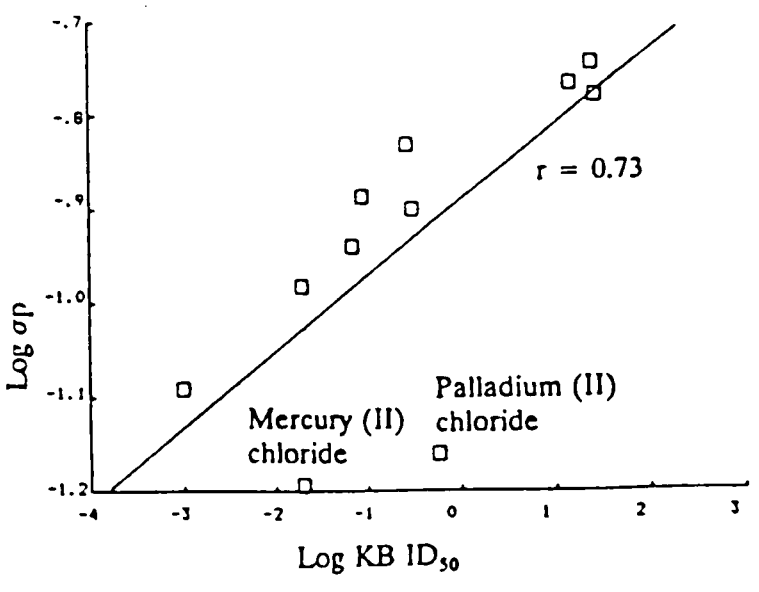

Figure 4.4(e)

r = the correlation coefficient; r(Hg) = the correlation coefficient when mercury (II) chloride is omitted from the comparison

If data set restrictions are removed, then comparison of the FRAME KB cytotoxicity assay with rat oral LD_{50} for all compounds with rat data (n = 27) gives an correlation coefficient of 0.73. Similarly, for all compounds with mouse data (n = 28), the KB ID_{50}/mouse i.p. LD_{50} comparison gives an correlation coefficient of 0.86.

4.2.2.4 **Comparison of the *in vivo* toxicity data with the softness parameter**

The comparisons of softness (σp) with rat oral LD_{50} data (Table 4.7, column A) gave higher correlation coefficients (0.77 and 0.81) than for the corresponding KB ID_{50}/rat oral LD_{50} comparisons (0.63 and 0.70; Table 4.6, column A), for the data sets 3 and 4. However, the comparisons of softness to mouse i.p. LD_{50} data (Table 4.7, column B) gave lower correlation coefficients (0.80 and 0.76) than the corresponding KB ID_{50} mouse i.p. LD_{50} comparisons (0.86 and 0.88; Table 4.6, column B).

The correlation coefficients for the softness/*in vivo* comparisons were not altered dramatically by the move from the divalents data set (set 3) to the divalent chlorides data set (set 4).

Omission of mercury (II) chloride from the softness/*in vivo* comparisons did not improve the correlation coefficients (Table 4.7, columns A and B). By contrast, omission of mercury (II) chloride from the KB ID_{50}/rat oral LD_{50} comparisons did improve them.

4.2.2.5 **Comparison of the *in vitro* toxicity data with the softness parameter**

The comparisons of the FRAME KB cytotoxicity assay results with softness values (Table 4.7, column C) gave among the poorest correlation coefficients in Tables 4.6 and 4.7. Figures 4.3 (e) and 4.4 (e) show that there are two

chemicals (mercury (II) chloride and palladium (II) chloride) which are distant from the regression line on both plots. Omitting these two points from both comparisons causes the correlation coefficients to rise from 0.70 to 0.96 (figure 4.3 (e)) and from 0.73 to 0.97 (figure 4.4 (e)).

4.2.2.6 **Conclusions**

The FRAME KB cytotoxicity assay data gave a better correlation with mouse i.p. LD_{50} values than with rat oral values, with all four data sets. However, in the FRAME KB cytotoxicity assay data/rat oral LD_{50} comparisons, the correlation coefficients were improved by the omission of mercury (II) chloride. The best correlation coefficients in Tables 4.6 and 4.7 were for the FRAME KB cytotoxicity assay/mouse i.p. LD_{50} comparisons.

Softness (σp) values predicted both rat oral and mouse i.p. LD_{50} values equally well. The softness parameter gave a better correlation with rat oral data than did the FRAME KB cytotoxicity assay. Conversely, the FRAME KB cytotoxicity assay gave a better correlation with the mouse i.p. data than did the softness parameter. Omission of mercury (II) chloride from the softness/*in vivo* comparisons worsened the correlations, unlike the KB ID_{50}/rat oral LD_{50} comparisons where the correlations were improved with the omission of mercury (II) chloride.

Comparison of the softness parameter with the FRAME KB cytotoxicity assay data serves to demonstrate that the two alternatives are not providing identical information, despite the fact that they both predict the rat and mouse data reasonably well. The regression plots of the KB ID_{50}/softness comparisons show basically good correlations, except for the outlying points of mercury (II) chloride and palladium (II) chloride. The softness values of these two compounds predict that they would be more toxic than the FRAME KB

cytotoxicity assay indicated. Examination of the softness/*in vivo* plots shows that mercury (II) chloride lies close to the regression lines, i.e. softness predicted mercury (II) chloride toxicity well. However, palladium (II) chloride remains an out-lier in these plots, so softness did not predict its *in vivo* toxicity correctly. The FRAME KB cytotoxicity assay correctly predicted the *in vivo* toxicity of palladium (II) chloride. Williams *et al.* (1982) expressed doubt of the accuracy of the palladium (II) softness value quoted by Ahrland (1968), and it may be "inconsistent" with other divalent metal ion softness values. Palladium (II) is also an out-lier in the softness/LD_{50} correlations of Williams *et al.* (1982).

4.3 DISCUSSION

A wide range of metal compounds was tested using the FRAME KB cytotoxicity assay, including some which are regarded as having low toxicity (for example, sodium chloride), some of low aqueous solubility and some compounds of less common metals, such as tellurium, yttrium and gadolinium. While other *in vitro* assay methods have also been used to assess the rank order of metal toxicity, they have tended to concentrate on small groups of the more toxic metals (Tan *et al.*, 1984; Babich *et al.*, 1986; Borenfreund and Puerner, 1986). It is desirable that, during the validation and evaluation of an *in vitro* toxicity test, applied to a chosen group of chemicals, a wide range of toxicities should be spanned. This ensures that, when further chemicals from this group are to be evaluated, there is a sufficiently large database with which to compare the new result (Clothier *et al.*, 1988; Balls *et al.*, 1990a). The results for the group of 52 metal compounds tested in the FRAME KB cytotoxicity assay spanned six orders of magnitude and hence provide a satisfactory spread, and a sufficient number of chemicals for such a database (Balls *et al.*, 1990a).

The expression of the ID_{50} values of the compounds in mM units raised questions concerning which component of the least toxic compounds was causing toxicity. The ID_{50} of potassium sulphate (K_2SO_4) is not half that of potassium chloride (KCl), as would be expected if the K^+ ion was alone responsible for the toxicities of these potassium compounds. It is apparent that the sulphate and chloride ions have different toxic effects.

The rank order of the cytotoxicities of the metal compounds generally reflected the known human toxicities of the compounds. For example, cadmium (II) chloride, mercury (II) chloride and thallium (I) sulphate are known to be severe poisons, while sodium (I) chloride, potassium (I) chloride and calcium (II) chloride are not normally considered poisonous to humans (Friberg *et al.*, 1986).

Although selecting metal chlorides (thereby avoiding "toxic" anions such as fluoride) allows direct comparison of the toxicities of the metal ions, this is only true for the more toxic metals (i.e. those with an ID_{50} of less than approximately 20mM). With chlorides of the least toxic metals (such as sodium and potassium), the possibility of anion effects contributing to the toxicity of a compound must also be considered, because of the high concentrations involved. Further examination of anion effects could be undertaken by the testing of additional anions (such as nitrate, phosphate or carbonate) with the alkali metals, and also a range of anions with divalent and trivalent metal ions of relatively low toxicity (such as chromium (III), strontium (II), and magnesium (II)). When testing metal compounds *in vitro*, in order to evaluate toxicity of the metal ions and not the whole compounds, the predominant anion in the medium (usually chloride) must be considered. However, some metal chlorides are insoluble or highly reactive, hence an alternative anion of low toxicity must be chosen.

The overall conclusion must be that the nature of the anion does not affect the toxicities of the majority of the metal compounds tested. The exceptions are the compounds involving intrinsically toxic anions, such as fluoride or tetrathionate, and when metal compounds of very low toxicity are tested. In the latter situation, the anion may contribute to the toxicity of the compounds by (along with the cation) being at a high concentration in the medium. This high concentration may affect the ionic strength and/or osmolality of the medium to such an extent that the tolerance limits of the cells to high ionic strength could be exceeded (Freshney, 1987) and result in inhibition of growth. The concentration of metal salts already present in the culture medium should also be taken into account (e.g. 3T3-L1 medium contains over 100mM sodium ions). However, as with *in vivo* toxicity tests, the FRAME KB cytotoxicity assay measures the effects of the *added* compound, and controls take into account the presence of some test compounds within the test system.

Of the two alternatives assessed, i.e. the FRAME KB cytotoxicity assay and the softness parameter, the cytotoxicity assay was found to be the more useful predictor of rat oral and mouse i.p. LD_{50} values, for the metal compounds examined, when linear regression analysis was used and other factors were considered.

The FRAME KB cytotoxicity assay data gave a better correlation with mouse i.p. LD_{50} values than with rat oral LD_{50} values. Absorption of metal compounds from the gut following oral administration is known to vary widely according to the metal and anion concerned, the solubility of the compounds and the state of the subject animal (Camner *et al.*, 1986; Nordberg *et al.*, 1986). Continuous exposure of cells in culture to a compound, as in the FRAME KB cytotoxicity assay, possibly mimics parenteral dosage better, where there are fewer absorption barriers to tissue exposure (Fry *et al.*, 1988a; Clothier *et al.*, 1989; Fry *et al.*, 1990b). Other workers have also found good

correlations between metal toxicity *in vitro* and *in vivo*. For example, the toxicity of sixteen compounds to Chinese hamster ovary cells correlated well with both mouse and *Drosophila* acute toxicity parameters (Tan *et al.*, 1984), and the NRU_{50} values for six compounds tested on bluegill (fish) cells compared favourably with the LC_{50} values from whole bluegill sunfish (Babich *et al.*, 1986).

The alterations in the correlation coefficients which occurred when only one compound (mercury (II) chloride) was omitted from KB ID_{50}/rat oral LD_{50} and softness/rat or mouse comparisons, could reduce the confidence placed in the accuracy and strength of the coefficient values. Further information can be gained from the regression plots. It is important to take into account the scatter of data points on either side of the regression line and at its furthermost lefthand and righthand edges. Outlying points can skew a regression line and make a reasonable correlation look worse by reducing the correlation coefficient (as demonstrated with mercury (II) chloride and the KB ID_{50}/rat oral LD_{50} comparisons). However, single data points within a small data set, at the lefthand or righthand edge of a regression line, can disproportionately influence the positioning of the regression line. This can make a mediocre correlation look reasonable (as demonstrated with softness/*in vivo* comparisons).

Mercury (II) chloride was an out-lier in the KB ID_{50}/rat oral LD_{50} comparisons, because it appeared to be more toxic by rat oral LD_{50} criterion than would be predicted by the FRAME KB cytotoxicity assay. A possible explanation for this is the fact that mercury (II) chloride is corrosive to the gastro-intestinal tract, which results in enhanced uptake of the Hg^{2+} ions into the circulation (Berlin, 1986). This compound is equally toxic by oral (LD_{50} 0.004 mmol/kg) and intravenous (LD_{50} 0.01 mmol/kg) administration to rats (Lewis and Tatken, 1982).

Because it refers to the metal ion only, the softness parameter is incapable of predicting the toxicities of compounds consisting of "toxic" anions and "non-toxic" cations, such as sodium tetrathionate and potassium fluoride. In addition, softness/*in vivo* comparisons in this study could only be made among divalent metal compounds, which excluded a high proportion of the compounds tested. Further limitations of the softness alternative are the variability and availability of published softness values for metals in all their valency states, and the suggestion that the alkaline earth metals do not fit into the softness scale (Williams *et al.*, 1982). However, existing softness values can be readily obtained from the literature, and, when put into the correct context, may offer additional or supportive information to that supplied by an *in vitro* cytotoxicity assay. Nevertheless, there is no indication that the softness parameter is of any real value on its own as an alternative means of predicting metal compound toxicity. There are, however, many other physico-chemical parameters of metals that may better predict (either alone or in combination) *in vivo* metal toxicity (Kaiser, 1985). Simple, inorganic metal compounds are closely related structurally, and the majority exert their toxicity by a common, basic mechanism, i.e. binding of the metal cation to electron-donating ligands at the target site. Hence, they are a suitable group of compounds for developing QSARs. QSARs are not as applicable with less closely-related groups, but may be useful tools in the initial phase of elucidating a new chemical's toxicological profile (Balls and Horner, 1985; Enslein, 1988).

The FRAME KB cytotoxicity assay has demonstrated considerable potential for the prediction of the rodent acute toxicities of simple metal compounds. It is not possible to conclude that the assay could also predict human toxicity, because of the lack of suitable human data with which to perform comparisons. However, the assay did, in general terms, find the rank order toxicity corresponded with the acknowledged toxicity of the compounds to humans.

Most of the 52 metal compounds tested were readily soluble in aqueous medium, and hence were not problematical to test in the FRAME KB cytotoxicity assay. Comparisons with *in vivo* toxicity parameters were also relatively straightforward, because of the availability of a reasonable quantity of LD_{50} data for the compounds. In an industrial situation, however, the test compounds may not be readily soluble, and may come from a set of compounds for which little or no toxicity data is available. It is therefore appropriate that the FRAME KB cytotoxicity assay should be assessed for its usefulness in testing more complex, commercial compounds and formulations.

CHAPTER 5 AN ASSESSMENT OF THE APPLICABILITY OF THE FRAME KB CYTOTOXICITY ASSAY FOR TESTING COMPLEX COMMERCIAL CHEMICALS AND FORMULATIONS

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5.2.1.7 Consideration of *in vivo* and *in vitro* data for the structurally related groups of chemicals

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5.3 Discussion

5.1 INTRODUCTION

As one of the industrial sponsors of the FRAME Research Programme, Rhone-Poulenc Ltd. (formerly May and Baker) had provided several pure chemicals which were tested in the first interlaboratory trial of the FRAME KB cytotoxicity test (Knox *et al.*, 1986). These chemicals (Butylated hydroxyanisole, Chloroquine sulphate and Ioxynil) were also tested on 3T3-L1 cells and the results were published as part of a set of 150 unrelated industrial and laboratory chemicals and human pharmaceuticals (Chapter 3) (Clothier *et al.*, 1988).

As part of an ongoing project to assess the applicability of the FRAME KB cytotoxicity assay in the prediction of acute lethal toxicity of industrial chemicals, Rhone-Poulenc Ltd. provided a further twenty-eight pure chemicals and twenty liquid formulations for testing. The samples were tested in the standard FRAME KB cytotoxicity assay protocol and the results are presented in two sections: pure chemicals and formulations.

5.2 RESULTS

5.2.1 Pure Chemicals

5.2.1.1 **Description of the chemicals**

The pure chemicals (Table 5.1) encompass fungicides, herbicides, an insecticide, anti-microbial agents, human pharmaceuticals, and chemicals in development (use unknown). Some are in commercial use, while some in development were found unsuitable for commercial use, or are metabolites of drugs. The chemicals also encompass a wide range of structural formulae, and cannot, therefore, be considered to be related to one another as a complete

Table 5.1. *In vitro* toxicities of twenty eight Rhone-Poulenc Ltd. pure chemicals

Rank Order No.	TOX No.	Chemical	ID_{20} μg/ml ± s.e.m.	ID_{50} μg/ml ± s.e.m.	ID_{80} μg/ml ± s.e.m.
1	*22*	coccidiostat	0.02 ± 0	0.03 ± 0	0.04 ± 0
2	*40*	Chlorothalonil (fungicide)	<1	2 ± 0	4 ± 0
3	*30*	Dichlorophen (biocide)	7 ± 0	13 ± 1	22 ± 1
4	*25**	Propranolol (cardiovascular drug)	7 (n=2)	22 ± 2	33 ± 5
6=	*43*	Neburon (herbicide)	16 ± 6	35 ± 9	ND
6=	*33*	DXH 1421 (chemical series A)	10 ± 2	35 ± 15	99 ± 22
7	*42*	MCPA acid (herbicide)	10 ± 2	36 ± 3	81 ± 7
8	*29*	anti-protozoal	14 ± 2	40 ± 7	71 (n=1)
9	*31*	JJW 2027 (chemical series A)	20 ± 9	43 ± 18	76 ± 25
10	*24*	herbicide	22 ± 3	52 ± 9	133 ± 19
11	*32*	JJW 2092/1 (chemical series A)	8 ± 1	81 ± 54	49 (n=1)
12	*39*	Cyanazine (herbicide)	27 ± 3	95 ± 7	>100
13	*46*	Trifluralin (herbicide)	143 ± 19	210 ± 19	383 ± 90
14	*44*	Simazine (herbicide)	24 ± 10	232 ± 70	ND
15	*41*	Dimethoate (insecticide)	175 ± 48	317 ± 44	628 ± 92
16	*45*	Mecoprop (herbicide)	169 ± 56	361 ± 31	623 ± 78
17	*35*	RRB 2306 (chemical series B)	266 ± 33	508 ± 77	877 ± 208
18	*38*	RRB 2347A (chemical series B)	355 ± 107	515 ± 83	817 ± 11
19	*26**	metabolite of a cardiovascular drug	302 (n=2)	544 ± 9	668 ± 11
20	*20**	8-Chlorotheophylline (lot B, 1% dimer)	234 ± 46	682 ± 67	1495 ± 262
21	*21**	8-Chlorotheophylline (lot A, 0.05% dimer)	209 ± 56	750 ± 76	1656 ± 197

22	*34*	RRB 2316 (chemical series B)	540 ± 186	844 ± 199	>1000
23	*27**	metabolite of a cardiovascular drug	557 (n=2)	990 ± 31	1171 ± 24
24	*23**	Asulam (herbicide)	1986 (n=2)	3560 ± 149	4794 ± 60
25	*16*	Thalidomide	>500†		
27=	*36*	RRB 2309/1 (chemical series B)	>1000†		
27=	*37*	BDH 202/1 (chemical series B)	>1000†		
28	*28*	intermediate of TOX 29	insoluble		

* = Were dissolved directly into culture medium for FRAME KB cytotoxicity assay. Remainder were dissolved initially in DMSO (DMSO at 1% (v/v) final concentration in test chemical treatment solution).

† = Limit of solubility in culture medium

ND = Not determined

group. Within the group, there are five sets of related chemicals. These are:

(a) TOX 20 and TOX 21 - two batches of 8-Chlorotheophylline (a human anti-emetic drug) with different levels of a dimer;

(b) TOX 42 and TOX 45 are [2-((4-chloro-o-tolyl)oxy)] groups with an acetic and a propionic side chain, respectively (Figure 5.1);

(c) TOX 39 and TOX 44, which are structurally identical except for a C=N (nitrile) side group in TOX 39 (Figure 5.1).

(d) TOX 31, 32 and 33 are from the same chemical series (named series A); and

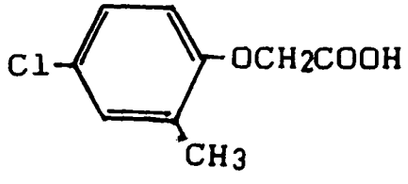
(e) TOX 34, 35, 36, 37 and 38 are from the same chemical series (named series B).

5.2.1.2 *In vitro* toxicities of pure chemicals

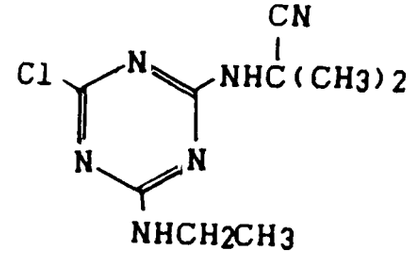
The 28 pure chemicals gave a wide range of cytotoxicities in the FRAME KB cytotoxicity assay, with ID_{50} values from 0.03 to over 3000 μg/ml (Table 5.1). The cytotoxicity results were not converted to millimolar concentrations, because of the need to be able to make comparisons with the results for the formulations. Molecular weights, for those chemicals for which the data were available, were all in the region of 250. Conversion of the results to millimolar concentrations would therefore not greatly alter the toxicity ranking (Table 5.1).

Definitive ID_{50} values were not obtained for four of the chemicals. This was due to partial or complete insolubility of the substances in culture medium or in any of the organic solvents recommended by FRAME (ethanol, methanol, acetone, DMSO). Twenty-one chemicals were not readily soluble in aqueous medium, and so were dissolved in DMSO at 100mg/ml, and then the DMSO was diluted to 1% (v/v) in the medium. Many of the chemicals from TOX 31

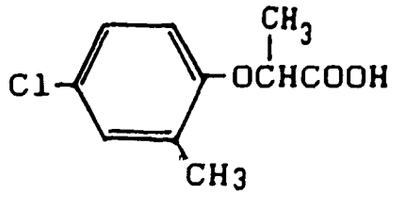
TOX 42 MCPA
(2-((4-chloro-o-tolyl)oxy)acetic acid)



TOX 39 Cyanazine
(2-((4-chloro-6-(ethylamino)-s-triazin-2-ylamina)-2-methyl)propionitrile)



TOX 45 Mecoprop
(2-((4-chloro-o-tolyl)oxy)propionic acid)



TOX 44 Simazine
(2-chloro-4, 6-bis (ethylamino)-s-triazine

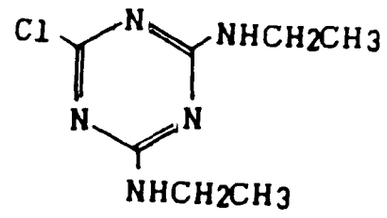


Figure 5.1 Chemical structures for 4 Rhone-Poulenc Ltd. pure chemicals

to 46 precipitated out when diluted to 1mg/ml in the medium. This stock solution in medium was used to prepare the dose range-finding solutions. No attempt was made to prepare solutions from a stock in which the highest concentration of test substance was fully soluble, i.e. which showed no precipitate.

5.2.1.3 *In vitro* toxicities of the related chemical groups

(a) The two batches of 8-Chlorotheophylline, with different levels of a by-product dimer, gave virtually identical ID_{50} results in the KB assay (Table 5.1).

(b) The ID_{50} for MCPA acid was ten times lower than that for Mecoprop (Table 5.1).

(c) The ID_{50} for Simazine was twice that for Cyanazine (Table 5.1).

(d) In chemical series A, TOX 31 to 33 all gave ID_{50} values close to 50μg/ml (Table 5.1).

(e) In chemical series B, TOX 34, 35 and 38 gave ID_{50} values between approximately 500 and 1000 μg/ml (Table 5.1). No results were obtained for TOX 36 and 37, due to lack of detectable toxic effects at the highest dose tested (1000 μg/ml).

5.2.1.4 Toxicity rank according to chemical use

There is no clear separation within the toxicity ranking according to chemical use. Herbicides, such as Neburon and Asulam, and human drugs, such as Propranolol and Thalidomide, were among the most and the least toxic substances in Table 5.1. However, the three most toxic chemicals (TOX 22, 30 and 40) were those designed to kill or inhibit the growth of bacteria and/or fungi.

5.2.1.5 **Comparison of new data with published data**

One chemical (TOX 22, Propranolol) was included to test whether the FRAME KB cytotoxicity assay could reproduce the published value (Clothier *et al.*, 1988). The ID_{50} value obtained (22±2 μg/ml) agreed well with the published result (12±0 μg/ml), when one considers that the full range of cytotoxic concentrations measured with the FRAME KB cytotoxicity assay is 0.01 μg/ml to 140,000 μg/ml (Clothier *et al.*, 1988).

5.2.1.6 **Comparison of *in vivo* and *in vitro* toxicity data for the pure chemicals**

The FRAME KB cytotoxicity assay ID_{50} results were compared with rat oral LD_{50} data provided by Rhone-Poulenc Ltd. (Table 5.2) and with parenteral LD_{50} data, where available. Rat oral LD_{50} values are used for classification of pesticides according to European Community requirements (Brian Ingham, personal communication). When the *in vivo* LD_{50} data were given as an approximate or "greater than" value in comparisons, these LD_{50} values were taken as being equal to the figures given. There is very little obvious similarity between the toxicity ranking by the FRAME KB cytotoxicity assay or by the rat oral LD_{50} (Table 5.2). Indeed, there are some striking discrepancies. For example, chlorothalonil is highly cytotoxic to 3T3-L1 cells, but is non-toxic to the rats by oral administration.

As the FRAME KB cytotoxicity assay results showed a better correlation with mouse i.p. LD_{50} data than with rat oral LD_{50} data for the metal compounds (Chapter 4), mouse i.p. data was sought from the Registry of Toxic Effects of Chemical Substances (RTECS) (Lewis and Tatken, 1982). Values were available for only three of the named chemicals, hence rat i.p., rat i.v. and mouse i.v. LD_{50} data was also sought (Table 5.2). Chlorothalonil is, in fact, highly toxic to mice by i.p. administration. Dichlorophen, Neburon and MCPA

Table 5.2. *In vitro* and *in vivo* toxicities of twenty eight Rhone-Poulenc Ltd. pure chemicals

TOX No.	Chemical name (if known)	KB ID_{50} (μg/ml)	Rat oral LD_{50} (mg/kg)*	Other LD_{50} (mg/kg)⊛
22	-	0.03	120	-
40	Chlorothalonil	2	>10000	2.5 (mo⊙ i.p.)
30	Dichlorophen	13	1506	17 (rat i.v.)
25	Propranolol	22	HCl 515	42 (mo i.p.); 23 (mo i.v.); 27 (rat i.v.)
43	Neburon	35	>10000	180 (mo i.v.)
33	DXH 1421 (chemical series A)	35	218	-
42	MCPA acid	36	700	28 (mo i.p.); 7 (rat i.v.)
29	-	40	>2000	-
31	JJW 2027 (chemical series A)	43	580	-
24	-	52	>2000	-
32	JJW 2092/1 (chemical series A)	81	97	-
39	Cyanazine	95	182-380	-
46	Trifluralin	210	>10000	>1500 (mo i.p.)
44	Simazine	232	>5000	100 (mo i.v.)
41	Dimethoate	317	500-600	45 (mo i.p.); 100 (rat i.p.); 250 (rat i.v.)
45	Mecoprop	361	930	402 (rat i.p.)
35	RRB 2306 (chemical series B)	508	413	-
38	RRB 2347A (chemical series B)	515	>2000	-
26	-	544	-	-
20	8-Chlorotheophylline (lot B)	682	~500	149 (mo i.p.); 200 (mo i.v.)‡
21	8-Chlorotheophylline (lot A)	750	~500	149 (mo i.p.); 200 (mo i.v.)‡

34	RRB 2316 (series B)	844	948	-
27	-	990	-	-
23	Asulam	3560	>5000	-
16	Thalidomide	>500†	113	-
36	RRB 2309/1 (series B)	>1000†	>5000	-
37	BDH 202/1 (series B)	>1000†	>5000	-
28	-	insoluble	>5000	

* = Data supplied by Rhone-Poulenc Ltd.
⊗ = Values obtained from RTECS (Lewis and Tatken, 1982).
⊙ = mouse.
† = Limit of solubility in culture medium.
‡ = Data is for the pure chemical.

acid are also more toxic to rats or mice by i.p. or i.v. administration than orally, and these chemicals are also among the most toxic to the 3T3-L1 cells (ranked number 2, 3, 6= and 7 respectively) (Table 5.1). Simazine (ranked 14) was less harmful to 3T3-L1 cells, non-toxic by rat oral LD_{50} (>5000mg/kg), but was 50 times more toxic by mouse i.v. LD_{50} (100 mg/kg). Dimethoate, Mecoprop and 8-Chlorotheophylline, which have similar cytotoxicities to Simazine and to each other in the FRAME KB cytotoxicity assay, have, unlike Simazine, similar *in vivo* toxicities by either the oral, i.p. or i.v. routes. Linear regression analysis of log KB ID_{50} (μg/ml) compared to log rat oral LD_{50} (mg/kg) for the chemicals in Table 5.1 excluding TOX 36, 37 and 28 (i.e., chemicals for which no *in vivo* or precise *in vitro* data was available), gave a correlation coefficient of 0.19, i.e. no correlation could be inferred.

5.2.1.7 **Consideration of *in vivo* and *in vitro* data for the structurally related groups of chemicals**

The *in vitro* and *in vivo* data for the five groups of structurally-related chemicals were examined. Both the FRAME KB cytotoxicity assay and the rat oral LD_{50} assay found the 8-Chlorotheophylline with different levels of dimer (TOX 20 and 21) gave essentially the same toxicities. Both assays found MCPA acid to be more toxic than Mecoprop, and Simazine to be less toxic than Cyanazine. However, there were no clear patterns with the chemicals in series A or B (Table 5.1). Between the two series, the FRAME KB cytotoxicity assay ranked the chemicals in series A more toxic than those in series B, and this was also true for the rat oral LD_{50} (Table 5.2). There was no obvious correspondence between the toxicity ranking by the FRAME KB cytotoxicity assay and by rat oral LD_{50} within series A. However, within series B, it appears that, with the exception of RRB 2347/A, the lower the rat oral LD_{50}, the lower the KB ID_{50}.

5.2.1.8 **Consideration of chemical solubility**

The aqueous solubilities of the ten named agrochemicals with *in vivo* data (Table 5.2) were compared with their *in vitro* and *in vivo* toxicities (Table 5.3). When the chemical are ranked in order of increasing solubility, it is apparent that the least soluble are also the least toxic to rats orally. The more soluble compounds mostly gave definitive LD_{50} results. There is, however, no correspondence between the aqueous solubility limits and the FRAME KB cytotoxicity assay ID_{50} results.

5.2.1.9 **Conclusions**

About half of the pure chemicals had an ID_{50} below 500 μg/ml in the FRAME KB cytotoxicity assay, hence, when compared with the spread of cytotoxicities of the 150 chemicals (Chapter 3), these would be considered to be moderately harmful. One chemical, Asulam, with an ID_{50} of 3560 μg/ml, would be considered to be of low toxicity. The three bactericidal/fungicidal chemicals were highly toxic, while the herbicides demonstrated a whole range of cytotoxicity from 35 μg/ml to 3560 μg/ml. Only two chemicals gave an ID_{50} below 10 μg/ml, with the coccidiostat by far the most toxic. Explanation of its high cytotoxicity is not possible, because of lack of information on its chemical nature.

It can be concluded that there is no direct correlation between rat oral LD_{50} data and the FRAME KB cytotoxicity assay ID_{50} results for the 25 chemicals compared. In most cases, the FRAME KB cytotoxicity assay predicted the toxicity to be higher than that obtained in the rat by the oral route.

For the ten agrochemicals listed in Table 5.3, there is a reasonably close rank order correlation between their rat oral toxicities and their aqueous solubilities.

Table 5.3 *In vivo* and *in vitro* toxicities of ten Rhone-Poulenc Ltd. agrochemicals and their aqueous solubility data

Chemical	KB ID_{50} (μg/ml)	Rat oral LD_{50} (mg/kg)	Solubility limit in water (μg/ml)*
Chlorothalonil	2	>10000	0.6
Trifluralin	210	>10000	<1
Neburon	35	>10000	4.8
Simazine	232	>5000	5
Dichlorophen	13	1506	30
Cyanazine	95	182-380	171
Mecoprop	361	930	620
MCPA acid	36	700	825
Asulam	3560	>5000	5000
Dimethoate	317	500-600	25000

*Data obtained from the Pesticide Manual (Martin and Worthing, 1974).

The four poorly soluble chemicals (i.e. 5 μg/ml solubility limit or less) gave undefinably high rat oral LD_{50} values, but a variety of ID_{50} values. Some of the group TOX 31-46 were toxic in the FRAME KB cytotoxicity assay at concentrations above their quoted aqueous solubility limits (Table 5.3). However, it cannot be concluded that the chemicals were not in solution at these levels. This is because of the presence of 1% (v/v) DMSO as well as the ingredients of the culture medium, which may have enhanced their solubilities. The FRAME KB cytotoxicity assay, as with the rat oral LD_{50}, was unable to detect the higher level of impurity in lot B of 8-Chlorotheophylline. Among the other related groups there is some indication that the FRAME KB cytotoxicity assay could predict the toxicity rank of the closely related pairs. However, it is not known how closely related the chemicals in series A and B were, and for these the FRAME KB cytotoxicity assay was less able to rank them correctly.

5.2.2 Formulations

5.2.2.1 **Description of the formulations**

Thirteen of the formulations were thick, opaque suspensions or emulsions, of various colours. The remaining seven were clear and pale brown or yellow in colour.

Seven of the pure chemicals (Table 5.1) were present as active ingredients in thirteen of the formulations. These formulations were for fungicidal, herbicidal or insecticidal use. Three formulations contained active ingredients which were not tested as pure chemicals (eglumine salt of Nitroxynil and Dimefuron). Four formulations (TOX 02, 03, 05 and 15) contained unknown active ingredients and one contained none. Information on the concentrations of the active ingredients in the majority of the formulations was not supplied,

as it is proprietary. However, it is known that some actives may be present at up to approximately 50% (w/v).

5.2.2.2 *In vitro* toxicities of the formulations

The results for the 20 formulations in the FRAME KB cytotoxicity assay (Table 5.4) spanned a narrower range of ID_{50} doses than the results for the pure chemicals (6 to 609 μg/ml).

As with the pure chemicals, solubility problems were encountered with some of the formulations. The opaque suspensions gave cloudy solutions upon dilution into culture medium. These often resulted in particles (probably of the active chemical) settling out at the bottom of culture wells during the 72-hour exposure period. In all cases, except TOX 13, the deposit of particles was present in wells at doses under or close to the ID_{50} doses. TOX 13 gave a higher toxicity than the other suspensions, and showed no particles in the well at the toxic dose level.

TOX 01 to 15 were tested as one project. TOX 17 to 19, and the two Trodax samples, underwent separate investigations.

Formulations TOX 01 - 15

The ID_{50} values for most of TOX 01 to 15 lie between about 100 and 600μg/ml (Table 5.4). The exception was TOX 13 (ID_{50} 6 μg/ml). Because of its high toxicity, TOX 13 was investigated further. The active ingredient, Chlorothalonil, was at the time untested as a pure chemical, but was known to be non-toxic when given to rats orally. Therefore, the preservative in the formulation, Dichlorophen, was suspected as having caused the high toxicity of TOX 13. Pure Dichlorophen (Table 5.1) was found to be less toxic than TOX 13 itself in the FRAME KB cytotoxicity assay. Chlorothalonil was then

Table 5.4. *In vitro* toxicities of twenty Rhone-Poulenc formulations

TOX No.	Active ingredient	ID_{20} μg/ml ± s.e.m.	ID_{50} μg/ml ± s.e.m.	ID_{80} μg/ml ± s.e.m.
13	Chlorothalonil	3 ± 1	6 ± 1	6 (n=2)
18	none	54 ± 2	81 ± 3	118 ± 15
17	Dimethoate	71 ± 5	96 ± 7	146 ± 14
14	Trifluralin	79 ± 16	111 ± 13	163 ± 14
04	Trifluralin	87 ± 11	128 ± 5	185 (n=2)
01	Neburon	12 (n=1)	136 ± 15	489 ± 16
-	Nitroxynil, eglumine salt 37% (w/v) (field sample of Trodax)	85 (n=1)	157 ± 20	242 ± 15
11	MCPA acid	91 ± 21	161 ± 10	239 (n=2)
05	not known	103 ± 9	170 ± 13	238 (n=2)
12	MCPA acid	98 (n=2)	173 ± 12	393 (n=2)
-	Nitroxynil, eglumine salt 37% (w/v) (lab sample of Trodax)	124 (n=1)	183 ± 37	316 ± 49
09	Cyanazine	75 ± 42	188 ± 37	176 (n=2)
19	Dimethoate	54 (n=2)	223 ± 36	1168 (n=2)
06	Mecoprop	139 ± 10	312 ± 35	582 ± 91
15	not known	70 (n=1)	325 ± 30	552 (n=2)
08	Mecoprop	192 ± 71	353 ± 46	565 (n=2)
02	not known	144 (n=2)	400 ± 26	678 ± 33
03	Dimefuron (40% (w/v))	<250	570 ± 67	1800 (n=1)
10	Cyanazine	220 (n=2)	579 ± 92	1040 ± 178
07	Mecoprop	299 ± 73	609 ± 71	636 (n=2)

tested and found to be the most likely source of the toxicity to the 3T3-L1 cells (Table 5.1).

Formulations TOX 17 - 19

These three liquids were tested in order to investigate the toxicity of the vehicles used to dissolve or suspend the active ingredients. TOX 17 and 18 gave very similar ID_{50} values, while TOX 19 was slightly less toxic (Table 5.4). It was revealed that TOX 18 was the vehicle for the formulation TOX 17, and TOX 19 contained the same active ingredient as TOX 17, but in a different vehicle.

Trodax Formulations

The two Trodax samples were tested because one (the "field" sample) had been returned to Rhone-Poulenc Ltd., with complaints about adverse effects to sheep (Trodax is an antihelmitic veterinary drug). The laboratory sample was from the same batch as the "field" sample. The two samples were equally toxic to cells in the FRAME KB cytotoxicity assay (Table 5.4). It is not known whether Rhone-Poulenc Ltd. were able to detect any difference between the samples.

5.2.2.3 **Comparison of *in vivo* and *in vitro* toxicity data for the formulations**

The FRAME KB cytotoxicity assay ID_{50} results for the first 15 formulations are shown in Table 5.5 with rat oral LD_{50}, rat dermal LD_{50} and skin and eye irritancy data (provided by Rhone-Poulenc Ltd.). There were no i.p. or i.v. LD_{50} data available, since the regulatory requirements are for rat oral data. There is no obvious relationship between ID_{50} rank order or actual values with any of the *in vivo* data sets shown. The rabbit eye and skin irritancy data appear to be completely random when compared to the KB assay results (Table 5.5).

Table 5.5. *In vitro* and *in vivo* toxicities of fifteen Rhone-Poulenc Ltd. formulations (TOX 01-15)

TOX No.	KB ID_{50} (μg/ml)	Rat oral LD_{50} (mg/kg)*	Rat dermal LD_{50} (μl/kg)*	Skin irritancy (rabbit)*	Eye irritancy (rabbit)*
13	6	>5000	>2000	V.slight	Slight
14	111	2000	>2000	Irritating	Moderate
04	128	>2500<5000μl/kg	>2000	Slight	Severe
01	136	3033	>2000	None	Slight
11	161	480μl/kg	>2000	V.slight	Moderate
05	170	697	>2000	Irritant	-
12	173	1500μl/kg	>2000	Slight	Moderate
09	188	1000μl/kg	>2000	None	None
06	312	552	1430	Slight	Severe
15	325	2135	1871mg/kg	Slight	Severe
08	353	1098	1974mg/kg	None	Severe
02	400	1000μl/kg	>2000	-	-
03	570	10000μl/kg	>2000	None	None
10	579	1250μl/kg	>2000	None	None
07	609	963	1952mg/kg	Slight	Severe

*Data supplied by Rhone-Poulenc Ltd.

5.2.2.4 **Consideration of active ingredients and their formulations**

A comparison of the *in vitro* and *in vivo* toxicities of seven pure chemicals and their formulations is shown in Table 5.6.

(a) Chlorothalonil and TOX 13 are both highly damaging to cells in the FRAME KB cytotoxicity assay, but appear non-toxic to rats orally, while Chlorothalonil is highly toxic to mice intraperitoneally.

(b) Pure Dimethoate is moderately damaging in the FRAME KB cytotoxicity assay, but both its formulations (TOX 17 and 19) are more toxic than the pure chemical. Both *in vivo* and *in vitro* values show that TOX 17 is more toxic than Dimethoate.

(c) Similarly, TOX 04 and 14 are more toxic than their main active ingredient, Trifluralin, in both the *in vivo* and *in vitro* assays.

(d) Neburon is more toxic to cells *in vitro* than its formulation TOX 01, while being less toxic than TOX 01 *in vivo*.

(e) MCPA acid is more toxic *in vitro* than its formulations TOX 11 and 12. One formulation was less toxic *in vivo* than MCPA acid, and the other was more toxic. TOX 11 and 12 gave very similar ID_{50} values but their rat oral LD_{50} values differed by a factor of three.

(f) Conversely, the two ID_{50} values of the formulations of Cyanazine (TOX 09 and 10) differ by a factor of three, while the rat oral LD_{50} values are quite similar. Both formulations are less toxic *in vitro* than Cyanazine itself, and this is also true *in vivo*.

(g) Mecoprop has the same toxicity to cells in the FRAME KB cytotoxicity assay as two of its formulations (TOX 06 and 08), with TOX 07 being slightly less toxic. The rat oral LD_{50} data shows TOX 07 and 08 being equally toxic to Mecoprop, with TOX 06 more toxic.

Table 5.6. *In vitro* and *in vivo* toxicities of seven Rhone-Poulenc Ltd. agrochemicals and twelve Rhone-Poulenc Ltd. formulations which contain them

Pure Chemical			Formulation		
Active Ingredient	ID_{50} (μg/ml)	Rat oral LD_{50} (mg/kg)*	TOX No.	ID_{50} (μg/ml)	Rat oral LD_{50} (mg/kg)*
Chlorothalonil	2	>10000	*13*	6	>5000
Dimethoate	317	500-600	*17*	96	200-250
			19	223	Not known
Trifluralin	210	>10000	*04*	128	2500-5000μl/kg
			14	111	2000
Neburon	35	>10000	*01*	136	3033
MCPA acid	36	700	*11*	161	480μl/kg
			12	173	1550μl/kg
Cyanazine	95	182-380	*09*	188	1000μl/kg
			10	579	1250μl/kg
Mecoprop	361	930	*06*	312	552
			07	609	963
			08	353	1098
Unknown	-	-	*05*	170	697
Unknown	-	-	*02*	400	1000μl/kg
Dimefuron	-	1000	*03*	570	>10000μl/kg

*Data supplied by Rhone-Poulenc Ltd., except for Dimefuron (RTECS (Lewis and Tatken, 1982).

5.2.2.5 **Consideration of vehicle toxicity**

Rhone-Poulenc Ltd. provided some information on components of the vehicles of formulations TOX 02 to 14 (Table 5.7). Wetting agents, preservatives, emulsifiers and other ingredients were not specified, but were known to be present. There were essentially four types of vehicle base:-

(a) TOX 05, 11 and 12 were prepared in similar vehicles, all containing N-methyl pyrrolidone and a light aromatic C10 solvent. These formulations gave essentially identical ID_{50} values;

(b) The C10 solvent with 5% (v/v) water was the vehicle for TOX 04 and 14, and these too, gave essentially identical ID_{50} values;

(c) The four formulations containing propylene glycol (TOX 03, 09, 10 and 13) gave widely differing ID_{50} values; and

(d) The three Mecoprop formulations (TOX 06, 07 and 08) were in vehicles containing triethylene glycol and potassium hydroxide at varying levels. The ID_{50} values appear to increase with decreasing triethylene glycol and concurrent increasing potassium hydroxide concentrations. The concentration of Mecoprop in these formulations is not known, however, so the differential toxicities could be due to varying amounts of Mecoprop.

5.2.2.6 **Conclusions**

The majority of the formulations would be considered only moderately harmful in the FRAME KB cytotoxicity assay, with the one formulation containing Chlorothalonil being much more harmful. However, all the formulations would be classified as "non-harmful" based on the basis of *in vivo* rat oral or dermal LD_{50} values. It is possible that the deposits left in the culture wells by some of the formulations, when tested at cytotoxic concentrations, may have

Table 5.7. Some vehicle ingredients of Rhone-Poulenc Ltd. formulations TOX 02 to 14, with *in vitro* toxicities

TOX No.	Vehicle Ingredients (excluding emulsifiers, wetting agents)	Active Ingredient	ID_{50} (μg/ml)
13	Propylene glycol, 7.5% (w/v); water	Chlorothalonil	6
10	Propylene glycol, 7.5% (w/v); water	Cyanazine	579
09	Propylene glycol, 6% (w/v); water	Cyanazine	188
03	Propylene glycol, 5% (w/v); water	Not known	570
06	Triethylene glycol, 25% (w/v); water; 50% potassium hydroxide solution, 16.5% (v/v)	Mecoprop	312
08	Triethylene glycol, 20% (w/v); water; 50% potassium hydroxide solution, 21% (v/v)	Mecoprop	353
07	Triethylene glycol, 15% (w/v); water; 50% potassium hydroxide solution, 23% (v/v)	Mecoprop	609
02	Triethylene glycol, 25% (w/v); water	Not known	400
11	N-methyl pyrrolidone, 33% (w/v); light aromatic C10 solvent	MCPA acid	161
12	N-methyl pyrrolidone, 30% (w/v); light aromatic C10 solvent	MCPA acid	173
05	N-methyl pyrrolidone, 25% (w/v); light aromatic C10 solvent	Not known	170
14	Water, 5% (w/v); light aromatic C10 solvent	Trifluralin	111
04	Water, 5% (w/v); light aromatic C10 solvent	Trifluralin	128

interfered with the growth of cells. However, the ID_{50} values of those formulations showing deposits ranged from 113 to 609 μg/ml, indicating that the presence of a deposit did not cause a consistent effect. If phagocytosis of particles did occur, it did not cause the 3T3-L1 cells to detach, or decrease their division rate, in any predictable fashion.

The importance of the vehicle toxicity was demonstrated with TOX 17 to 19. The addition of the active ingredient (Dimethoate) to the vehicle (TOX 18) did not alter its toxicity. Dimethoate in an alternative vehicle was less toxic. This proves that it must not be assumed that it is only the active chemical(s) in a formulation which is (are) responsible for the toxicity *in vitro*. The vehicle alone may be damaging, or synergistic effects between the active and its vehicle may take place.

The FRAME KB cytotoxicity assay did not reproduce the difference in toxicity to sheep reported between the two samples of Trodax. No information was provided as to how serious the instance of adverse *in vivo* effects was, or whether Rhone-Poulenc Ltd. were able to reproduce the difference.

As with the pure chemicals, there was no correlation between the toxicity of the formulations *in vitro* (as a whole) and *in vivo* by the rat oral route. In addition, there was no correlation with rat dermal LD_{50}, or rabbit skin or eye irritancy. The rat oral LD_{50} values covered a relatively small range, as did most of the ID_{50} values.

When the rat oral LD_{50} values of the pure chemicals were compared with the rat oral LD_{50} values of the formulations that contained them, the same relationship between the toxicity values (i.e. whether the pure chemical was more toxic than its formulation(s) or vice versa) was generally also reflected in the *in vitro* values.

5.3 DISCUSSION

Both the pure chemicals and the formulations were a considerable challenge to the FRAME KB cytotoxicity assay, due to their chemical and physical properties. Solubility problems were encountered with the pure chemicals and the formulations. This led to some chemicals providing no result, and to some formulations having their results questioned, because of the presence of deposits settled out in culture wells. It is a commercial advantage for some of the agrochemicals (e.g. herbicides) to be of low aqueous solubility, so that they are not easily washed off foliage by the first shower of rain or into water courses. Aqueous insoluble substances can be readily administered to laboratory animals by the oral route, in the form of a suspension. If the FRAME KB cytotoxicity assay is to be useful with this type of chemical, then it may be necessary to devise new ways of solubilizing them. Otherwise, it must be acknowledged that a proportion of these chemicals will provide results only up to their maximum solubility in medium. This is a methodological problem for *in vitro* assays, which has yet to be adequately addressed (Bernson *et al.*, 1986; Stark *et al.*, 1986). The current FRAME KB cytotoxicity assay protocol is designed for use with solubilised substances only.

The chemical nature of the pure compounds and the formulations led to problems with interpretation of results. The identity of half of the chemicals was unknown. Although some of these were stated as being chemically related, it was not known how closely. Hence, the comparisons of *in vivo* and *in vitro* data amongst all the chemicals or amongst related groups was hampered by lack of knowledge about chemical structures. In addition, lack of information about the ingredients of the formulations meant that only limited conclusions could be drawn from the results. Both the active ingredients and their vehicles were responsible for cytotoxic effects *in vitro*. The extent to which each plays a role in the cytotoxicity of a formulation could

only be elucidated if all the ingredients could be tested separately and in various combinations. The complexity of some of the formulations means that many interactions, such as synergistic effects, could occur.

At first glance, the FRAME KB cytotoxicity assay appears unable to predict the rat oral LD_{50} values for the pure chemicals or for the formulations. There were several distinct discrepancies, i.e. materials toxic to cells *in vitro*, but virtually non-toxic to rats orally. These discrepants were, in general, very poorly soluble and also much more toxic by parenteral administration. It appears doubtful, therefore, that the rat oral assay was detecting a true lack of intrinsic toxic properties in these particular chemicals. It is more likely that their low solubilities prevented adequate absorption from the GI tract. This is most striking with Chlorothalonil, Trifluralin, Neburon and Simazine (Table 5.3). Trifluralin is of low toxicity parenterally, and this is possibly due to low intrinsic toxicity, or to its degradation in the body to metabolites of low toxicity (Gosselin *et al.*, 1976). Trifluralin is known to be poorly absorbed from the GI tract in the rat (Gosselin *et al.*, 1976) and this probably explains its low oral toxicity. One soluble chemical, Asulam, also gave low toxicity to rats orally. Although no parenteral LD_{50} data were available, it was of low toxicity *in vitro* (Table 5.3) and hence it probably has low intrinsic toxicity. This may explain its low oral toxicity, although it, too, may be poorly absorbed from GI tract, despite its solubility. This demonstrates how the degree of absorption of a chemical, from the GI tract into the systemic circulation, must be considered when performing *in vitro*/oral LD_{50} data comparisons. Although laboratory animals can be easily dosed orally with suspensions of insoluble chemicals, the amount that actually enters the circulation may bear no relation to the administered dose. In these cases, the oral *in vivo* assay may not detect any toxic effects. Cells in the *in vitro* assay are continuously bathed in the test substance. This equates better to a parenteral dose (Fry *et al.*, 1988a), and is therefore more likely to detect intrinsic toxic properties of the substance. It

is acknowledged that *in vitro* cytotoxicity assays cannot simulate the pharmacokinetic processes involved in the uptake and distribution of a chemical administered orally (Ekwall, 1983a; Rowan, 1983).

The relevance of performing rat oral LD_{50} assays with very high doses of herbicides of known poor solubility is, therefore, highly questionable. In the modern agrochemical industry, herbicides are selected for their activity at low treatment levels (250 mg or less per hectare) and for their low aqueous solubility. As it appears that poorly soluble substances do not elicit toxic effects when given to rats orally, it would be more relevant to perform a toxicity assay which would detect intrinsic toxic properties of a chemical. However, the rat oral LD_{50} value must be obtained for classification purposes and, because this type of regulation is so inflexible, the assay is still conducted, whatever the nature of the chemical.

Closer inspection of individual related sets of chemicals, and individual chemicals with their formulations, showed that, in some cases, reasonable similarity between toxicity rank did exist between the ID_{50} and the rat oral LD_{50}.

The overall lack of correlation between the FRAME KB cytotoxicity assay and the rat oral LD_{50} assay for the Rhone-Poulenc Ltd. substances can, therefore, be attributed mainly to their insolubility. There appears to be a stronger correlation between ID_{50} results and LD_{50} values from rat or mouse by i.p. or i.v. dose, but with the small amount of data available, firm conclusions cannot be drawn.

Agrochemicals, in general, must have low oral and dernal toxicities to be acceptable, and yet must still possess highly specific lethal activities on a wide diversity of target organisms. It is possible that it is these specific toxic effects

which were, in some cases, detected by the FRAME KB cytotoxicity assay. For example, Chlorothalonil, the fungicide, was found to be highly toxic *in vitro,* but is virtually non-toxic to rats orally. This compound is accumulated rapidly by yeast cells, and exerts its toxicity ultimately by inhibition of thiol-dependent enzymes (Tillman *et al.*, 1973). A similar mechanism could have caused the potent inhibition of growth of the 3T3-L1 cells seen in the *in vitro* assay.

Overall, it is difficult to draw any firm conclusions from the FRAME KB cytotoxicity assay results for the Rhone-Poulenc Ltd. chemicals and formulations. Although the correlation with rat oral LD_{50} does not look too promising at this stage, this is not surprising, because of the diverse chemical and physical properties of the test materials. Further work with related chemicals and their vehicles and formulations may provide additional information. At present, pesticides are classified according to their rat oral and/or dermal LD_{50} values (Brian Ingham, personal communication). Based on these results, the current FRAME KB cytotoxicity assay could not, therefore, be used to test chemicals and formulations for classification purposes. It may be more useful for predicting i.p. or i.v. acute toxicity, or for predicting the rank order of oral toxicity among closely structurally related chemicals.

The problem of aqueous insolubility of test substances is not confined to *in vitro* toxicology. The high rat oral LD_{50} values for the poorly soluble agrochemicals have little meaning as measures of toxic potency. It is therefore of no merit to attempt to develop an alternative assay method to predict these oral LD_{50} results. Once the methodological and interpretative difficulties involved with testing poorly-soluble substances in both *in vivo* and *in vitro* acute toxicity assays are overcome, then further progress toward a new approach to assessing acute toxicity for classification purposes may be possible. This should have a more logical and scientific basis (Balls, 1991d), and hopefully would place much less reliance on formal *in vivo* studies.

CHAPTER 6 AN ASSESSMENT OF THE APPLICABILITY OF THE FRAME KB CYTOTOXICITY ASSAY FOR TESTING PURE SURFACTANTS AND TOILETRY FORMULATIONS

6.1 INTRODUCTION

A dilemma currently exists in the cosmetics and toiletries industry between providing adequate safety assurance for their products, whilst not being seen to test them on animals (Balls, 1991a). Safety assessments need to be conducted, particularly with new ingredients and new types of formulations, in order to ensure that the consumer is not harmed. Safety assessments have traditionally been based on results of toxicity tests on animals (Balls, 1991e). Tests such as the Draize skin and eye irritancy assays have become increasingly unpopular with the public in recent years, and many cosmetics and toiletries are now labelled as "not tested on animals" in order to attract purchasers. Such claims are not easily substantiated, because, although a final product may not have been tested *in vivo*, it is very likely that some or all of its ingredients will have been. Some companies rely on human volunteers to test their products. This method of safety assessment cannot, however, be used for every newly-developed ingredient or every product, because of the large number of volunteers required and the potential risk of permanent damage to a person from a new compound which may have inadequate background safety data.

A way out of this dilemma is to use scientifically-validated non-animal alternative methods which have been accepted by regulatory authorities. As yet, no validated alternative method exists which could provide adequate data upon which companies could make safety assessments for cosmetics and toiletries (van den Heuvel and Fielder, 1990). A wide range of alternative methods do exist, however (Balls *et al.*, 1991a), but progress towards their regulatory acceptance is slow. The goal of a testing strategy for toiletry products and ingredients is to select non-irritants. This has proved more problematical for some methods than has the selection (i.e. screening out) of strong irritants (Reinhardt, 1990). Two types of alternative which are

undergoing extensive evaluation are:-

(a) physicochemical tests, e.g. the EYTEX™ method, based on the breakdown of proteins in solution (Gordon and Bergman, 1987); and

(b) cytotoxicity tests, e.g. the FRAME KB cytotoxicity assay (Knox *et al.*, 1986), the Neutral Red Uptake assay (Borenfreund and Puerner, 1985), the FRAME Neutral Red Release assay (Reader *et al.*, 1989) and the FRAME fluoroscein dye leakage assay (Shaw *et al.*, 1990)

Initial evaluations have been conducted using a large number of pure chemicals. The tests are now being evaluated with both products in development and final products of the cosmetic/toiletry type.

This chapter considers the potential use of the FRAME KB cytotoxicity assay in the testing of surfactant-containing toiletries and pure surfactants, and two other categories of toiletry product which do not contain surfactants as their principal ingredient.

6.2 RESULTS

6.2.1 Pure surfactants

6.2.1.1 **Description of the surfactants**

The cytotoxicity of surfactants *in vitro* has been well studied, (e.g. Ferguson and Prottey, 1976; North-Root *et al.*, 1982; Borenfreund and Borrero, 1984; Bracher *et al.*, 1989), often in attempts to correlate the *in vitro* results to Draize eye irritancy scores. Some studies show good rank correlations (North-Root *et al.*, 1982; Borenfreund and Borrero, 1984), while some gave poorer

comparisons (Scaife, 1982; Flower, 1987). Few, if any, assays have been proposed as *in vitro* acute lethal toxicity tests for surfactants, despite the fact that soaps, detergents and cleaners are frequently accidentally swallowed by children (Seabaugh, 1977), and are released into the environment in vast quantities, causing damage to animal and plant life (Lewis, 1990; Lewis, 1991).

The pure surfactants were selected for testing as a group, because many are active ingredients in a very wide range of cosmetics, toiletries and household and industrial cleaning products. A high proportion of the population is exposed to them every day. They were also selected because of their known physicochemical activity in disrupting cell membranes (Helenius and Simons, 1975; Womack *et al.*, 1983).

The chemicals tested are related by their surface-active properties and not by similarity of chemical structure. However, these surface-active properties are a result of a hydrophobic portion and a hydrophilic portion of the surfactant molecule. There is, therefore, a tenuous chemical-structure link between the surfactants. Their common chemical activity does mean that, when tested in *in vitro* toxicity assays, the results are more likely to correlate well with *in vivo* toxicity than would result for a group of completely unrelated chemicals (Fry *et al.*, 1990b).

Four types of charged surfactants are available: cationic, anionic, amphoteric and non-ionic (Young and Coons, 1945). Amphoteric surfactants become cationic or anionic, depending on the pH of the solution. Within each charged group, at least two types of chemical structure were tested. Two small sets of structurally-related surfactants were included - the non-ionic Tweens (polyoxyethylene sorbitans) and the cationic alkyl ammonium bromides. Most of the surfactants tested were synthetic, although some were naturally occurring gastro-intestinal salts, or derivatives of them. All were dissolved

directly into culture medium and hence were tested in the absence of organic solvents.

6.2.1.2 *In vitro* toxicities of twenty-nine pure surfactants

The cytotoxicities of 29 surfactants, measured in the FRAME KB cytotoxicity assay, ranged from 1 to 4120μg/ml (Table 6.1). This is a narrower range than that covered by the group of metal compounds described in Chapter 4. The ID values have not been converted to millimolar concentrations for two reasons. Firstly, several of the surfactants are polymers of indeterminate molecular weight, which makes an accurate molarity calculation impossible, and secondly, the μg/ml concentrations allow direct comparisons of the cytotoxicities of these pure chemicals with the formulations which contain them. The formulations and mixtures obviously have no defined molecular weights.

It can be clearly seen that the cationic (positively charged) surfactants are the most toxic of the four surfactant groups tested, following the 72-hour exposure period (Table 6.1). The three alkyl ammonium bromides (cetyldimethylethyl - , tetradecyltrimethyl - and hexadecyltrimethyl-ammonium bromide) are equally toxic, and appear to be, in μg/ml terms, more toxic than another cationic surfactant, benzethonium chloride.

The ionic charge of the remaining surfactants does not appear to influence their relative positions in the toxicity ranking (Table 6.1). The non-ionics fall into two groups, with Triton X-100, Brij 35 and Nonidet P40 the most toxic, giving ID_{50} values close to those for the least toxic cationics. The remaining non-ionics comprise the Tween series (polyoxyethylene sorbitans). These gave cytotoxicities ranging from 160 μg/ml to 620 μg/ml. The ID_{50} values for Tween 20, 40 and 60 are similar, with Tween 80 and 85 being less toxic. The five

Table 6.1. *In vitro* toxicities of twenty nine pure surfactants

Charge	Surfactant	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
C	Cetylpyridinium bromide	0.1 ± 0	1 ± 0	3 ± 1
C	Cetyldimethylethyl ammonium bromide	0.3 ± 0	2 ± 1	6 ± 1
C	Tetradecyltrimethyl ammonium bromide	0.3 ± 0	2 ± 0	4 ± 0
C	Hexadecyltrimethyl ammonium bromide	0.6 ± 0	2 ± 0	4 ± 0
C	Benzalkonium chloride	4 ± 1	7 ± 0	9 ± 2
C	Dodecyltrimethyl ammonium bromide	2 (n=2)	10 ± 1	17 ± 1
N	Triton X-100	2 ± 2	14 ± 3	50 ± 12
C	Benzethonium chloride	6 ± 2	17 ± 2	24 ± 5
N	Nonoxynol 11	15 ± 1	35 ± 5	68 ± 8
N	Brij 35	15 ± 2	52 ± 7	126 ± 19
N	Nonidet P-40	27 (n=2)	55 ± 7	105 ± 12
A	Sodium dodecyl sulphate	66 ± 11	94 ± 12	162 ± 17
A	N-Lauroyl sarcosine, sodium salt	104 ± 1	124 ± 2	139 ± 1
A	Dodecylbenzenesulphonic acid, sodium salt	120 ± 6	137 ± 1	150 ± 2
A	Deoxycholic acid, sodium salt	101 (n=2)	141 ± 6	199 ± 20
N	Tween 40	53 ± 5	159 ± 49	295 ± 50
Z	Miranol (38%)	100 ± 27	179 ± 29	261 ± 27
A	1-Decanesulphonic acid, sodium salt	130 ± 7	223 ± 2	275 ± 3
N	Tween 60	146 ± 31	244 ± 27	345 ± 9
N	Tween 20	123 ± 5	262 ± 43	396 ± 42

A	Teals (40%)	275 ± 30	306 ± 31	344 ± 32
N	Tween 85	253 ± 149	507 ± 50	821 ± 7
A	Caprylic acid, sodium salt	261 ± 48	560 ± 44	843 ± 44
N	Tween 80	134 ± 25	619 ± 69	836 ± 9
A	Cholic acid	565 ± 34	802 ± 28	1110 ± 27
A	1-Heptanesulphonic acid, sodium salt	625 ± 86	1520 ± 92	2560 ± 367
Z	CHAPSO	1850 ± 90	2150 ± 12	2360 ± 11
Z	CHAPS	2020 ± 99	2380 ± 32	2640 ± 90
A	Taurocholic acid, sodium salt	2690 ± 272	4120 ± 72	4670 ± 34

C = Cationic
N = Non-ionic
A = Anionic
Z = Zwitterionic (amphoteric)

Tweens are related in structure, having sidechains of different lengths, but their cytotoxicities showed no direct correspondence with this.

The anionic (negatively charged) surfactants are distributed from the centre to the low toxicity end of the ranking. The three anionic bile salts tested, deoxycholic acid, cholic acid and taurocholic acid, had markedly different toxicities, ranging from 141 to 4120 μg/ml, i.e. they were found spread throughout the anionics range.

Three amphoteric (zwitterionic) surfactants were tested. Again, there was no pattern to their positions in the toxicity ranking, although CHAPS and CHAPSO (two cholamidopropyl dimethyl ammonio sulphonates) gave similar ID_{50} results, and were placed at the least-toxic end of the ranking. The third zwitterionic tested was a 38% solution of Miranol. Correcting the ID_{50} for this dilution factor would give a value of approximately 70 μg/ml for the pure chemical, i.e. it would be 30 more toxic than CHAPS or CHAPSO.

Regardless of the toxicity of the surfactants, it is evident that the toxicity increases rapidly with increasing concentration. In most cases the ID_{20} and ID_{80} are approximately symmetrical on either side of the ID_{50} dose.

6.2.1.3 **Conclusions**

The cationic surfactants were consistently more toxic than the other charged groups. There was no clear rank order of toxicity among the anionics, non-ionics and amphoterics. As a group, the surfactants did not cover as wide a spread of toxicities as the metal compounds (Chapter 4) or the miscellaneous set (Chapter 3). The majority would be placed in the upper (more toxic) half of the toxicity ranking in Table 3.1, and therefore would be considered of high toxicity *in vitro*.

6.2.2 Toiletry Formulations

6.2.2.1 **Description of the formulations**

The majority of the toiletry products tested were supplied by manufacturing companies, who support the FRAME Research Programme and who wished to evaluate the usefulness of FRAME *in vitro* toxicity assays as part of product and ingredient safety assessment. Other products were bought from shops in order to provide a reference database. Several skin gels from one manufacturer were tested blind, in order to evaluate whether products which had unexpectedly caused irritancy in consumers could be detected in the *in vitro* assays.

Shampoos and hair conditioners contain a high proportion of surfactants as their active ingredients. A typical shampoo might contain up to 50% anionic surfactants, as these provide good foam and are of low cost (Alexander, 1971). Auxiliary surfactants may be of any of the four charged types. Non-ionic auxiliary surfactants, such as the Tweens, provide cleansing power and help to improve the solubility of the anionics. Amphoterics, such as Miranols or amino propionates, are the basis of non-irritant shampoos for children. Cationic surfactants have hair conditioning properties; for example, amidoamine salts or quaternary ammonium salts, which may be present at up to 3% in hair conditioners. Hair products are highly complex formulations and contain many non-surfactant additives, such as viscosity enhancers, clarifying agents, preservatives, dyes, perfumes and medications. Any of these additives may contribute toward the *in vivo* or *in vitro* toxicity of a shampoo or conditioner. Some additives may help to reduce irritant properties of other ingredients.

Hair sprays typically contain a small proportion of the hair-fixing ingredients,

high proportions of an alcohol as solvent, and a propellant gas (Beaven, 1988). Some modern pump sprays may not contain aerosol gases, but probably contain about the same proportion of hair fixatives. All the hair sprays tested were provided by one manufacturer (A), and were not in aerosol cans. All were readily miscible with culture medium.

The ingredients of facial, body and eye gels and skin liquids tested are unknown, but it is likely that they have a high water content and low levels of "active" ingredients, such as sunscreen chemicals. The five sun-protective and aftersun skin gels were provided by one manufacturer (D) in order to assess whether the FRAME KB cytotoxicity assay, or other FRAME *in vitro* toxicity assays, could detect those products which had caused unexpected skin and eye irritancy in some consumers. The remaining skin products were from a manufacturer (B) who professes to provide cosmetics from well-tried existing and "natural" ingredients, with alleged very low risk of adverse skin reactions.

All the products were readily miscible with aqueous medium. One product, an aftersun gel with aloe vera, gave a deposit of small particles in culture wells at all the toxic dose ranges tested. The avocado and aloe vera factor 8 sun protective gel reacted with the plastic of Universal containers after approximately ten minutes of contact, causing it to whiten and roughen.

6.2.2.2 *In vitro* toxicities of 29 hair cleaning and conditioning products

Over all, the 29 hair cleaning and conditioning products gave a range of cytotoxicity values (Table 6.2) similar to that shown by the pure surfactants (Table 6.1), i.e. 1 to 2530 μg/ml compared with 1 to 4120 μg/ml. However, the majority of the formulations would be placed in the middle to bottom of the toxicity ranking in Table 6.1.

Table 6.2. *In vitro* toxicities of twenty nine hair cleaning and conditioning products

Manufacturer*		Identity	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
Boots	S	Anti-dandruff	1 ± 0.1	1 ± 0.2	1 ± 0.3
A	C	Rinse-out, code D87	10	70 ± 12	170
A	C	Jojoba moisturising, factor 3 deep conditioning	27 ± 11	170 ± 40	926 ± 200
A	C	For permed, coloured extra dry hair (batch A437)	62 ± 3	173 ± 20	300 ± 33
Revlon	C	Flex	19 ± 2	174 ± 43	463
A	C	For permed, coloured extra dry hair (batch A435)	80 ± 6	244 ± 31	397 ± 25
Boots	S	Silk protein dandruff shampoo	148 ± 15	282 ± 49	381 ± 66
Beechams	S	Vidal Sassoon, Wash 'n' Go conditioning frequent use	190 ± 113	337 ± 118	537 ± 154
A	C	Nutrient henna, for dark hair	113 ± 20	344 ± 41	677 ± 76
A	S	Jojoba, factor 3, deep cleansing	255 ± 31	408 ± 33	516 ± 41
A	C	Nutrient evening primrose, frequent use	112 ± 25	449 ± 21	777 ± 62
A	C	Nutrient camomile, for blonde hair	167 ± 53	477 ± 20	838 ± 33
A	S	Volumising, code F8	331	512 ± 31	672 ± 33
B	S	Grapefruit, frequent wash	390 ± 26	531 ± 11	623 ± 20
A	S	Jojoba, factor 2, balanced cleansing	255 ± 31	536 ± 33	628 ± 25
A	S	Medicated	445 ± 23	553 ± 42	678 ± 16
A	S	Mild, code E7	445 ± 44	598 ± 22	709 ± 24
Hennara	S	Henna	482 ± 90	632 ± 113	832 ± 89

A	S	Nutrient enriched, henna	505 ± 26	668 ± 37	776 ± 45
A	S	Apple, for greasy hair	412 ± 99	704 ± 17	833 ± 32
A	S	Balsam, conditioning, all hair types	428 ± 19	748 ± 21	890 ± 35
Revlon	S	Flex	290 ± 97	763 ± 68	924 ± 47
A	S	Nutrient enriched, evening primrose, frequent use	486 ± 141	810 ± 23	951 ± 15
A	S	Balsam, conditioning, for frequently washed hair	481 ± 90	959 ± 130	>1000
C	S	Code 4613/6	644 ± 30	1120 ± 21	1350 ± 36
Johnson & Johnson	S	Baby	874 ± 134	1300 ± 83	1630 ± 53
C	S	Zwitsal baby	890 ± 31	1420 ± 96	1750 ± 118
A	C	Leave-in, Code F5e	1350 ± 278	2530 ± 402	3910 ± 820

*The identities of manufacturers A, B and C must be concealed due to confidentiality

C = Conditioner
S = Shampoo

One shampoo was very damaging to cells in the assay. This was an anti-dandruff shampoo, which was four hundred times more toxic than the other shampoos tested, and as cytotoxic as pure cetylpyridinium chloride. Another medicated (not anti-dandruff) shampoo gave an ID_{50} very similar to the majority of the other, non-medicated shampoos, as did a second anti-dandruff shampoo. The highly toxic anti-dandruff shampoo contained zinc pyrithione as its active anti-dandruff ingredient. Zinc pyrithione itself gave an ID_{50} of 0.06μg/ml in the FRAME KB cytotoxicity assay.

The Johnson and Johnson baby shampoo was the least toxic shampoo tested. Several "mild" and "frequent use" shampoos for adults were tested, but were overall neither more or less toxic than the other shampoos. Two conditioning shampoos were placed at the lower end of the toxicity ranking in Table 2, but their ID_{50} values were still close to those for other shampoos for adults.

In general, the hair conditioners were more toxic to 3T3-L1 cells than the shampoos, but the differences in ID_{50} values were not great. The conditioners also tended to give relatively shallower toxicity curves than the shampoos. One leave-in conditioner (i.e. it is not meant to be rinsed out of the hair as normal hair conditioners are) was much less toxic (ID_{50} 2530 μg/ml) than a rinse-out conditioner from the same manufacturer (ID_{50} 70 μg/ml).

6.2.2.3 *In vitro* toxicities of 14 hair spray products

Table 6.3 shows the cytotoxicities of fourteen hair sprays and gel sprays tested in the FRAME KB cytotoxicity assay. The range of ID_{50} values covered is small (14900 to 40000 μg/ml) and the values themselves are considerably larger than those for the pure surfactants and surfactant-containing formulations. The gel sprays appear in general to be slightly more toxic than the hair sprays.

Table 6.3. *In vitro* toxicities of fourteen hair sprays and gel sprays

Manufacturer*	Product range	Identity	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
A	Gel spray	Tresemme 4 + 4	7580 ± 730	14900 ± 1990	28700 ± 8490
A	Gel spray	Bold Salon formula	7140 ± 1900	15500 ± 2660	25300 ± 2940
A	Gel spray	Indola extra strong	7430 ± 1440	17000 ± 1820	25700 ± 4150
A	Gel spray	V05 Mega Hold	4530 ± 1630	18500 ± 1840	27300 ± 3520
A	Hair spray	Indola lite, no gas	6520 ± 572	21200 ± 4700	29700 ± 3900
A	Gel spray	Salon formula ultimate hold	8870 ± 2160	22600 ± 2440	33200 ± 5500
A	Hair spray	V05 Extra Hold	10800 ± 1810	25200 ± 1660	37700 ± 4580
A	Hair spray	V05 Ultra Hold	11800 ± 3900	27100 ± 6340	37800 ± 6230
A	Hair spray	Salon formula strong hold	12000 ± 2040	29100 ± 6290	35200 ± 10700
A	Gel spray	Code F9	9570 ± 766	29800 ± 1390	37200 ± 611
A	Hair spray	Pure and clear, extra hold	13400 ± 2760	32300 ± 4990	42800 ± 5610
A	Hair spray	Pure and clear, ultra hold	17000 ± 3540	32300 ± 3310	43800 ± 4310
A	Hair spray	Code E1c	21400 ± 854	38600 ± 1840	48400 ± 3140
A	Hair spray	Code E1d	19900 ± 3640	40000 ± 1840	48200 ± 1690

*The identity of manufacturer A must be concealed due to confidentiality.

6.2.2.4 *In vitro* toxicities of 10 facial, body and eye gels and skin liquids

A variety of product types, designed for application to the skin at regular intervals without being rinsed off, were tested (Table 6.4). These included body gels, eye gels, a facial skin tonic and an eye make-up removing liquid. A wide range of ID_{50} values was obtained (143 to 16400μg/ml).

Five of the body gels were sun-care products from the same manufacturer (D), and three of these appear to be considerably more damaging to the 3T3-L1 cells than any of the other products in Table 6.4. The factor 2 and factor 8 sun-protective gels gave ID_{50} values similar to those for Tween 40 (Table 6.1), or a Jojoba moisturising hair conditioner (Table 6.2). The aftersun skin gel gave an ID_{50} value similar to that for Tween 80 or a conditioning shampoo. The remaining two skin gels by this manufacturer (another aftersun gel and a factor 2 sun-protective gel) gave ID_{50} values which would place them amongst other weakly damaging substances, such as magnesium (II) chloride hexahydrate and sodium (I) iodide in the FRAME KB cytotoxicity assay toxicity rank order (Table 3.1).

The remaining five products were from a second manufacturer (B) and all gave cytotoxicities which also placed them in the toxicity rank amongst substances of low toxicity. Of these, a perfumed and an unperfumed version of the same eye gel gave essentially identical cytotoxicities.

6.2.2.5 Conclusions

It is apparent that, in general, the hair conditioners contain ingredients which cause them to be slightly more damaging to 3T3-L1 cells than the shampoos. The two conditioning shampoos were not more toxic than other, regular, shampoos. One anti-dandruff shampoo gave unexpectedly high toxicity *in vitro*.

Table 6.4. *In vitro* toxicities of ten facial, body and eye gels and skin liquids

Manu-facturer*	Product range	Identity	ID_{20} (μg/ml ± s.e.m.)	ID_{50} (μg/ml ± s.e.m.)	ID_{80} (μg/ml ± s.e.m.)
D	Sun protective skin gel	Factor 2, honey and wheatgerm	24 ± 4	143 ± 25	232 ± 28
D	Sun protective skin gel	Factor 8, avocado and aloe vera	47 ± 18	165 ± 32	243 ± 25
D	Aftersun skin gel	Cucumber and royal jelly	234 ± 65	748 ± 149	1270 ± 285
D	Aftersun skin gel	Cooling with aloe vera	2010 ± 367	5510 ± 455	>10000
B	Eye makeup remover	Camomile, for eyes	4450 ± 757	7070 ± 437	8770 ± 481
B	Skin gel	Cooling foot and leg (GP/088D)	5830 ± 82	8650 ± 715	13000 ± 2080
B	Skin tonic	Salad wipes (AF/041)	5710 ± 1430	12200 ± 1020	20300
D	Sun protective skin gel	Factor 2, with carrot extract	7650 ± 1970	12900 ± 1220	13300 (n=1)
B	Eye gel	Elderflower, unperfumed	9150 ± 512	13300 ± 1170	17200 ± 2080
B	Eye gel	Elderflower	12600	16400 ± 2620	16700

*The identities of manufacturers B and D must be concealed due to confidentiality.

The ingredient responsible for this was almost certainly zinc pyrithione. The baby shampoos were, as expected, less damaging than those formulated for adult use.

The hairspray products gave *in vitro* toxicities similar to one another, and to those for the lower alcohols (Table 3.1), and were therefore considerably less toxic than the shampoos or conditioners.

The three body gels which were unexpectedly damaging in the FRAME KB cytotoxicity assay were those which had generated customer complaints. They appear to be unusually cytotoxic for this category of skin toiletry product.

6.3 DISCUSSION

The cationic surfactants were consistently more toxic than the other charged groups in the FRAME KB cytotoxicity assay, as has been found in other *in vitro* toxicity studies with surfactants (Borenfreund and Borrero, 1984; Kato *et al.*, 1988; Gajjar and Benford, 1989). However, whilst some studies have found anionics and amphoterics to be more toxic than non-ionics (North-Root *et al.*, 1982; Shopsis and Eng, 1985), there was no clear distinction between these groups in the FRAME KB cytotoxicity assay. This result may reflect the differences in the assay method employed in these studies. Benoit *et al.* (1987) found sodium dodecyl sulphate (SDS) (anionic) to be less toxic than Triton X-100 (non-ionic) in a 72 hour exposure cytotoxicity assay using 10% FCS in the culture medium. With 0 or 1% FCS, however, SDS was more toxic. Bianchi and Fortunati (1990) also found the cytotoxicity of an anionic surfactant increased when FCS was omitted from the treatment medium. Brij surfactants (non-ionic) were more cytotoxic than anionics over 2, 24, 48 or 72 hours exposure in an *in vitro* study by Cornelis *et al.* (1991). Anionics are known to bind to serum albumin with higher affinity than other detergents (Helenius and

Simons, 1975). Hence, with assays which involve dissolving the test surfactants in serum-containing medium (such as the FRAME KB cytotoxicity assay), it must be acknowledged that some surfactants may be partially "de-toxified" by binding to the serum proteins (Stark *et al.*, 1986).

This phenomenon is only of significance where the binding is of such high affinity that a chemical's toxicity rank position is radically altered by testing in the presence of little or no serum. It would not be straightforward to test chemicals in the FRAME KB cytotoxicity assay using low-serum medium. Although Benoit *et al* (1987) used a 72 hour exposure period, the endpoint measured was 100% cell death (i.e. the assay did not require the cells to multiply). The FRAME KB cytotoxicity assay measures inhibition of growth, and as 3T3-L1 cell growth would normally be markedly reduced in the absence of serum, any cell growth that did occur would probably not be inhibited (in the presence of test chemical) in the same manner as in the standard assay.

Serum-free or low-serum media are commercially available for certain attached cells, and 3T3-L1 cells might grow satisfactorily in them. However, in order that results would be comparable, all chemicals tested to date in the FRAME KB cytotoxicity assay would also have to be tested using the serum-free or low-serum medium.

The surfactants, which have rapid toxic effects on cells in culture (Riddell *et al.*, 1986a; Garle *et al.*, 1987; Balls *et al.*, 1991), can produce meaningful results in short-term exposure assays such as the SIRC test (North-Root *et al.*, 1982) or the FRAME Neutral Red Release (NRR) assay (Reader *et al.*, 1989). Cell growth during the exposure period is not required in these assays, and hence the question of serum binding effects may be examined. Some cytotoxicity studies on surfactants have specifically omitted serum from the medium due to concern about differential binding (Scaife, 1982) or have omitted testing the

cationics and anionics completely, in order to avoid potentially misleading results (Ernst and Arditti, 1980).

It is not possible to conclude definitely that it is the surfactants alone in the hair shampoos and conditioners which are responsible for inhibiting cell growth in the FRAME KB cytotoxicity assay. There may be both potentiation and inhibition of toxicity by other non-surfactant or surfactant ingredients. Some surfactants are known to increase cell membrane permeability, thereby causing the cells to become more susceptible to toxic chemicals (Parekh and Chitnis, 1990). However, the majority of the ID_{50} values of the shampoos are very similar and are at a level which would suggest they contain similar amounts of anionics, with little effect being caused by ingredients such as conditioners or simple medications.

Although the conditioners contain cationic surfactants, which are known to be toxic *in vitro,* it is not possible to conclude definitely that it is the cationics which are responsible for the higher toxicities of the conditioners. The low-toxicity, "leave-in" conditioner probably contains a much lower amount of the hair-conditioning agent(s) than the "rinse-out" version. This suggests that the conditioning agent is the prime cause of the toxicity of the hair conditioners, and is probably a cationic surfactant. Unfortunately, the actual identity of the hair conditioning agent(s) present in these formulations is not known, owing to trade secrets. Although it is likely that the conditioning shampoos contained cationic surfactants, they may also have contained less anionics than other shampoos, so their toxicity was not increased. Alternatively, their cationic ingredients may have been of lower *in vitro* cytotoxicity than the types used in pure conditioners.

Zinc pyrithione is reportedly an inhibitor of DNA synthesis (North-Root *et al.*, 1985) and was the active ingredient in the high toxicity shampoo tested. North-

Root *et al.* (1985) tested two "dandruff" shampoo formulations in a one-hour exposure, colony survival cytotoxicity assay, and found, in contrast to the results in this study, that both gave very similar toxicities, despite the fact that one formulation did not contain zinc pyrithione, or any other anti-dandruff ingredient. In their study, the "dandruff" formulations gave LC_{50} concentrations over ten times lower than those for two "regular" shampoos, which were in turn more toxic than two baby shampoos. Muir (1983) and Flower (1987) also found a decrease in effect from anti-dandruff to adult to baby shampoos in *in vitro* assays. Further anti-dandruff and baby shampoos must be tested in the FRAME KB cytotoxicity assay, before it can be confirmed that this toxicity ranking also holds true in this assay.

In the absence of any propellant chemicals, the hairspray products tested were probably almost totally made up of one or more of the lower alcohols. This explains their low toxicity to 3T3-L1 cells. The slight differences in the toxicities of the gel sprays and hair sprays may therefore reflect either inclusion of a greater amount of a more toxic alcohol or different toxic effects of different hair fixing ingredients.

The "irritant" suncare skin gels contained well-tested ingredients, but one was at a higher concentration than formerly used. The identity of this ingredient is not known due to trade secrets. It is not possible to say whether any sun-screening chemical was involved in the cytotoxic effects of the honey and wheatgerm or the avocado and aloe vera sun-protective gels. As with the shampoo and conditioner formulations, additional testing of individual ingredients and mixtures is required before these results can be put into their proper perspective. The results from the body and eye gels from manufacturer B suggest that manufacturer D's toxic gels were indeed unusually damaging for this type of skin product.

The question remains as to how the *in vitro* data would be used, in the context of safety assessment. Can cell growth inhibition tests, such as the FRAME KB cytotoxicity assay, provide relevant information on chemicals and products where the prime concern is whether they are irritant?

The results for the pure surfactants could be compared to *in vivo* acute lethal toxicity data in order to assess whether the *in vitro* assay could predict *in vivo* toxicity for this type of chemical. A direct comparison with *in vivo* toxicity values was not possible, due to a lack of data in the literature. The human acute poisoning rank order for surfactants is cationic > anionic/non-ionic (Gosselin *et al.*, 1976) and this is reflected in the rank order in the FRAME KB cytotoxicity assay. When considering systemic toxicity, the problem of surfactants binding to proteins in the cell culture medium is of less significance, because they may also bind to proteins in the gut or in the circulation of the subject animal. It can therefore be argued that low or no-serum conditions *in vitro* are unphysiological when compared to those facing cells inside an animal's body. If an outer skin layer is exposed to a chemical, no serum is present, because epithelial cells are not naturally bathed in a protein solution. The 3T3-L1 cells used in the FRAME KB cytotoxicity assay are fibroblastic, not epithelial, and therefore it seems logical that they should be exposed to test chemicals in the presence of serum. Although the FRAME KB cytotoxicity assay by itself has never been proposed as a test to detect irritant properties of chemicals, the results for the surfactants partially correlate with acknowledged *in vivo* irritancy rankings, i.e. cationic > anionic/amphoteric > non-ionic (Shopsis *et al.*, 1985). If the surfactants are considered as a group of surface-active chemicals among a large database of unrelated chemicals (Chapter 3; Clothier *et al.*, 1988), they may be seen as being relatively highly placed in the overall rank. This is primarily as a result of their specific ability to disrupt cell membranes (Helenius and Simons, 1975).

The FRAME KB cytotoxicity assay results for the surfactants are likely to become useful when they can be used as a reference database for product formulations which contain them, such as shampoos or household cleaners. Non-surfactant ingredients of these formulations may not produce rapid toxic effects like the surfactants, and thus the FRAME KB cytotoxicity assay may be more appropriate for these longer-acting substances. In this case the results for all the ingredients are required in order to assess which are primarily responsible for the toxicity of a formulation. It is necessary to increase the database of types of pure surfactants which are included in shampoos, conditioners and other cleaning products.

The results for the hairspray, body gel and liquid products are of a preliminary nature only, because there is no database of results for their ingredients and no *in vivo* data available with which to make comparisons. Further work would have to be performed in conjunction with the manufacturers in order to assess the usefulness of the FRAME KB cytotoxicity assay with these and other product types. With the surfactants and shampoos, however, the assay has demonstrated potential for providing relevant information for safety assessment. It could be of considerable use as a cell growth inhibition assay within a battery of *in vitro* toxicity assays designed to predict *in vivo* irritancy (Bracher *et al.*, 1989; Balls, 1991a). Such a battery has yet to be completely devised, but the increased collaboration between academic and industrial laboratories could lead to one (or more) being developed in the near future (Balls, 1989).

CHAPTER 7 MODIFICATION OF THE FRAME KB CYTOTOXICITY ASSAY FOR TESTS ON VOLATILE CHEMICALS

7.1 INTRODUCTION

The first interlaboratory evaluation of the FRAME KB cytotoxicity assay was conducted as a blind study in four laboratories (Knox *et al.*, 1986). Fifty test chemicals, which had been selected with the advice of the FRAME Research Advisory Committee on Cytotoxicology, were tested on a human embryo-derived cell line, BCL-D1. "Difficult" chemicals, i.e. types of chemicals known to pose problems for *in vitro* toxicology, were deliberately included. These included volatile, unstable, and insoluble chemicals, and chemicals which require metabolic activation to exert an enhanced effect *in vivo*. As expected, the "difficult" chemicals did pose some problems. For example, little confidence was expressed in the results for acrylonitrile, due to its high volatility. The ID values for this chemical showed both large intralaboratory and interlaboratory variation. It was suggested that volatile compounds could not be tested in the 24-well plates, unless a system for reducing evaporation of test chemicals was devised. Garle *et al.* (1987) also expressed low confidence in results for acrylonitrile in a 4-hour and a 72-hour exposure cytotoxicity assay, due to its volatility.

Clothier *et al.* (1988) noted that, even when using 96-well plates with the FRAME KB cytotoxicity assay, evaporation of test chemicals from the culture medium still occurred. The consequence of this evaporation would be that the cell growth inhibitory doses (ID values) calculated might not be a true reflection of the concentrations of the chemical which interacted with the target cells. If the chemical evaporated before it came into contact with the cells, then its toxicity would be drastically underestimated. It was also pointed out that care must be taken to prevent such vaporised chemicals from re-dissolving in the untreated control wells, thereby inhibiting control cell growth. This latter problem was most prevalent with chemicals of high volatility and low toxicity, as the plates had to be treated with high concentrations of the test

material.

Stark *et al.* (1986) found that "modular incubator chambers" were effective for containing volatile agents being tested in the Neutral Red Uptake cytotoxicity assay (Borenfreund and Puerner, 1984). Other workers (Tyson *et al.*, 1983; Clemedson *et al.*, 1989; Ghantous *et al.*, 1990) have developed sophisticated culture apparatus in order to minimise losses of volatile compounds from culture media during cytotoxicity tests. These are not suitable, however, for adaptation for use in a simple cytotoxicity test such as the FRAME KB cytotoxicity assay, due to their complexity, cost and low rate of test substance throughput.

The difficulty with handling volatile chemicals in *in vitro* toxicology has therefore been perceived as principally that of evaporation of the test chemical from the culture medium. The subsequent problem is to decide how much confidence can be placed in the results, as there are many degrees of volatility and toxicity, and hence some chemicals are more problematical than others. The question arises as to what extent different degrees of volatility and toxicity in a chemical could cause a researcher to express lack of confidence in a result. To date, only a small number of liquids have been acknowledged in the literature to represent a problem for simple cytotoxicity assays (Knox *et al.*, 1986; Garle *et al.*, 1987; Clothier *et al.*, 1988), but it is likely that others also cause difficulties. It is also pertinent to know whether the FRAME KB cytotoxicity assay could be adapted to include a sealing system for use with volatile chemicals, and whether the results would correlate better with *in vivo* toxicities than the results obtained in the standard FRAME KB cytotoxicity assay protocol.

There is no clear definition of volatility. Liquids which evaporate rapidly at

room temperature, such as acetaldehyde or methanol, are generally regarded as "volatile". However, liquids such as propan-l-ol or hydrochloric acid are less clearly volatile, but still evaporate rapidly. The volatility of a chemical depends mainly on its boiling point and latent heat of vapourisation. Trouton's rule indicates that the molar heat of vapourisation divided by the boiling point (in degrees absolute) is approximately a constant. Hence, volatility can be related directly to the boiling point.

The rate of evaporation of a test chemical from culture medium during a cytotoxicity assay is affected by several factors (Table 7.1). All or some of these factors will contribute to the interlaboratory and intralaboratory variations in cytotoxicity values obtained when testing volatile liquids in the FRAME KB cytotoxicity assay. Standardisation of some of the factors listed in Table 7.1 might be effective in reducing these variations. A system whereby treated wells are sealed immediately after test chemical application might help to reduce the evaporation rates, and hence also reduce variation. Sealing the wells also decreases the risk of transfer of volatile chemicals from treated to untreated control cultures. In addition, sealing may help to reduce the problem of evaporation of the culture medium itself. This can occur in the peripheral wells of 96-well plates and therefore renders them unsuitable for inclusion in the standard FRAME KB assay protocol (INVITTOX Protocol 36, Warren *et al.*, 1990).

Two methods for sealing 96-well plates were considered. In order to fit in with the overall objectives of the FRAME KB cytotoxicity assay, the methods should be inexpensive and easy to manipulate, and the sealing itself should not interfere with the performance of the standard assay. The systems investigated were:

1. Light paraffin oil, layered over the medium in each well (Figure 7.1b);

Table 7.1. Potential sources of variation in interlaboratory and intralaboratory FRAME KB cytotoxicity assay results with regard to volatile chemicals

		Example of how evaporation rate of liquid test chemical may be variably affected
1.	Environment in which the pure liquid test chemical is dispensed	Warm, dry environment with flow of air may cause evaporation of the chemical during transfer from stock bottle, resulting in pipetting error.
2.	Method of preparation of chemical dilution series in culture medium	(a) Potential evaporation of initial small-volume dilutions in volatile solvents. (b) Potential evaporation of chemical from culture medium solutions if left for extended periods before treatment of plates.
3.	Design of multi-well culture plate	Well size and shape, and design of lid, affect evaporation rates. Small wells (e.g. in 96-well plates) generally have lower rates of evaporation of both chemical and culture medium.
4.	Treatment protocol	(a) Volume of culture medium in each well affects surface area to medium volume ratio. Low surface area to volume ratios increase the potential for evaporation of both chemical and medium. (b) Proximity of treated wells to plate edge. Wells near edge suffer more evaporation than central wells.
5.	Incubator environment	(a) Low humidity in incubator increases evaporation rates. (b) Plates close to incubator internal fan will potentially suffer greater evaporation rates.

and

2. A self-adhesive, carbon dioxide-permeable plastic film, designed for use in microplate immunoassays (Figure 7.1c).

Selling and Ekwall (1985) employed both paraffin oil and a plastic film in their 96-well plate cytotoxicity assay. The purpose of these sealants was not stated, but appears to have been prevention of carbon dioxide exchange between the medium and the atmosphere.

The adhesive film and the paraffin oil were assessed for ease of use and for potential interference with the performance of the FRAME KB cytotoxicity assay. Eleven non-volatile (i.e. solid) chemicals were tested initially, using both sealants. Subsequently, the sealants were evaluated for use with liquids, including a series of straight-chain alcohols, which have varying degrees of volatility. The liquids were deemed to be of varying volatilities because of the range of their boiling points.

The results are presented as mM values, rather than μg/ml values, to allow for comparison of test chemicals by the number of test chemical molecules, rather than their weight, in solution.

Note: The experiments described in this chapter were all conducted in the laboratories of Northumbria Biologicals Ltd., Nelson Industrial Estate, Cramlington, NE23 9BL.

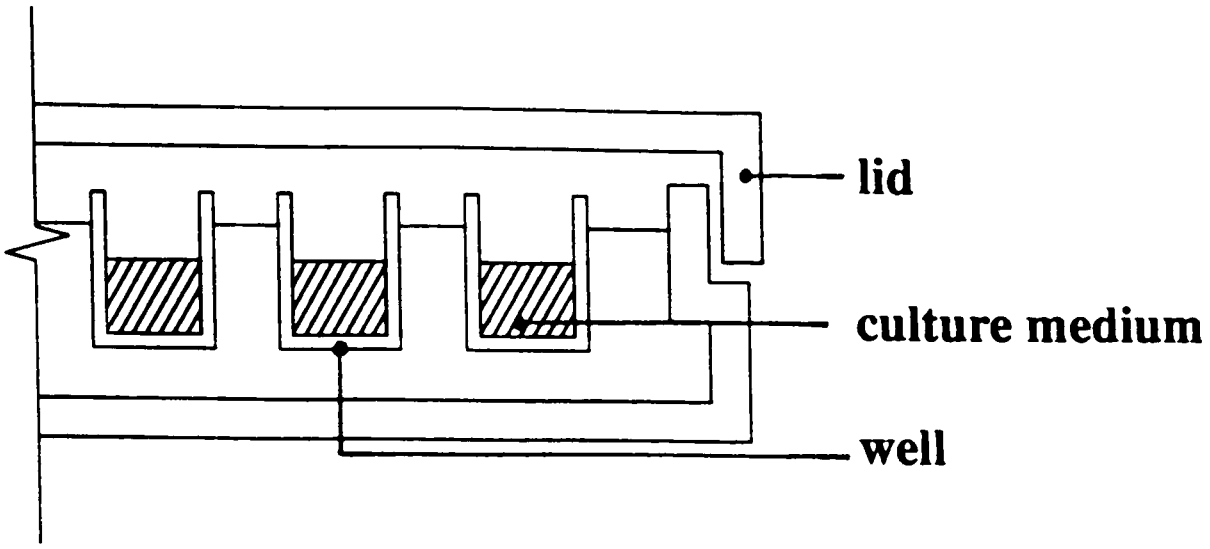


Figure 7.1(a): Unsealed.

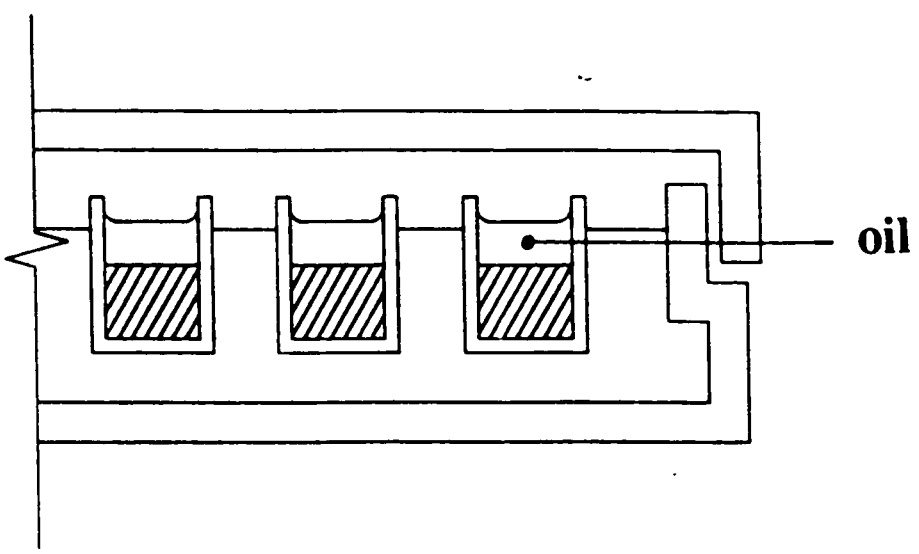


Figure 7.1(b): Paraffin oil as sealant.

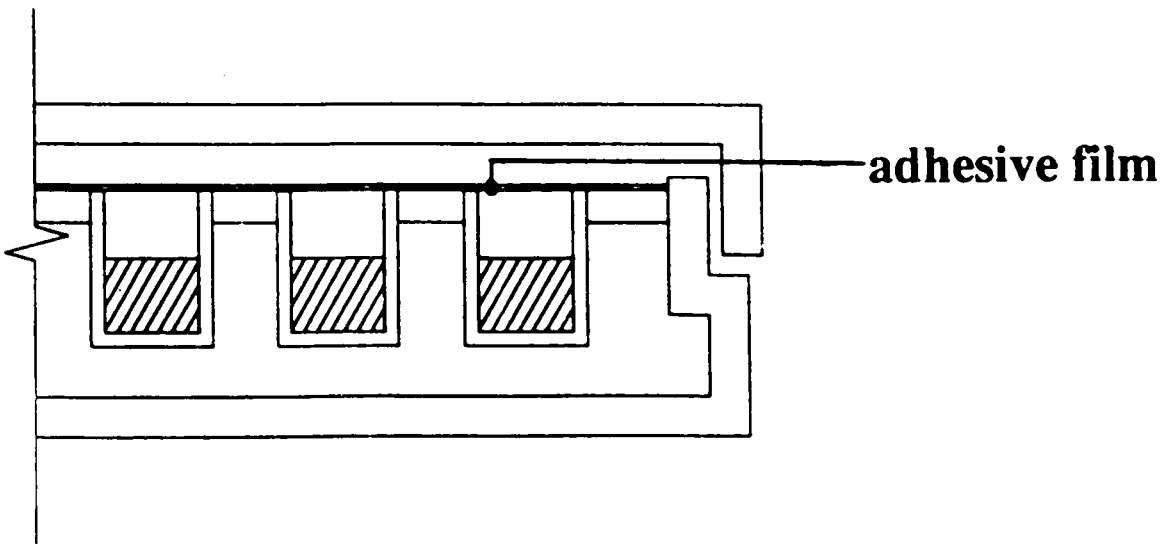


Figure 7.1(c): Adhesive film as sealant.

Figure 7.1 Diagram of cross section of culture plates with sealants in position.

7.2 RESULTS

7.2.1 Initial assessment of the two sealants

7.2.1.1 **Effects of sealants on cell growth**

3T3-L1 cells were plated at 0.5 x $10^4/cm^2$ (0.15 x 10^4 cells in 0.15ml of medium) in identical 96-well culture plates and were allowed to adhere overnight. The plates were then either left unsealed, or sealed with the adhesive film or with 100μl per well of light paraffin oil. The mean total cellular protein in the central 60 wells of the unsealed and sealed plates was measured by the FRAME KB cytotoxicity assay at 0, 24, 48 and 72 hours after sealing (or not) of the plates.

The total protein at the 72 hour endpoint was the same in both unsealed (US) and oil-sealed (OS) wells (Table 7.2). The adhesive film-sealed (FS) wells, however, showed approximately 40% less total protein at 72 hours. Examination of the cells in the FS plates using phase-contrast microscopy, both before and after assay fixation, indicated that a proportion of the cells became detached and were lost during the washing/fixation step. This occurred rarely in US and OS plates. Daily observation of the FS cultures revealed that the film sealing did affect cell growth, because fewer living cells were observed than in US or OS plates at the 48 and 72 hour timepoints. This is reflected in the growth curves (Figure 7.2). Cell growth in FS wells appeared to be equivalent to US wells at 24 hours after sealing, but was less rapid thereafter.

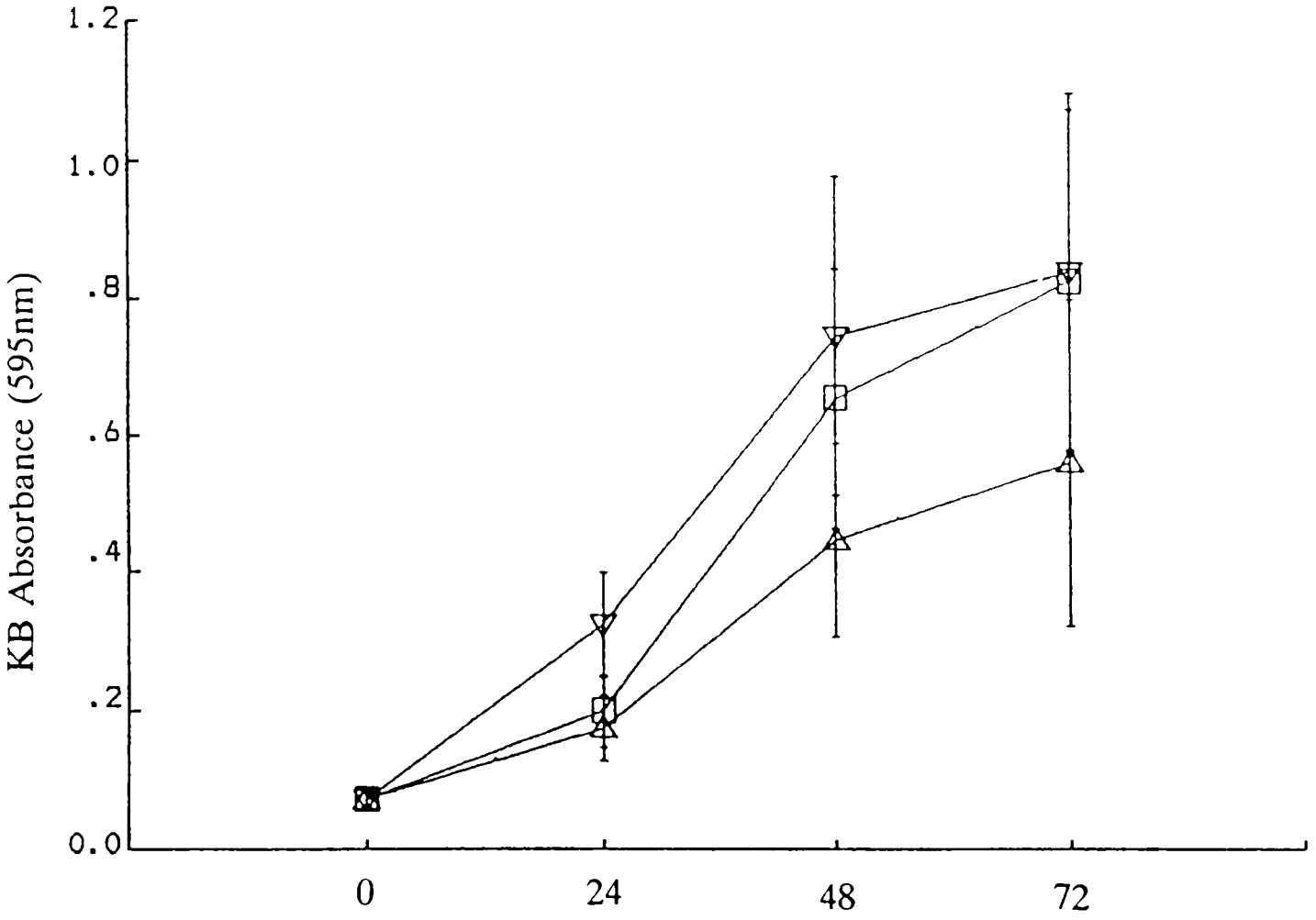


Figure 7.2 Growth of 3T3-L1 cells in unsealed or sealed culture plates (total protein measured by Kenacid blue (KB) staining).

□ Unsealed culture plate

▽ Oil-sealed culture plate

△ Film-sealed culture plate

Table 7.2. Effects of sealants on the growth of 3T3-L1 cells

Time after sealing	Total protein in the sealed wells, as a percentage of total protein in unsealed wells ± s.e.m.	
	Oil-sealed wells	Film-sealed wells
0 hr	100 ± 0	100 ± 0
24 hr	166 ± 14	87 ± 10
48 hr	113 ± 7	68 ± 3
72 hr	101 ± 1	63 ± 9

7.2.1.2 **Effects of sealants on culture medium pH**

The ability of the culture medium in the sealed plates to buffer to the correct pH (7.2 - 7.4) was examined. The colour due to the pH indicator (phenol red) was the same in unsealed and sealed plates throughout the incubation periods. DMEM has a high bicarbonate level and loses carbon dioxide rapidly when removed from the 5% carbon dioxide atmosphere of the incubator. This causes a noticeable colour change in the phenol red, from orange to pink. When plates were removed from the incubator, the medium in US plates began to become pinker within a few minutes, whilst medium in OS and FS plates did not, indicating that the pH remained at between 7.2 and 7.4. This suggested that both sealants reduced the rate of loss of carbon dioxide from the culture medium.

7.2.1.3 **Effects of sealants on the protein level within the blank (medium only) wells**

The blank well in each plate is used as the blank when the KB absorbance

values are read. The well is given medium, but no cells, for the duration of the assay. Protein from the serum in the medium adheres to the well plastic. Since the blank well was always the corner (position A1) well, there was the possibility that medium evaporation could cause a deposit of protein at the air/medium interface. The effect of the sealants on the KB absorbance values of the blank wells was therefore examined. Ten each of the US, OS and FS plates used for cytotoxicity assays were randomly selected. The absorbance values for the blank wells were meaned and the results are shown in Table 7.3.

Table 7.3. Mean absorbance of blank wells on unsealed, oil sealed or film sealed plates

	Mean absorbance ± s.e.m.
Unsealed plates	0.23 ± 0.01
Oil sealed plates	0.20 ± 0.01
Film sealed plates	0.23 ± 0.01

The OS blank wells had a slightly lower protein content than the US and FS wells.

7.2.1.4 **Effects of adhesive film immersed in culture medium**

A small piece of adhesive film (5-10mm^2) was immersed upright in culture well medium after cells had adhered overnight. Following incubation for a further 72 hours, cytotoxicity was observed. In fact, no living cells remained in these wells. By contrast, the paraffin oil did not appear to inhibit cell growth.

7.2.1.5 **Conclusions**

It was found that both the paraffin oil and the adhesive film were very easy to handle, and added only minimal time and cost to performance of the standard

FRAME KB cytotoxicity assay protocol. Neither sealant caused KB absorbances to exceed normal (unsealed plate) values, or caused inhibition of the process of cellular protein staining.

Although cell growth was inhibited by the adhesive film, this was not a sufficient reason for rejecting use of the film at this stage.

Neither sealant affected the ability of the culture medium buffering system to maintain the correct pH, indicating that the sealants did not adversely affect gaseous exchange in the 96-well plates.

Despite the apparent lower KB absorbance in blank wells on OS plates, this may not have any significance in terms of indicating reduced evaporation of medium. It was possibly due to oil adhering to the sides of the culture well and thus preventing the protein from binding to the plastic.

As both sealants showed no major disadvantages during this initial phase, their ability to affect the cytotoxicities of chemicals in the FRAME KB cytotoxicity assay was examined.

7.2.2 Effects of sealants on the *in vitro* toxicities of chemicals of low volatility

7.2.2.1 **Effects of sealants on chemicals of low volatility**

In order to establish whether either the paraffin oil or the adhesive film altered the toxicities of test substances of low volatility, eleven solids were tested for cytotoxicity by the FRAME KB cytotoxicity assay in US, OS and FS wells. The eleven chemicals (Table 7.4) included eight of the ten FRAME-recommended "standard" chemicals (Clothier *et al.*, 1988). They were also tested in order to check whether the assays run on US plates (i.e. by the

Table 7.4. *In vitro* toxicities of eleven solid chemicals on unsealed and sealed culture plates

		ID_{20} mM ± s.e.m.	ID_{50} mM ± s.e.m.	ID_{80} mM ± s.e.m.
Colchicine	US	<0.00003	0.00008 ± 0.00001	0.006 ± 0.002
	OS	0.00003 ± 0	0.00008 ± 0.00000	0.0008 (n = 2)
	FS	<0.00003	<0.00003	0.008 ± 0.001
Cadmium chloride	US	0.0008 ± 0.0002	0.001 ± 0.000	0.002 ± 0.000
	OS	0.0006 ± 0.0001	0.001 ± 0.000	0.001 ± 0.000
	FS	0.0007 ± 0.0001	0.001 ± 0.000	0.002 ± 0.002
Pentachlorophenol	US	0.06 ± 0.00	0.09 ± 0.00	0.11 ± 0.00
	OS	0.04 ± 0.01	0.09 ± 0.00	0.12 ± 0.00
	FS	0.02 ± 0.00	0.05 ± 0.00	0.08 ± 0.01
2,4-Dinitrophenol	US	0.10 ± 0.01	0.21 ± 0.10	0.30 ± 0.01
	OS	0.08 ± 0.02	0.21 ± 0.01	0.32 ± 0.02
	FS	0.03 (n = 2)	0.09 ± 0.02	0.18 ± 0.03
Sodium dodecyl sulphate	US	0.22 ± 0.01	0.27 ± 0.01	0.32 ± 0.01
	OS	0.25 ± 0.00	0.30 ± 0.00	0.33 ± 0.00
	FS	0.17 ± 0.02	0.23 ± 0.01	0.28 ± 0.01

Sodium fluoride	US	0.51 ± 0.13	1.4 ± 0.1	2.0 ± 0.1
	OS	0.61 ± 0.13	1.4 ± 0.1	2.1 ± 0.1
	FS	0.63 ± 0.22	1.3 ± 0.1	1.9 ± 0.0
Isoniazid	US	<0.73	4.2 ± 0.4	17 ± 0
	OS	1.0 (n = 2)	5.5 ± 0.8	17 (n = 1)
	FS	<0.73	1.5 ± 0.1	5.3 ± 0.6
Ammonium chloride	US	2.9 ± 0.7	4.9 ± 0.6	5.9 ± 0.6
	OS	3.0 (n = 1)	3.5 ± 0.7	4.8 ± 0.5
	FS	1.9 (n = 1)	3.7 ± 0.6	5.0 ± 0.4
Trichloroacetic acid	US	3.2 ± 0.7	6.3 ± 0.4	8.9 ± 0.7
	OS	2.7 ± 0.4	6.0 ± 0.8	8.6 ± 0.8
	FS	1.4 (n = 2)	2.8 ± 0.7	5.1 ± 1.0
Sodium hydroxide	US	17 ± 4	28 ± 3	40 ± 2
	OS	6.2 ± 0.3	10 ± 1	13 ± 0
	FS	7.8 ± 1.8	10 ± 2	13 ± 3
D-Glucose	US	139 (n = 2)	200 ± 13	269 ± 15
	OS	138 (n = 2)	215 ± 16	278 ± 16
	FS	133 (n = 1)	188 ± 8	252 ± 9

US = Unsealed culture plates
OS = Oil-sealed culture plates
FS = Film sealed culture plates

standard FRAME KB cytotoxicity assay protocol) in the laboratories at Northumbria Biologicals Ltd. provided results close to the published values (Clothier *et al.*, 1988).

2,4-Dinitrophenol and pentachlorophenol were dissolved initially in DMSO and ethanol, respectively, prior to dilution into the culture medium to a final solvent concentration of 0.1% (v/v). This is less than the solvent concentration normally used in the FRAME KB cytotoxicity assay (1% (v/v)), and was used because of the inhibition of cell growth by 1% (v/v) solvent on the sealed plates.

The dose-response curves of five of the chemicals were not significantly different in the sealed or unsealed plates. However, six chemicals (colchicine, 2,4-dinitrophenol, isoniazid, sodium dodecyl sulphate, sodium hydroxide and trichloroacetic acid) were all significantly more toxic on FS plates than on US plates (Table 7.5). Of these six, sodium dodecyl sulphate and sodium hydroxide also gave significantly increased toxicity on OS plates.

Where chemicals gave increased toxicity on sealed plates, the ID_{20}, ID_{50} and ID_{80} were all reduced by a similar factor (Table 7.4), indicating that the sealants did not affect the shape of the dose response curves. The ratio of standard error values to their ID values was fairly constant on the sealed and unsealed plates, indicating that the sealants did not affect the normal experiment to experiment variability of the results for these chemicals.

The toxicities of the eleven solids on US plates were no different from the FRAME published values for these substances (Clothier *et al*, 1988) (Table 7.6). Comparison of the logged ID_{50} (mM) values by linear regression gave a correlation coefficient of 0.93.

Table 7.5. Comparison of ID_{50} values for eleven solid chemicals on unsealed and sealed culture plates

	ID_{50} (mM)		
	Unsealed plate	Oil-sealed plate	Film-sealed plate
Colchicine	0.00008	0.00008	<0.00003*⊗
Cadmium chloride	0.001	0.001	0.001
Pentachlorophenol	0.09	0.09	0.05
2,4-Dinitrophenol	0.21	0.21	0.09*⊗
Sodium dodecyl sulphate	0.27	0.30*	0.23*⊗
Sodium fluoride	1.4	1.4	1.3
Isoniazid	4.2	5.5	1.5*⊗
Ammonium chloride	4.9	3.5	3.7
Trichloroacetic acid	6.3	6.0	2.8*⊗
Sodium hydroxide	28	10*	10*
D-Glucose	200	215	188

* = ID_{50} significantly different from unsealed plate ID_{50}

⊗ = ID_{50} significantly different from oil-sealed plate ID_{50}

Table 7.6. Comparison of FRAME published ID_{50} values with those obtained at Northumbria Biologicals Ltd. (NBL) for eleven solid chemicals, tested on unsealed culture plates

Chemical	FRAME ID_{50} (mM)	NBL ID_{50} (mM)
Cadmium chloride	0.001	0.001
Colchicine	0.02	0.00008
Pentachlorophenol	0.12	0.09
Sodium dodecyl sulphate	0.33	0.27
2,4-Dinitrophenol	0.39	0.21
Sodium fluoride	1.3	1.4
Trichloroacetic acid	3.2	6.3
Ammonium chloride	6.3	4.9
Isoniazid	16	4.2
Sodium hydroxide	37	28
D-Glucose	269	200

7.2.2.2 **Conclusions**

The paraffin oil provided more-acceptable results in the FRAME KB cytotoxicity assay than the adhesive film, as it altered the toxicity of only one solid, compared to five under the film. The small increase in toxicities of solid chemicals on sealed plates was unexpected, and does not have a simple explanation. While sodium dodecyl sulphate is statistically significantly more toxic on OS and FS plates than on US, the difference in the ID_{50} values between US, OS and FS plates is marginal.

Due to increased toxicities of organic solvents on sealed plates, the solvent concentration was reduced to 0.1% (v/v). This would prevent the testing of chemicals requiring a solvent, which were toxic at doses above 100μg/ml (assuming a maximum solubility of 100mg/ml of the chemical in the solvent).

Interlaboratory variation in results for the solids was low, indicating the reproducibilty of the FRAME KB cytotoxicity assay method.

Although the adhesive film revealed a major drawback in its effect on solid chemical cytotoxicity, it was decided to evaluate its use with liquid chemicals in order to complete the evaluation of whether the two sealants could provide different information with the liquid chemicals. Both sealants were therefore tested with a set of related volatile chemicals, the straight-chain alcohols.

7.2.3 Effects of sealants on the *in vitro* toxicities of structurally-related volatile chemicals

7.2.3.1 **Effects of sealants on structurally-related volatile chemicals**

A set of eight straight-chain alcohols were tested. The increase in carbon

chain length from methanol to octanol results in increased viscosity, a higher oil/water partition coefficient (Borenfreund and Babich, 1987b) and an increased boiling point (and hence reduced volatility). These chemicals were also selected because, although they are closely related structurally, they have a wide range of cytotoxicities to 3T3-L1 cells (Clothier *et al.*, 1988).

The toxicity curves on US plates showed that the toxicities of the alcohols increased with increasing chain length from methanol (1 carbon) up to octanol (8 carbons) (Table 7.7). The same pattern of increasing toxicity from methanol to octanol is also seen on the sealed plates. With the lower alcohols (methanol to propan-l-ol), both the sealants caused increased toxicity to a significant degree (Table 7.8), as is shown in the dose-response curves (Figure 7.3). With the higher alcohols, a variety of effects were noted. The toxicities of butan-l-ol and pentanol were the same on US and OS plates, but were increased significantly by the film sealant (Table 7.8, Figure 7.4). With hexanol, the oil sealant decreased toxicity significantly, but the film did not affect it (Table 7.8, Figure 7.5). The toxicity of heptanol was significantly decreased by both the oil and the film (Table 7.8), but to a greater degree by the oil (Figure 7.5). The toxicity of octanol appeared to be similar in sealed and unsealed plates (Table 7.5), but the dose-response curves (Figure 7.4) show that the shapes of the curves across the dose range are not the same. The curve from the OS plates has a distinct "elbow", which is also noticeable on the curve for heptanol on OS plates. This "elbow" is not apparent in the curves for the other alcohols. None of the alcohols showed any evidence of cross-contamination of control wells, i.e. control cell growth was not affected by the presence of an alcohol in wells on the same plate.

7.2.3.2 **Conclusions**

Sealing the plates with adhesive film or paraffin oil increased the toxicities of

Table 7.7. *In vitro* toxicities of eight straight chain alcohols in unsealed and sealed culture plates

		ID_{20} mM ± s.e.m.	ID_{50} mM ± s.e.m.	ID_{80} mM ± s.e.m.
Octanol	US	1.0 ± 0.3	1.7 ± 0.2	2.4 ± 0.4
	OS	1.7 ± 0.0	2.1 ± 0.1	2.5 ± 0.2
	FS	1.1 ± 0.4	1.8 ± 0.1	2.1 ± 0.0
Heptanol	US	1.0 (n = 1)	2.3 ± 0.3	3.6 ± 0.5
	OS	4.2 ± 0.8	6.3 ± 0.5	7.2 ± 0.6
	FS	2.7 ± 0.4	4.6 ± 0.3	5.8 ± 0.3
Hexanol	US	2.7 ± 0.6	7.3 ± 0.5	9.2 ± 0.5
	OS	6.9 ± 1.0	14 ± 1	21 ± 3
	FS	3.3 ± 0.6	7.3 ± 0.5	10 ± 0
Pentanol	US	25 ± 2	37 ± 32	44 ± 3
	OS	22 ± 4	36 ± 4	47 ± 2
	FS	7.4 ± 4.3	17 ± 4	30 ± 5
Butan-l-ol	US	21 ± 6	45 ± 4	72 ± 6
	OS	23 ± 7	42 ± 4	59 ± 4
	FS	12 ± 2	25 ± 2	45 ± 3
Propan-l-ol	US	68 ± 14	114 ± 10	169 ± 19
	OS	49 (n = 2)	79 ± 4	103 ± 9
	FS	39 (n = 1)	62 ± 3	108 ± 11
Ethanol	US	287 ± 32	418 ± 27	518 ± 10
	OS	129 (n = 1)	224 ± 19	320 ± 26
	FS	169 (n = 1)	223 ± 32	320 ± 31
Methanol	US	942 (n = 1)	1099 ± 53	1344 ± 43
	OS	312 (n = 1)	571 ± 67	842 ± 79
	FS	551 (n = 1)	705 ± 107	937 ± 68

US = unsealed culture plates
OS = oil-sealed culture plates
FS = film-sealed culture plates

Table 7.8. Comparison of ID_{50} values for eight straight chain alcohols on unsealed and sealed culture plates

	ID_{50} (mM)		
	Unsealed plate	Oil-sealed plate	Film-sealed plate
Octanol	1.7	2.1	1.8
Heptanol	2.3	6.3*	4.6*⊗
Hexanol	7.3	14*	7.3⊗
Pentanol	37	36	17*⊗
Butan-l-ol	45	42	25*⊗
Propan-l-ol	114	79*	62*
Ethanol	418	224*	223*
Methanol	1099	571*	705*

* = ID_{50} significantly different from unsealed plate ID_{50}

⊗ = ID_{50} significantly different from oil-sealed plate ID_{50}

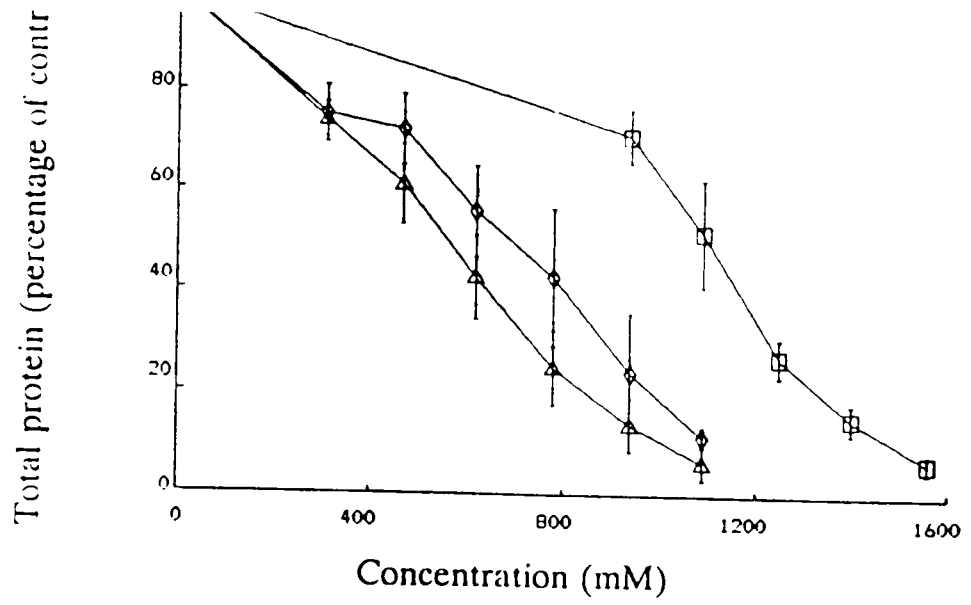


Figure 7.3(a)

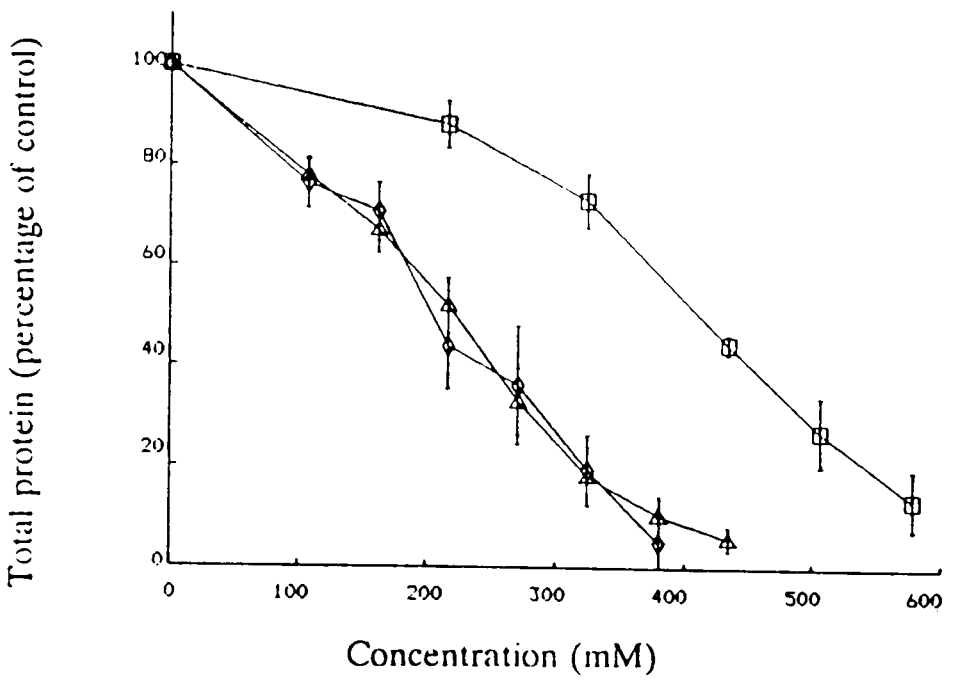


Figure 7.3(b)

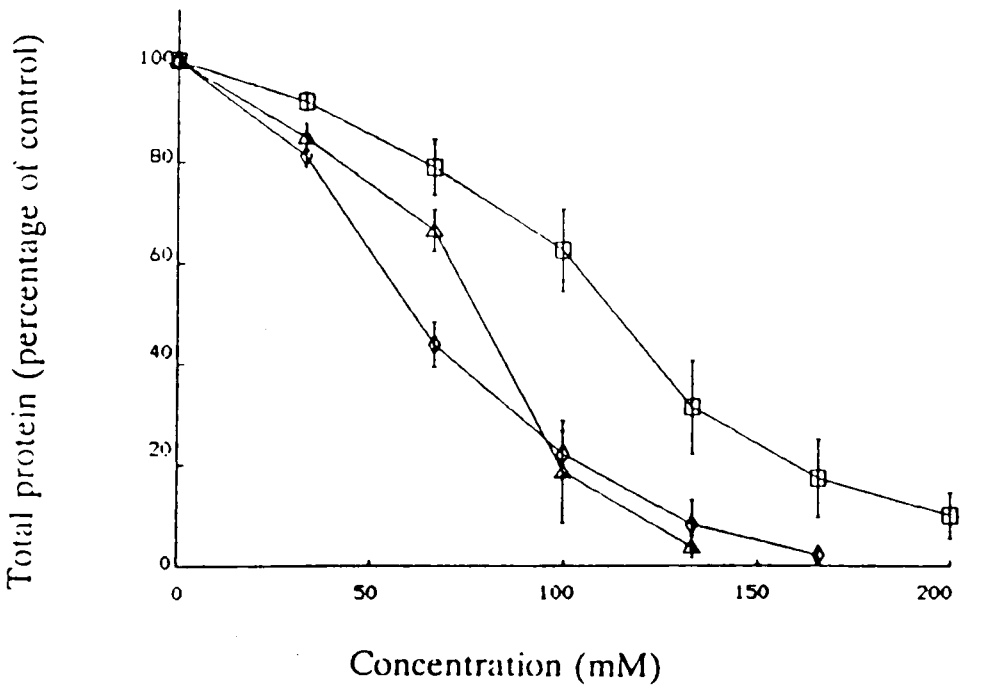


Figure 7.3(c)

Figure 7.3 *In vitro* toxicity of (a) methanol, (b) ethanol and (c) propan-l-ol in the FRAME KB cytotoxicity assay, on unsealed (□), oil-sealed (△) and film-sealed (◇) culture plates.

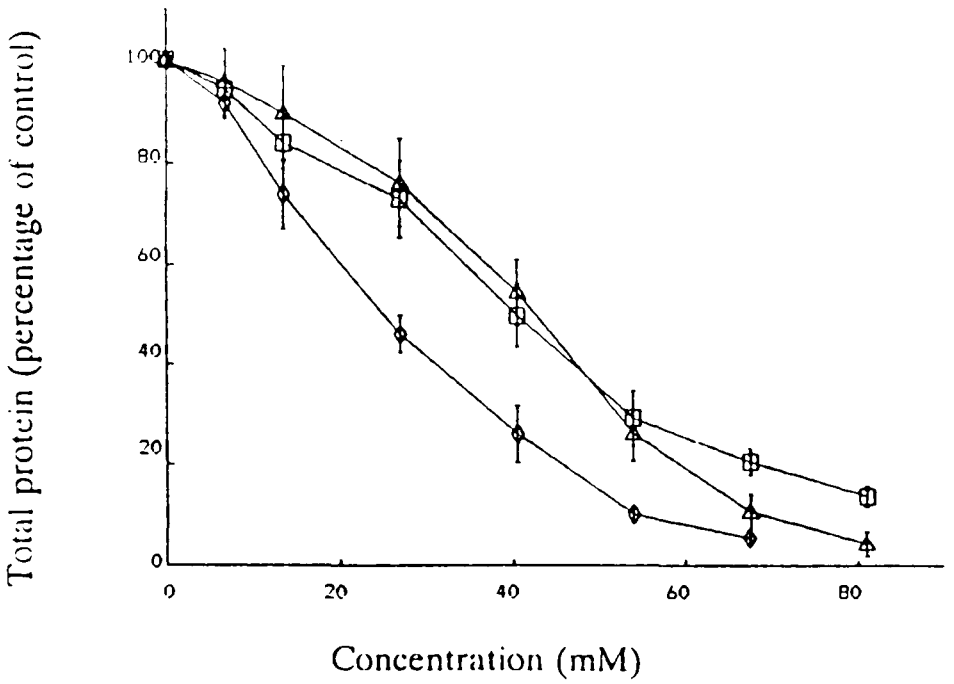


Figure 7.4(a)

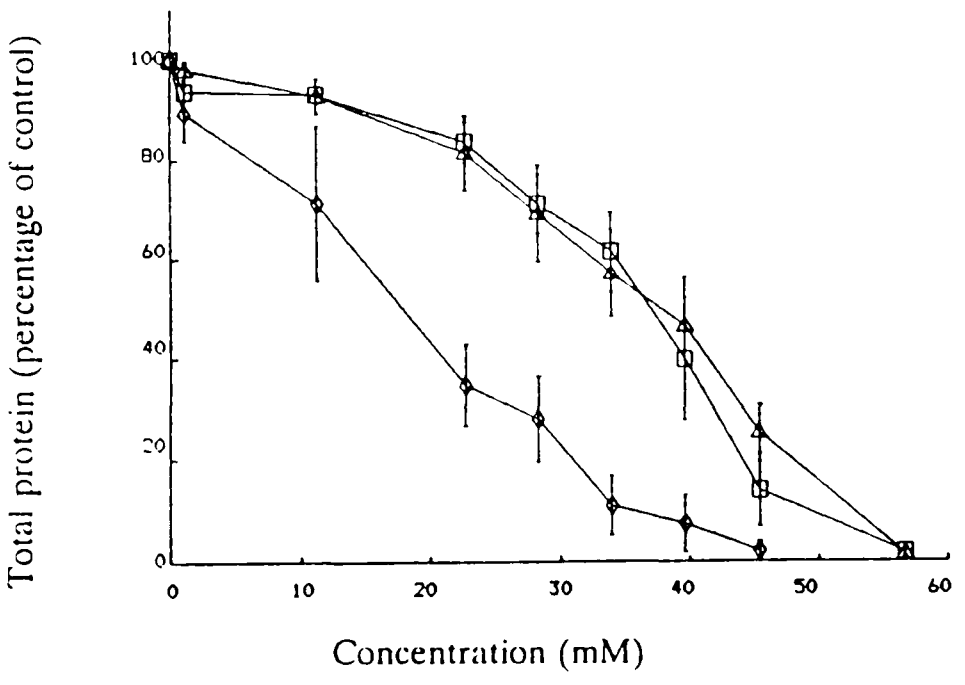


Figure 7.4(b)

Figure 7.4 *In vitro* toxicity of (a) butan-l-ol and (b) pentanol in the FRAME KB cytotoxicity assay, on unsealed (⊡), oil-sealed (△) and film-sealed (◇) culture plates.

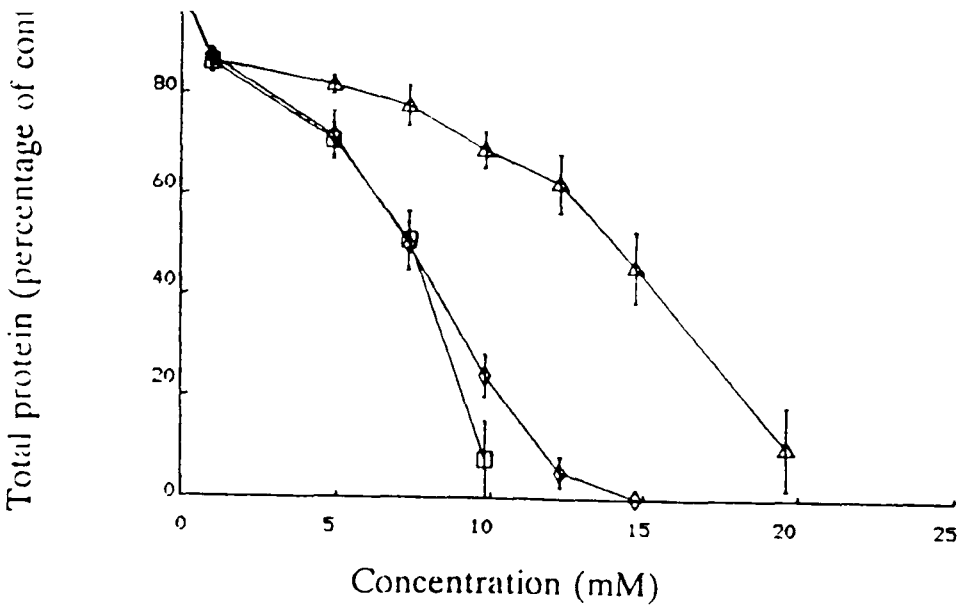


Figure 7.5(a)

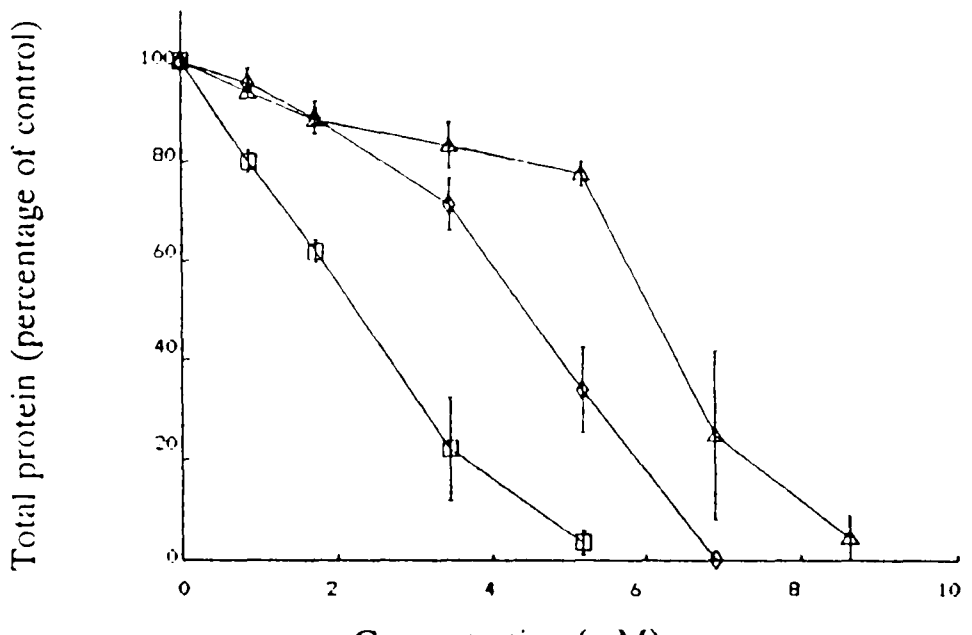


Figure 7.5(b)

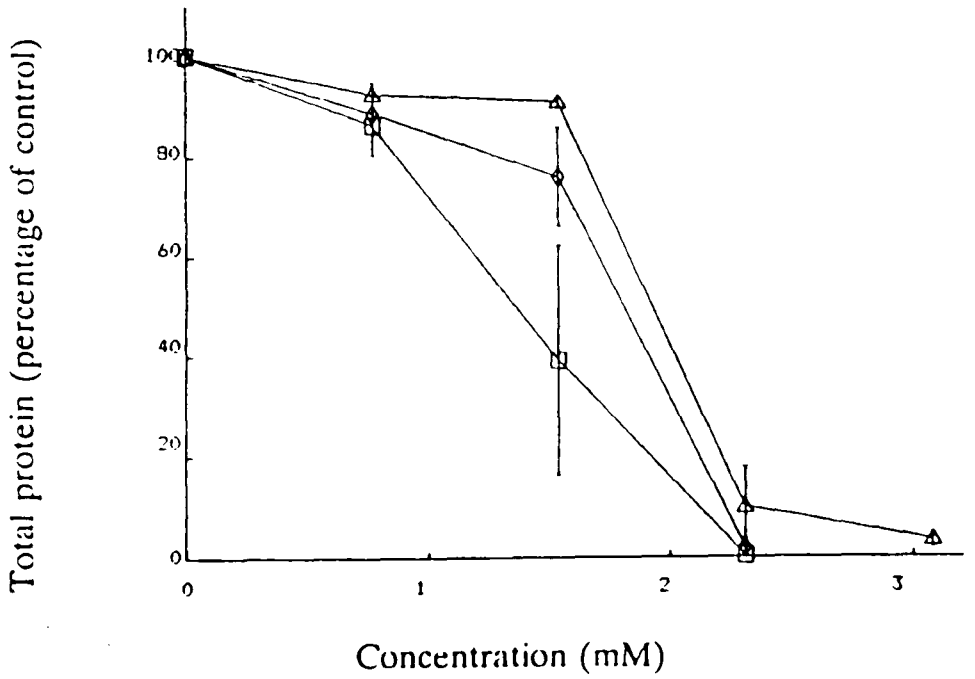


Figure 7.5(c)

Figure 7.5 *In vitro* toxicity of (a) hexanol, (b) heptanol and (c) octanol in the FRAME KB cytotoxicity assay, on unsealed (□), oil-sealed (△) and film-sealed (◇) culture plates.

the more volatile alcohols. The toxicities of the less volatile alcohols were not affected or were even reduced by the sealants, but the two sealants produced different levels of effects.

As the adhesive film did not show any different effects to those of the oil with regard to the toxicities of the more volatile alcohols, and as it is also inhibitory to cell growth and can adversely alter the toxicities of non-volatile chemicals, it was decided not to further evaluate its use.

7.2.4 Effects of the paraffin oil sealant on the *in vitro* toxicities of unrelated liquid chemicals

7.2.4.1 **Effects of the paraffin oil sealant on unrelated liquid chemicals**

A further nineteen unrelated liquid chemicals were tested on US and OS plates. Most of the liquids were selected from those previously tested by FRAME in the FRAME KB cytotoxicity assay (Clothier *et al.*, 1988).

As with the straight-chain alcohols, sealing the plates with oil altered the toxicity of some of the unrelated liquids (Table 7.9). The change in toxicity does not appear to be related to the actual ID_{50} concentration on US plates. Acetone and glycerol have the same toxicities in US plates (approximately 725mM), yet oil sealing has no effect on the toxicity of glycerol, but enhances the toxicity of acetone.

More liquids had their toxicity increased in the presence of the oil sealant (nine to a statistically significant degree) than had their toxicity significantly decreased (three) (Table 7.10). Liquids generally regarded as volatile (such as acetaldehyde and acetone) have their toxicities increased in the presence of the oil sealant, while those regarded as having low volatility (such as Table

7.9. *In vitro* toxicities of nineteen liquid chemicals on unsealed and oil-sealed culture plates

		ID_{20} mM ± s.e.m.	ID_{50} mM ± s.e.m.	ID_{80} mM ± s.e.m.
Tri-n-butyltin chloride	US	0.0006(n=1)	0.0007±0.0001	0.009±0.000
	OS	0.001 ± 0.001	0.002±0.000	0.003 (n=2)
Tween 20	US	0.10 ± 0.04	0.20 ± 0.04	0.29 ± 0.04
	OS	0.13 (n=2)	0.15 ± 0.04	0.27 ± 0.04
1,2,4-Trichloro-benzene	US	0.62 ± 0.05	0.72 ± 0.08	0.82 ± 0.08
	OS	0.63 ± 0.06	0.74 ± 0.10	0.88 ± 0.09
1,2-Dibromo-methane	US	1.8 ± 0.7	3.7 ± 0.4	4.0 (n=1)
	OS	1.8 ± 0.6	4.1 (n=2)	>5.3
Acetaldehyde	US	2.9 ± 0.7	6.0 ± 0.6	13 ± 1
	OS	0.72 (n=2)	0.81 ± 0.15	1.3 ± 0.2
Allyl alcohol	US	5.7 ± 1.2	9.4 ± 2.4	11 (n=2)
	OS	3.2 ± 0.6	4.7 ± 0.4	6.9 ± 0.4
Aniline	US	5.9 ± 1.8	10 ± 1	14 ± 2
	OS	7.0 ± 0.5	11 ± 1	15 ± 1
Sulphuric acid	US	8.7 ± 1.9	13 ± 0	15 (n=1)
	OS	9.3 ± 1.6	13 ± 0	>15
Trifluoroacetic acid	US	10 ± 2	18 ± 1	29 (n=2)
	OS	13 ± 2	23 ± 1	>26
Acetic acid	US	14 (n=2)	20 ± 1	25 ± 3
	OS	15 (n=1)	18 ± 2	24 ± 3
Pyrrole	US	37 ± 4	56 ± 3	71 (n=1)
	OS	18 (n=1)	26 ± 3	40 ± 1
Dimethylformamide	US	50 ± 6	108 ± 9	164 ± 12
	OS	69 ± 2	120 ± 9	180 ± 13
Hydrochloric acid	US	86 ± 10	126 ± 2	147 ± 3
	OS	83 ± 13	128 ± 0	142 (n=1)

Table 7.9. (contd)

		ID_{20} mM ± s.e.m.	ID_{50} mM ± s.e.m.	ID_{80} mM ± s.e.m.
2-Methoxyethanol	US	62 (n=2)	153 ± 7	223 ± 7
	OS	66 ± 17	150 ± 5	216 ± 8
Dimethyl-sulphoxide	US	148 (n=2)	229 ± 17	329 ± 16
	OS	186 ± 16	287 ± 14	483 ± 11
Ethyl acetate	US	187 ± 61	323 ± 13	359 ± 14
	OS	107 ± 21	228 ± 38	304 (n=2)
Glycerol	US	479 ± 183	708 ± 127	940 ± 137
	OS	438 ± 178	706 ± 126	967 ± 138
Acetonitrile	US	177 (n=1)	739 ± 174	842 (n=2)
	OS	57 (n=2)	137 ± 31	381 ± 54
Acetone	US	550 ± 11	774 ± 26	934 ± 26
	OS	134 (n=2)	249 ± 31	346 ± 19

US = Unsealed culture plates
OS = Oil-sealed culture plates

Table 7.10. ID_{50} values of twenty seven liquids on oil-sealed culture plates, expressed as percentages of ID_{50} values on unsealed culture plates

	$\frac{ID_{50}\text{ (oil-sealed)}}{ID_{50}\text{ (unsealed)}} \times 100\%$	
Acetaldehyde	14*	increasingly
Acetonitrile	19*	toxic on
Acetone	32*	oil-sealed
Pyrrole	46*	plates
Allyl alcohol	50*	
Methanol	52*	
Ethanol	54*	
Propan-l-ol	69*	
Ethyl acetate	71*	
Tween 20	75	
Acetic acid	90	
Butan-l-ol	93	as toxic on
Pentanol	97	unsealed as
Methoxyethanol	98	on oil sealed
Glycerol	100	
Sulphuric acid	100	
Hydrochloric acid	102	
1,2,4-Trichlorobenzene	103	
Aniline	110	
1,2-Dibromomethane	111	
Dimethylformamide	111	
Octanol	124	
Dimethyl sulphoxide	125	
Trifluoroacetic acid	128	decreasingly
Hexanol	192*	toxic on
Heptanol	274*	oil-sealed
Tri-n-butyltin chloride	286*	plates

* = ID_{50} of chemical on oil-sealed plates was significantly different from the ID_{50} on unsealed plates

glycerol and dimethyl sulphoxide) did not have their toxicities altered significantly.

Some of the liquids tested showed evidence of cross-contamination i.e., redissolving in surrounding, untreated culture wells and affecting their growth. This occurred most noticeably with acetaldehyde and pyrrole on unsealed plates, but was not apparent on oil-sealed plates. This lends support to the effectiveness of the use of oil as a sealant. Pyrrole and acetaldehyde are among the most volatile liquids tested, as judged by the increase in toxicity on OS plates (Table 7.10).

7.2.4.2 **Comparison of *in vivo* and *in vitro* toxicity of volatile liquids**

Rat oral and mouse i.p. LD_{50} data were available for 10 of the 27 liquids tested on US and OS plates (Table 7.11). When linear regression analysis was performed on logged data, the correlation coefficients for the KB ID_{50}/rat oral LD_{50} comparisons were 0.91 for the US ID_{50} values and 0.95 for the OS ID_{50} values. For the KB ID_{50}/mouse i.p. LD_{50} comparisons were 0.74 and 0.85 for the US and OS ID_{50} values, respectively.

7.2.4.3 **Comparison of new cytotoxicity data with published data**

Table 7.12 compares ID_{50} the results for 22 of the liquids, as published by FRAME (Clothier *et al.*, 1988) and those obtained at Northumbria Biologicals Ltd. The ID_{50} values are very similar in both laboratories, except for hexanol and heptanol, which were rather more toxic at Northumbria Biologicals Ltd.

7.2.4.4 **Conclusions**

As was found with the more volatile alcohols, the oil sealing increased the

Table 7.11 Comparison of *in vivo* and *in vitro* toxicity data for 10 liquids

	ID_{50} (mM) US plate	ID_{50} (mM) OS plate	Rat oral LD_{50} (mmol/kg)	Mouse i.p. LD_{50} (mmol/kg)
1,2-Dibromomethane	3.7	4.1	0.57	1.2
Allyl alcohol	9.4	4.7	1.1	0.7
Aniline	10	11	5.2	5.3
Propan-l-ol	114	79	323	52
Dimethyl sulphoxide	229	287	217	168
Ethyl acetate	323	228	125	8
Ethanol	418	224	289	130
Acetonitrile	739	137	91	8
Acetone	774	249	165	22
Methanol	1099	571	313	263

Table 7.12. Comparison of FRAME published ID_{50} values with those obtained at Northumbria Biologicals Ltd. (NBL) for twenty two liquid chemicals

	FRAME ID_{50} (mM)	NBL ID_{50} (mM)
Tri-n-butyltin chloride	0.001	0.0007
Tween 20	0.21	0.20
1,2,4-Trichlorobenzene	0.67	0.72
Octanol	2.3	1.7
1,2-Dibromomethane	3.4	3.7
Allyl alcohol	6.1	9.4
Heptanol	8.7	2.3
Acetaldehyde	11	6.0
Aniline	16	10
Acetic acid	21	20
Hexanol	35	7.3
Pentanol	40	37
Butan-l-ol	64	45
Pyrrole	98	56
Propan-l-ol	186	114
Dimethyl sulphoxide	287	229
Ethyl acetate	491	323
Acetonitrile	562	739
Ethanol	783	418
Glycerol	713	708
Acetone	617	774
Methanol	1441	1099

cytotoxicities of the most volatile liquids, presumably by reducing their evaporation and thus keeping higher concentrations in contact with the cells for longer than would have occurred in an unsealed plate. The most volatile liquid tested, acetaldehyde, had its ID_{50} decreased under oil by a factor of about seven. This will alter its toxicity rank position (in terms of ID_{50} (mM) value), and hence its relative toxicity compared to other chemicals. Therefore, acetaldehyde would change from being regarded as of low toxicity (result in US wells), to being of moderate toxicity *in vitro* (result in OS wells). However, other liquids did not have their toxicity altered to the same extent by the oil sealing, and therefore their rank position would not change as much.

Criteria need to be set, to allow a distinction to be made between chemicals whose toxicity was unchanged by a sealant, and those whose toxicity would be regarded as having been altered. Statistical tests can be employed, but may be misleading (for example, with sodium dodecyl sulphate). A reduction or increase of more than 25% in the mean ID_{50} is a strong indication that the toxicity has been altered by the sealing. In addition, if the ranges of individual ID_{50} values from the sets of experiments of unsealed and sealed plates do not overlap, then the toxicity may be considered altered. If the ranges do overlap then the toxicity may be regarded as unchanged.

Oil sealing, by virtue of slowing down evaporation, appears to be able to reduce or even prevent the phenomenon of cross-contamination of untreated or low-dose treated wells.

The *in vitro* toxicity results correlated well with rat oral and mouse i.p. values for the 10 liquids. There was no difference between correlation coefficients for comparisons using US and OS *in vitro* data and rat oral LD_{50} values. The mouse i.p. LD_{50}/KB ID_{50} correlation was slightly closer with OS *in vitro* data than with the US.

7.3 DISCUSSION

Sealing of culture plates was suggested by Knox *et al.* (1986) as a possible means of reducing intralaboratory and interlaboratory variations in FRAME KB cytotoxicity assay results for volatile liquids, such as acrylonitrile. Less variability would then permit greater confidence to be placed in assay results.

The introduction of 96-well plates, to replace 24-well plates in the assay probably helped to reduce intralaboratory variation, and possibly also interlaboratory variation. A new problem was introduced, however - that of well-to-well cross-contamination by volatile chemicals, as noted by Stark *et al.* (1986) and Clothier *et al.* (1988).

The two sealant procedures assessed for their ability to reduce variation in results, paraffin oil and adhesive film, were both easily adapted to the FRAME KB cytotoxicity assay protocol. They were found to be effective at reducing the evaporation of (and thereby increasing the toxicity of) the more volatile straight chain alcohols. However, the adhesive film was less suitable for general use than the oil, because it caused a slight inhibition of normal cell growth and also increased the toxicities of some solid (i.e. non-volatile) chemicals. The adhesive film appears to contain some kind of diffusible toxic substances, which were able to leach from the film during its incubation immersed in the medium at 37°C, and hence cause cell death. However, the film in use as a plate sealant does not come into direct contact with the culture medium (Figure 7.1). It is possible, therefore, that the humidified atmosphere between the culture medium and the film permitted the transfer of the leached toxic substances from the film into the medium. The slow-down of the cell growth rate in FS wells at 48 hours after sealing may have been due to a gradual build up of these toxins after the film was applied, until a threshold limit was reached between 24 and 48 hours, as this was where cell

division was first affected. It should be possible to develop a non-toxic adhesive film, if this was felt desirable (Srivastava *et al.*, 1990).

The adhesive film, by slowing the cell growth rate, may be placing the cells under stress, thus causing them to be more susceptible than normal to toxic insult from a test chemical. Alternatively, diffusible toxic substance(s) in the film may somehow potentiate the cytotoxicity of certain chemicals. It is not clear why only some of the solid chemicals tested were affected by the film. The increased toxicity of sodium hydroxide under both film and oil may be connected with the ability of the medium to buffer from alkaline pH back to normal pH on sealed plates. This could be examined by testing other alkaline chemicals which are likely to be toxic by altering the pH of the medium.

Neither sealant appeared able to improve experiment to experiment variation in ID_{20}, ID_{50} and ID_{80} results. The paraffin oil was effective in preventing cross-contamination of volatile chemicals into control wells, which occurred with some of the most volatile chemicals in unsealed plates. The oil also increased the toxicities of chemically-unrelated volatile liquids, while not altering the ID_{50} values of non-volatiles (such as DMSO).

The paraffin oil may have reduced the toxicities of hexanol and heptanol by adsorbing a proportion of these hydrophobic alcohols, thus reducing the concentration of alcohol in the medium. This also may have occurred with butan-l-ol and pentanol, but, by a combination of reducing their evaporation and partitioning into the oil, the overall toxicities were not affected. Several of the unrelated chemicals, whose ID_{50} values were unaltered on OS plates, may have undergone the same effect as was postulated for hexanol and heptanol, i.e. the oil was responsible for both keeping chemical in the culture well (by preventing its evaporation), and removing it (by partitioning). This may be more prevalent with hydrophobic liquids such as 1,2,4-

trichlorobenzene. Overall, the paraffin oil was the better of the two sealants for incorporation into a modified FRAME KB cytotoxicity assay.

The type of liquids whose toxicities would be significantly increased by the oil sealant constitutes a small proportion of the general population of test chemicals. However, liquid chemicals predominate in the group which are tested for irritancy. Some liquids will have their cytotoxicity altered to a greater degree than the others. It is important that this group can now be handled in the FRAME KB cytotoxicity assay, with greater confidence that their toxicities will not be underestimated.

In terms of the variation of results between laboratories, evaporation may still be of importance. Evaporation could occur after culture plate treatment, or as a result of methods of dose-range preparation (Table 7.1). This could only be resolved by further experiments in a number of laboratories and by the use of very specific protocols. It is possible that the use of 96-well plates alone could reduce both intralaboratory variation and lack of confidence in results obtained with 24-well plates (Knox *et al.*, 1986).

The sealing of 96-well plates with paraffin oil sealing may not therefore be useful in the context of reducing experiment to experiment variation with volatile chemicals, because this variation does not appear to be as great a problem with 96-well plates than was seen with 24-well plates. The oil may be effective in preventing well-to-well cross-contamination by volatiles, but it is also possible to achieve this by arranging treated wells to be surrounded by untreated wells.

The small number of liquids in the *in vivo/in vitro* comparison does not permit firm conclusions to be drawn. There was an uneven spread of toxic potency amongst the 10 liquids in the comparison (most were of low toxicity), thus

decreasing the validity of the comparison. It is therefore not possible to deduce whether the enhanced *in vitro* toxicity of some liquids on OS plates would give a more accurate prediction of *in vivo* lethal potency. A larger number of liquids, with a greater range of potencies and with available *in vivo* data, need to be examined in order to answer this question.

The similarity of results between FRAME and Northumbria Biologicals Ltd. is surprising because of several differences in the way the assays were performed at the two locations. For example, cell seeding density, medium manufacturer, incubator type and assay operator were different. It is therefore likely that the one important common factor, namely, the manufacturer and size (96-well) of the culture plates, was significant in keeping the interlaboratory variation low, particularly with the liquids (Table 7.11). The robustness and reproducibility of the FRAME KB cytotoxicity assay is also confirmed.

Finally, it can be concluded that paraffin oil sealing can be effective in reducing the evaporation of very volatile liquids from 96-well culture plates, so that there is less likelihood that the cytotoxicities of these liquids will be underestimated. It would be useful to include the use of the oil sealant in the FRAME KB cytotoxicity assay protocol whenever suspected volatile liquids require testing. The paraffin oil method could also be readily adapted to other cytotoxicity assays which use 96-well plates.

CHAPTER 8 AN EVALUATION OF THE F9 EMBRYONAL CARCINOMA CELL LINE FOR USE IN *IN VITRO* TOXICITY TESTING

8.1 INTRODUCTION

8.1.1 Current status of teratogenicity testing

Many *in vitro* cytotoxicity tests have been developed during the past 15 years. A number employ measurement of cell death or inhibition of cell growth as their endpoint and have been proposed as alternatives to various *in vivo* toxicity tests, e.g. the Draize rabbit eye irritancy test (Borenfreund and Borrero, 1984; Reinhardt, 1990), rodent oral LD_{50} tests (Ekwall, 1983b) and *in vivo* tests for teratogenicity (Pratt and Willis, 1985). The potential uses of one such *in vitro* test, the FRAME KB cytotoxicity assay, have already been discussed in chapters 3 to 7.

Toxicology, however, is not only concerned with extreme, acute effects leading to cell or organism death. Many subtle, sublethal effects can be exerted by chemicals, causing dysfunction of one or more processes. Two examples of such effects are mutagenesis and teratogenesis. Several *in vivo* and *in vitro* methods exist for the detection of mutagenic changes induced by chemical (or other) agents. Regulatory bodies have accepted the validity of some of the *in vitro* mutagenicity tests, such as the Ames test (Ames *et al.*, 1975). The use of such tests has contributed to a reduction in the use of animals in this area of toxicology (van den Heuvel and Fielder, 1990). The search for acceptable *in vitro* alternative tests for potential teratogens, however, continues, but progress has been hampered for a number of reasons, related both to scientific opinion and the interactive nature of teratogenicity.

Scientific and regulatory opinion has been very slow to accept that *in vitro* techniques have a place in toxicology, least of all in teratology. In addition, it seems that *in vitro* teratologists have been reluctant to tackle the "real" problems in human reproductive and developmental failure, and little visible

progress has been made by utilising *in vitro* studies (Brown, 1991). Teratogenesis itself is a highly complex phenomenon and is difficult to define precisely. Once considered as the induction of gross morphological abnormalities in a fetus during pregnancy (Lansdown, 1988), it is now regarded as encompassing a wider range of effects (Table 8.1; Timbrell 1982) and has been placed under the umbrella term of developmental toxicity. Several mechanisms of teratogenesis have been elucidated (Table 8.2), but their causes are not necessarily due to the presence of an exogenous agent (chemical, virus, physical phenomenon). The lack of certain nutritional substances, such as glucose in diabetic mothers, is also known to cause birth defects (Lansdown, 1988). This diversity of mechanisms is itself a stumbling block toward the development of successful *in vitro* teratogenicity tests, because the test (or battery of tests) should be able to detect effects based on all these mechanisms (Daston and D'Amato, 1989).

Current *in vivo* reproductive and developmental toxicity studies are based on tests recommended by the USA Food and Drug Administration 25 years ago (FDA, 1966). Few modifications to this system have occurred since then. The Segment II phase remains the standard teratogenicity protocol (Schmid, 1987). This requires the sacrifice of over 100 pregnant laboratory animals per test chemical, the fetuses of which are examined for embryotoxicity and gross abnormalities. This system has a number of drawbacks. It is lengthy, labour intensive, expensive, and could not realistically be used to test the thousands of chemicals in use today, for which no developmental toxicity data are available (Daston and D'Amato, 1989).

In circumstances where teratologists cannot even agree on terminology (Neubert *et al.*, 1985; Broecker and Sowinski, 1987), it is not easy to engender serious consideration for alternative tests. But the climate of uncertainty, coupled with the urgent need to provide data on previously untested

Table 8.1 Examples of adverse developmental events in humans

Intrauterine growth retardation

Death:

- of embryo (miscarriage)
- stillbirth
- neonatal

Morphological malformation, e.g.:

- palate anomalies
- skeletal abnormalities
- cardiac defects
- facial dysmorphia
- urogenital defects

Functional disorder, e.g.:

- mental retardation
- cerebral palsy
- psychoses
- epilepsy
- neurological abnormalities
- immunological dysfunction
- hormonal dysfunction
- metabolic dysfunction

Adapted from Schardein and Keller (1989)

Table 8.2 Mechanisms of teratogenesis

Mutation

Chromosomal aberrations

Mitotic interference

Alterations of nuclei acid synthesis and function

Lack of precursors, substrates and coenzymes for biosynthesis

Alteration of energy sources

Enzyme inhibition

Osmolar imbalance

Alteration of membrane characteristics

Other

From Beckman and Brent (1984)

chemicals, makes the investigation and validation of more-rapid alternative tests all the more important. No *in vitro* teratogenicity test has been adequately validated for regulatory purposes (Fielder, 1991) but a small number are used by pharmaceutical companies in-house, to select drugs for further development (Duffy *et al.*, 1991; Zijlstra, 1991), and some *in vitro* techniques are important in mechanistic studies (Peters and Piersma, 1990). Recently, progress has been made toward harmonising regulatory requirements for *in vivo* teratogenicity tests, and this will hopefully lead to a reduction in the wasteful practice of duplicating experiments for legal purposes (Duffy *et al.*, 1991).

8.1.2 *In vitro* approaches in teratology

Our incomplete understanding of the biochemical regulation of normal embryonic and fetal development has hampered research into the mechanisms of teratogenesis. Hence, the investigation of teratogenicity *in vitro* is hindered by a lack of basic knowledge of how well the *in vitro* models of development mimic the equivalent processes *in vivo*. Many such models exist, but only some of them are suitable for use in toxicological studies. Some consider that it is desirable, but not essential, for an *in vitro* teratogenicity test system to model one or more of the processes of embryonic development (Kimmel *et al.*, 1982). However, others (e.g. Flint, 1991) argue that mechanistic relevance to the *in vivo* situation is a pre-requisite for all *in vitro* toxicity tests.

Many *in vitro* teratogenicity tests have been proposed (Table 8.3), based on developmental models utilising dissociated cells through to complete embryos. Only those with close mechanistic similarities to mammalian embryogenic processes have generated serious interest (Brown, 1987; Neubert, 1989; Duffy *et al.*, 1991). These are the rat whole embryo culture technique (Sadler and Warner, 1984) and the rat embryo mid-brain and limb-bud cell micromass

Table 8.3 Alternative tests for teratogenicity

A. Established cell lines

Mouse ovarian tumour (MOT) cell attachment

Human embryonic palatal mesenchyme (HEPM)

Neuroblastoma differentiation

V79/HEPM intercellular communication

Pox-virus morphogenesis

B. Primary embryonic cell cultures

Drosophila neuroblasts and myoblasts

Rat embryo micromass - limb bud

Rat embryo micromass - neural crest

Chick embryo neural retina

C. Embryonic organ culture

Limb bud (mouse or chick)

Neural tube (chick)

D. Whole embryo culture

Mammalian whole embryo culture

Chick embryo culture (CHEST)

Frog embryo culture (FETAX)

Fish embryo culture

Hydra 'embryo' culture

Drosophila larvae culture

Planaria regeneration

Sea urchin embryo culture

Shrimp larvae culture

Adapted from Faustman (1988) and Daston and D'Amato (1989)

method (Flint, 1987). These techniques are undergoing interlaboratory validation (Parsons *et al.*, 1990; Piersma *et al.*, 1990; Uphill *et al.*, 1990) and may gain wider acceptance for use as pre-screens, as well as in studies of mechanisms of action of teratogens (Neubert, 1989).

The rat whole embryo method has an advantage, in that, although the culture period is limited, all the basic processes of development are represented (Daston and D'Amato, 1989). The embryos in culture develop in a way comparable to those *in utero*, although growth is retarded. However, this is a highly specialised technique, still requires the use of many pregnant rats to study one compound, and is classed as an animal experiment in Britain.

The micromass method uses primary cultures of rat embryo mid-brain and limb-bud cells as models of embryonic cell growth and differentiation. The cells multiply and differentiate into neurons and chrondocytes, respectively. The effects of a test chemical on inhibiting these processes are measured, and the relationship between the dose affecting growth on the one hand, and differentiation on the other, indicates the potential teratogenicity of the chemical. This method, also, requires a high degree of technical skill and uses pregnant rats. It would therefore be advantageous if a model of cell growth and differentiation which used a permanent cell line could be employed. Mummery *et al.* (1984) proposed the use of a neuroblastoma cell line, which could be induced to differentiate morphologically by removing bovine serum from the culture medium. Both induction and inhibition of differentiation were measured, and the assay was moderately successful at distinguishing teratogens from non-teratogens. However, a drawback of the method is the single, morphological endpoint, which is scored subjectively. This would put the test at risk of poor interlaboratory reproducibility.

8.1.3 Embryonal carcinoma cells

Teratocarcinoma, or embryonal carcinoma (EC), cells have been widely used as *in vitro* models of embryogenesis (Martin, 1975; Martin, 1980; Strickland, 1981). The use of EC cells in *in vitro* models of differentiation for teratogenicity studies has been proposed recently (Piersma *et al.*, 1988). Mouse embryo-derived EC cells can be induced to demonstrate certain characteristics also found in the early mouse embryo (Sherman, 1986). Hence, the effect of test chemicals on the appearance or degree of expression of these characteristics can be investigated. EC cells have the advantage of being permanent cell lines, removing the need for animal sacrifice. Their method of culture is fully defined, cellular characteristics are well documented, and the costs are lower than for primary cultures. Disadvantages include: their transformed nature; their lack of ability to metabolically activate chemicals (such as cyclophosphamide) whose metabolites exert the toxic effects *in vivo*; their murine, rather than human, origin; and the fact that certain EC lines need an added chemical if they are to be induced to differentiate.

One EC line which has been studied in detail as a model of mouse embryogenesis, is the F9 mouse testicular teratocarcinoma (Bernstine *et al.*, 1973; Hogan *et al.*, 1983). F9 cells were considered nullipotent (i.e. unable to differentiate) until it was discovered that retinoic acid (RA) induced them to differentiate into endoderm-like cells (Sherman and Miller, 1978; Strickland and Mahdavi, 1978). There is an extensive literature on F9 cells, and the cell line is available through the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK). Several marked biochemical, as well as morphological, changes occur during F9 cell differentiation (Strickland *et al.*, 1980). As well as parietal and visceral endoderm-like cells, it has also been suggested that neuron-like cells may be formed (Kuff and Fewell, 1980; Wartiovaara *et al.*, 1984), although this is less well documented. Recent

studies using F9 cells have included an evaluation of gene transcription during RA-induced differentiation (Rickles *et al.*, 1989).

The F9 cell line was therefore evaluated for potential use in *in vitro* teratogenicity or developmental toxicity studies, as part of the work for this thesis. The initial phase of the evaluation consisted of determining F9 cell growth characteristics, the establishment of a method for reproducible differentiation of a high proportion of the cellular population, and the establishment of reliable assay methods for measuring cell growth and the chosen biochemical marker of the degree of differentiation (production of laminin). A small number of chemicals (some with well-known teratogenic properties) were then tested for their cytotoxic effects on undifferentiated cells. The differential effects of selected chemicals on cell growth and the production of laminin by differentiating and differentiated cells were also examined.

8.2 RESULTS

8.2.1 Initial Experiments

8.2.1.1 **Establishment of the growth characteristics of F9 cells**

In general, the F9 EC cell line does not require any exceptionally specialised culture techniques. However, they do require more frequent attention than the 3T3-L1 mouse embryo fibroblast line used in the FRAME KB cytotoxicity assay (Clothier *et al.*, 1988). For the F9 cells to grow in monolayers, culture surfaces had to be pre-coated with 0.1% (w/v) gelatin solution (Rudnicki and McBurney, 1987). The culture medium contains 10% (v/v) fetal calf serum (Daly and Redfern, 1987) rather than the 10% (v/v) new-born calf serum which is sufficient for 3T3-L1 cells. Anti-fungal chemicals, such as Fungizone,

must not be added to the F9 culture medium, as they can retard growth at their effective concentrations (Rudnicki and McBurney, 1987). This resulted initially in frequent fungal contamination of cultures. The problem was overcome by improving basic laboratory management, such as increasing the regularity and extent of cleaning of incubators and work-surfaces with methanol, and by instigating more-rigorous standards of sterile technique when handling the cells.

F9 cells grew unreliably from seeding densities of less than 1 x $10^4/cm^2$, and, when seeded at lower densities, required a large surface area (greater than $10cm^2$) to provide a growing culture. Once a culture was established, however, the cells had a rapid growth rate, with a cell cycle time of approximately 14 hours, and hence were subcultured more frequently than 3T3-L1 cells, which have a cell cycle time of approximately 22 hours. Stock flasks were subcultured three times a week, as it was found that cultures were difficult to initiate from flasks that had been left confluent for more than one to two days.

8.2.1.2 **Establishment of a protocol for the differentiation of F9 cells**

For an F9 cell assay to be useful as a model of cell differentiation in *in vitro* toxicology testing, a reliable and reproducible protocol for inducing a high proportion of the cells to differentiate was required. The proportion should ideally be at least 70%, to allow for the detection of variations in the level of differentiation. A number of protocols for inducing morphological and biochemical differentiation of F9 cells have been published (see Strickland and Mahdavi, 1978; Moore *et al.*, 1986; Rudnicki and McBurney, 1987; Williams *et al.*, 1987). All the protocols require RA and some also use dibutyryl cyclic adenosine monophosphate (dbcAMP).

In early experiments, F9 cells were seeded at 1 x $10^4/cm^2$ in 24-well plates, in

order that growth could be followed for more than three days. The cells were treated 24 hours after plating with culture medium containing RA at 0.001, 0.01, 0.1 or 1μM, with or without 1mM dbcAMP. After 5 days of growth, during which time the medium was changed with fresh medium plus RA, and with added dbcAMP as appropriate, 0.001μM and 0.01μM RA alone were found to be ineffective in inducing significant morphological changes in the F9 cells. However, both 0.1μM and 1μM RA were equally effective, and caused noticeable morphological changes after 48 hours of treatment. The cells altered from the normal phenotype of small and round cells (tightly packed at confluency) (Figure 8.1) to a spindle shape, some with long processes (Figure 8.2). Neighbouring cells did not adhere closely, and the long processes sometimes overlapped, as previously described (Strickland and Mahdavi, 1978; Kuff and Fewell, 1980; Linder *et al.*, 1981; Moore *et al.*, 1986). The proportion of cells undergoing these changes was over 50%, but was difficult to assess accurately by phase-contrast microscopy. Treatment of cells with 0.1μM or 1μM RA plus 1mM dbcAMP induced the same type of morphological changes as had been induced by 0.1μM or 1μM RA alone, i.e. there was no synergistic effect and no increase in the proportion of cells induced to differentiate. 1mM dbcAMP alone caused no alterations in F9 cell morphology, but inhibited growth over 6 days, by up to 20% compared to untreated cultures. In addition, exposure of cells to 0.1μM or 1μM RA plus 1mM dbcAMP for 6 days caused growth inhibition by over 50%. In comparison, 0.1μM or 1μM RA caused no growth inhibition after 6 days. As it did not appear to offer any advantages, and in view of its toxic effect and high cost, dbcAMP was not included in the subsequent protocols for F9 cell differentiation.

The minimum concentration of RA required to induce satisfactory morphological changes was therefore 0.1μM. The lowest possible concentration was desirable, to minimise unwanted toxic effects which resulted

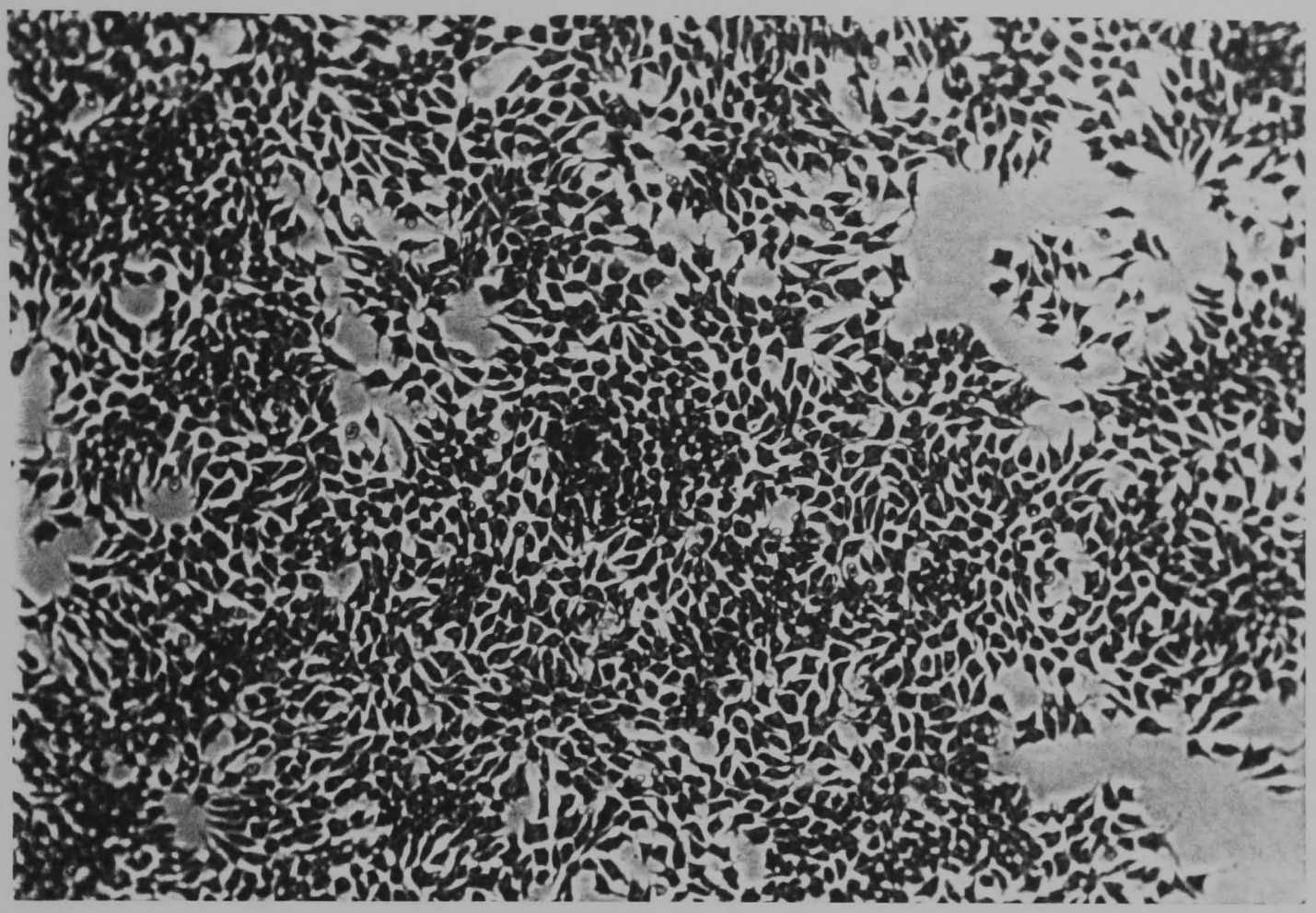


Figure 8.1 Undifferentiated F9 cells stained with Kenacid Blue R. Magnification x27

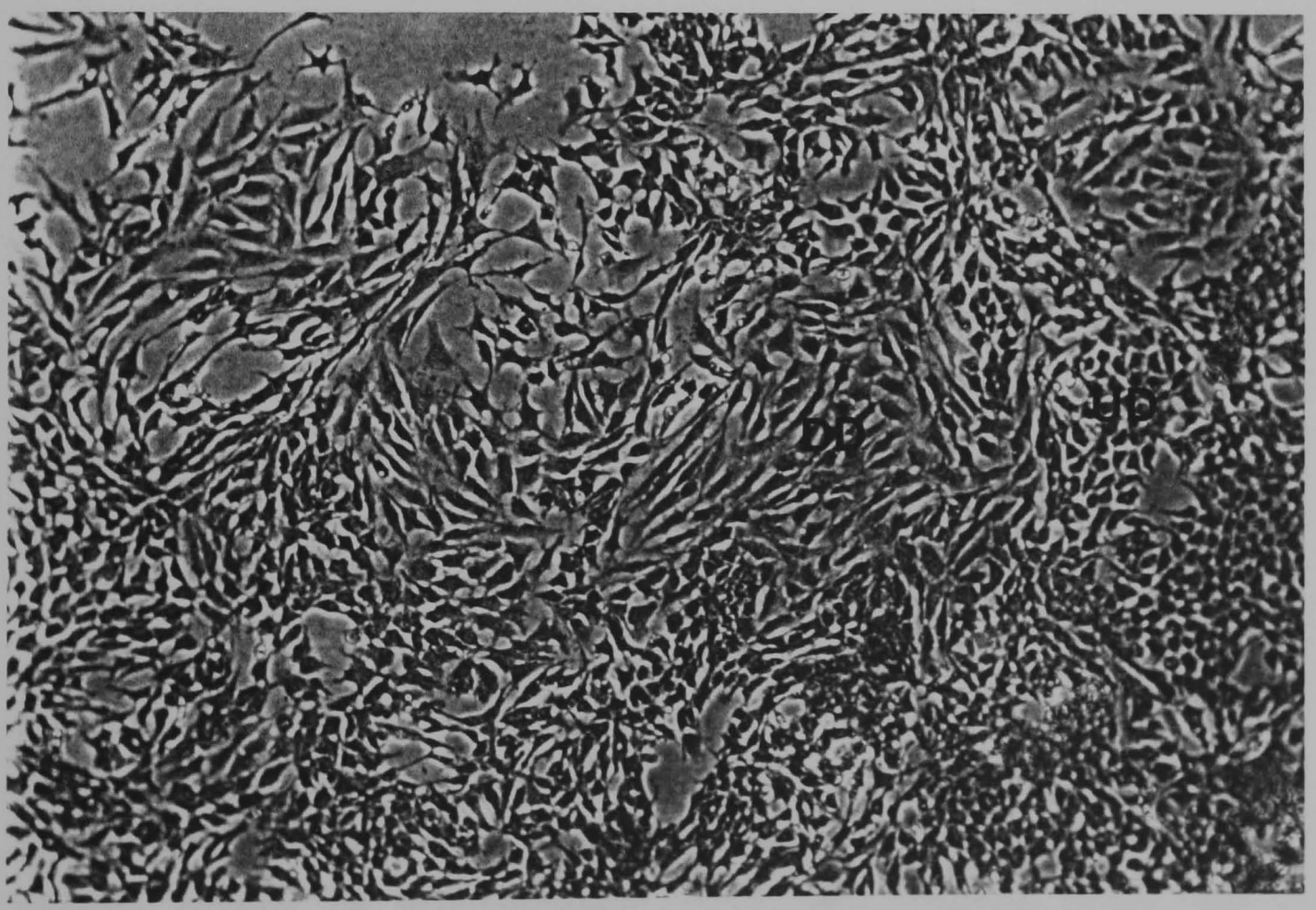


Figure 8.2 F9 cells undergoing differentiation in the presence of 0.1μM RA, stained with Kenacid Blue R. Magnification x27

in, for example, reduced growth rate (possibly causing differentiation in itself), morphological changes not associated with differentiation, or biochemical changes due to toxic perturbations. The concentration of RA which caused 20% inhibition of growth in the cytotoxicity assay was approximately 10μM. Phenotypic changes were visible after treatment of growing cultures with 0.1μM RA for 48 hours. During this first 48 hours, the F9 cells were undergoing the initial steps in the differentiation pathway (Williams *et al.*, 1987). The time period for the cytotoxicity assays was also 48 hours, due to the cell cycle time. Although no detectable laminin was produced by the cells during this time, important subcellular events were almost certainly occurring; for example, induction of mRNA for laminin subunits (Durkin *et al.*, 1986). F9 cells which had been seeded at 2.5 x $10^4/cm^2$ in 24-well plates, allowed to adhere overnight and then treated with fresh medium containing 0.1μM RA, were termed differentiating (DG) cultures.

It was found that by seeding cells in 75cm^2 flasks in medium containing 0.1μM RA, and then subculturing into fresh 0.1μM RA-containing medium at day 3, almost 100% morphological differentiation of cells could be reproducibly achieved by day 6. This method is very similar to the protocol described by Rudnicki and McBurney (1987). The treated cells could then be trypsinised on day 6 or 7 and seeded at 5 x $10^4/cm^2$ in 0.1μM RA-containing medium in 24-well plates. Morphological changes were not lost, and the cells continued to multiply over the following 72 hours (Figure 8.3). Cultures set up in this way were termed differentiated (DD).

8.2.1.3 **Establishment of cytotoxicity assay protocol**

Having determined the culture conditions required to obtain reproducible, growing cultures of undifferentiated (UD), differentiating (DG), and differentiated (DD) F9 cells, cytotoxicity assay protocols were developed.

These were based on the FRAME KB cytotoxicity assay, which measures inhibition of cell growth as the end point. The F9 cells could not be fixed consistently *in situ* on 96-well plates with standard KB assay fixative and, as a result, the cytotoxicity assay could not be miniaturised as has been achieved in the FRAME KB cytotoxicity assay using 3T3-L1 cells (Clothier *et al.*, 1988).

Hence, gelatin-coated 24-well plates were used. 24-well plates were advantageous when DG and DD cells were tested. They facilitated culture inspection by phase-contrast microscopy, and provided a suitable volume of culture medium for the subsequent assay of laminin production.

Both neutral red uptake (Borenfreund and Puerner, 1985) and KB staining (Knox *et al.*, 1986) were evaluated for the measurement of final cell number, this being the endpoint parameter of the cytotoxicity test. F9 cells took up little neutral red stain, possibly due to a low lysosome number. The KB stain binds to protein, and therefore measures relative total cellular protein. The intensity of KB staining of protein shows a linear relationship with BCL-D1 cell number and is known to be reproducible (Knox *et al.*, 1986).

F9 cells were stained efficiently with KB, and there was a constant relationship with cell number and with total protein until the cultures were just sub-confluent (Figure 8.3). Hence KB staining was chosen for the assay of relative total cellular protein in UD cultures.

In DD cell cultures stained with KB, the cells exhibiting altered morphology were often a much paler blue than UD cells, as observed by inverted microscopy. The KB stain estimates relative total cell number by assuming that each cell takes up an equal amount of stain. In homogeneous cultures, this holds true (for example, with UD F9 cells, Figure 8.1). However, the F9 cell cultures undergoing differentiation were often a heterogeneous mixture of

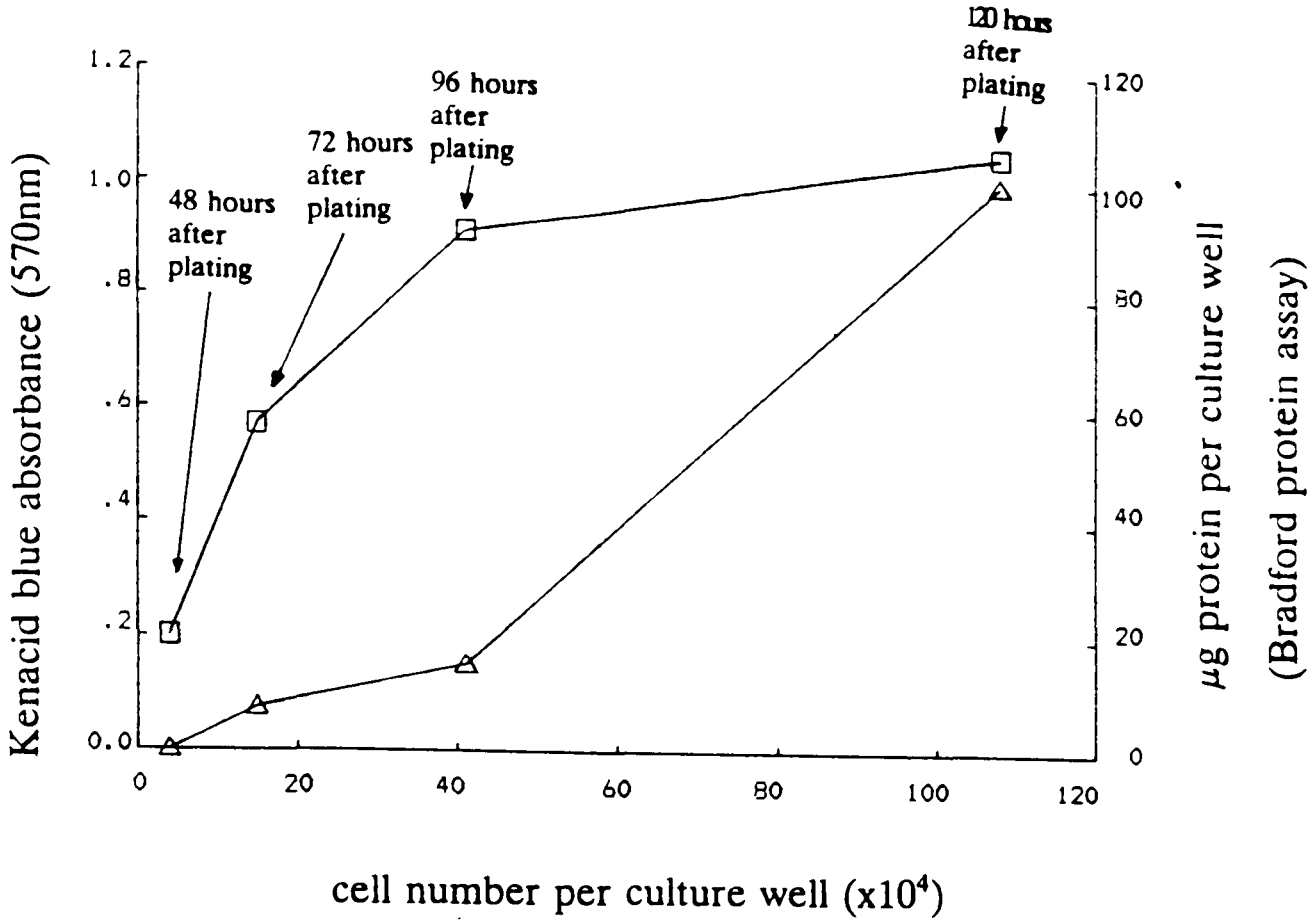


Figure 8.3 Growth of undifferentiated F9 cells over 72 hours, with comparison of the Kenacid blue protein assay (□) and Bradford protein assay (△) as measures of relative cell number.

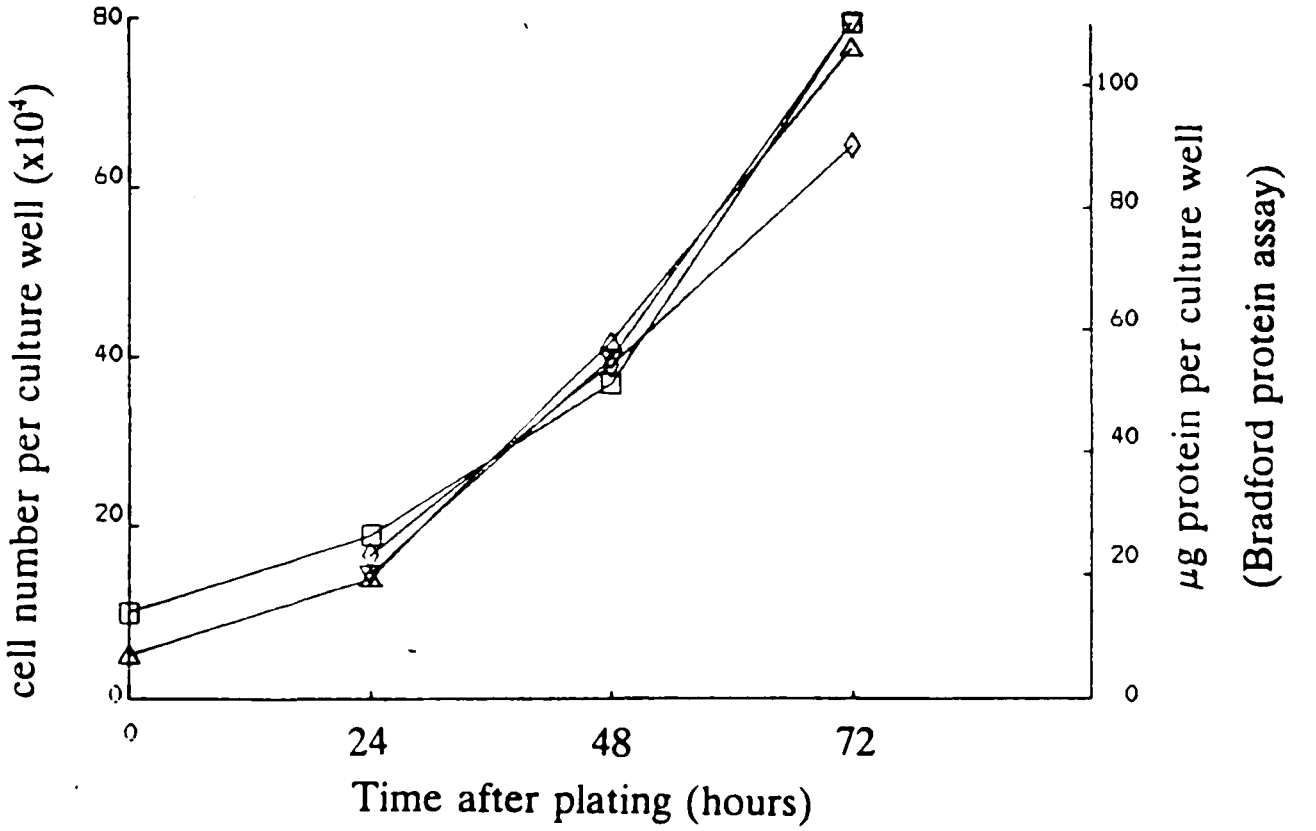


Figure 8.4 Growth of differentiating and differentiated F9 cells, measured by cell counting and the Bradford protein asay.

△ differentiating cells, cell count
▽ differentiating cells, Bradford assay
□ differentiated cells, cell count
◇ differentiated cells, Bradford assay

UD, DG and DD cells, which showed different morphologies and intensities of KB staining (Figure 8.2). Although the paler staining in DD cells may have been due to the cells being flatter and thinner than UD cells, there was also the possibility that DD cells took up less stain per cell than UD cells. In order to eliminate any potential inaccuracies caused by unequal staining, an alternative total protein assay was sought.

The Bradford protein assay method (Bradford, 1976) has been used reliably and successfully in cytotoxicity assays; for example, with V79 cells (Garle *et al.*, 1987) and Balb/c 3T3 cells (Shopsis and Eng, 1985). It was therefore evaluated with growing UD, DG and DD F9 cultures. UD F9 cells were seeded at low density (0.4 x $10^4/cm^2$) into 24-well plates, and quadruplicate wells were counted for cell number, and assayed for protein by the KB and the Bradford methods, daily for 7 days. The cultures were confluent at day 5, but continued to divide and pile up after this time. Over the exponential growth phase (prior to confluency), the Bradford assay was shown to be equivalent to the KB assay (Fig 8.3) and both provided a good estimation of relative cell number. At the later time points, the Bradford assay was found to provide a more accurate assessment of total cell number than the KB assay, possibly due to the inability of the KB dye to penetrate confluent, piled up cells.

DG and DD cultures were prepared as described in section 8.2.1.2, and triplicate wells were counted for cell number and assayed for protein by the Bradford method at 24, 48 and 72 hours after plating. The Bradford protein assay was a good estimator of relative cell number (Fig 8.4).

In summary, for cytotoxicity tests with all three cell types, cells were seeded, given 24 hours to attach and then treated with test chemicals for 48 hours. DG and DD cells were treated with test chemicals dissolved in medium containing 0.1μM RA. The RA was used to induce differentiation for DG

cultures, and to maintain and continue the process of differentiation in DD cultures. DD cells were seeded at a higher density than those for UD and DG cultures because of their initial slower initial division rate (Figure 8.4). UD cultures were assayed for protein by KB staining, while DG and DD cultures were assayed using the Bradford protein method (see section 2.1.3.2).

8.2.1.4 **Establishment of protocol for measuring laminin production by F9 cells**

It was very difficult to judge accurately the proportion of non-differentiated to differentiated cells in a culture well, and hence it was important to find a non-subjective (i.e. quantitative) method of assessing the extent of differentiation in a culture.

At the same time as differentiating morphologically, the F9 cells also alter biochemically in several ways (Moore *et al.*, 1986). One of the alterations which is relatively simple to assay is the production of laminin. Laminin is produced *de novo* during differentiation (Moore *et al.*, 1986) and is released into the culture medium. Using this biochemical indicator has the advantage that cells need not be disrupted in order to monitor the quantity produced. An enzyme-linked immunosorbent assay (ELISA) protocol was developed to measure laminin production by UD, DG and DD cell cultures. The protocol for the ELISA was based on that of Williams *et al.* (1987); see section 2.1.3.4.

8.2.1.5 **Conclusions**

F9 cells can, with attention to certain culture requirements, be grown in a reliable way from a constant seeding density. The addition of 0.1μM RA to the culture medium could be used to reproducibly induce the cells to alter morphologically. A high proportion of the cells were seen to change their

phenotype when they were seeded and then subcultured in 0.1μM RA-containing medium (as in DD cultures). As these methods closely matched published protocols, and as the morphology of altered cells was also very similar to descriptions in the literature, it was concluded that the cells had been induced to differentiate by the RA.

8.2.2 Cytotoxicity tests with UD F9 cells

8.2.2.1 *In vitro* toxicities of 24 chemicals to F9 cells

Twenty-four chemicals were tested for cytotoxicity to UD F9 cells over a 48 hour exposure period, using the KB assay. The chemicals were selected because of their *in vitro* toxicity values to 3T3-L1 cells in the FRAME KB cytotoxicity assay (Clothier *et al.*, 1988), and included chemicals recommended for use in validation trials of *in vitro* tests for teratogenicity (Smith *et al.*, 1983). Six of the chemicals were subsequently tested on DG and DD cultures.

The ID_{20}, ID_{50} and ID_{80} values for the chemicals are shown in Table 8.4. Good reproducibility was obtained, as is indicated by the small standard errors. The toxicity curves for some of the chemicals were not linear, making the ID_{80} value difficult to ascertain. With phenytoin and thalidomide, solubility problems prevented estimation of the ID_{80}. A number of the more toxic chemicals gave ID_{80} values in excess of fifty times the ID_{50} values, most notably 5-fluorouracil, dexamethasone, 6-methylcoumarin and acetylsalicylic acid.

A comparison of the cytotoxicity assay ID_{50} values obtained from F9 cells and 3T3-L1 cells for the 24 chemicals is shown in Table 8.5. The range of ID_{50} concentrations covered (approximately seven orders of magnitude) is similar for the two cell lines. In terms of absolute numerical values, the two cell lines

Table 8.4 *In vitro* toxicities of 24 chemicals to undifferentiated F9 cells

Chemical	ID_{20} (μg/ml $\pm$ s.e.m.)	ID_{50} (μg/ml $\pm$ s.e.m.)	ID_{80} (μg/ml $\pm$ s.e.m.)
5-Fluorouracil	0.005 $\pm$ 0.001	0.007 $\pm$ 0.002	>0.5
Colchicine	0.009 $\pm$ 0.007	0.02 $\pm$ 0.01	0.03 $\pm$ 0.02
6-Amino-nicotinamide	0.03 $\pm$ 0.01	0.3 $\pm$ 0.1	11 $\pm$ 2
Diethylstilboestrol	0.04 $\pm$ 0.02	0.3 $\pm$ 0.2	8 $\pm$ 2
Dexamethasone	0.2 $\pm$ 0.1	0.7 $\pm$ 0.2	>100
6-Methylcoumarin	0.3 $\pm$ 0.1	0.7 $\pm$ 0.1	54 $\pm$ 10(n=2)
Cadmium (II) chloride	0.5 $\pm$ 0	1 $\pm$ 0	2 $\pm$ 0
Acetylsalicylic acid	1 $\pm$ 0	3 $\pm$ 1	203 $\pm$ 92
Phenytoin	0.5 $\pm$ 0.1	7 $\pm$ 3	>200
Retinoic acid	3 $\pm$ 1	9 $\pm$ 1	15 $\pm$ 1
Pentachlorophenol	14 $\pm$ 1	20 $\pm$ 3	30 $\pm$ 3
Thalidomide	5 $\pm$ 0.3	33 $\pm$ 9	>500[a]
2,4-Dinitrophenol	28 $\pm$ 3	57 $\pm$ 6	86
Isoniazid	30 $\pm$ 7	60 $\pm$ 13	136 $\pm$ 33
Caffeine	62 $\pm$ 29	111 $\pm$ 46	333 $\pm$ 42
Chloramphenicol	57 $\pm$ 19	272 $\pm$ 26	614 $\pm$ 172
Saccharin	130 $\pm$ 20	330 $\pm$ 70	7800(n=1)
Trichloroacetic acid	390 $\pm$ 60	790 $\pm$ 80	>20000
Cyclophosphamide	1100 $\pm$ 150	1750 $\pm$ 210	2650 $\pm$ 310
Sodium	1300 $\pm$ 260	2600 $\pm$ 390	5900 $\pm$340(n=2)
Sodium (I) chloride	2400 $\pm$ 350	3600 $\pm$ 540	5300 $\pm$ 610
Methanol	10000 $\pm$ 1500	20900 $\pm$ 1400	70300 $\pm$ 3400
Ethanol	12000 $\pm$ 2000	21000 $\pm$ 3400	38800 $\pm$ 5700
Dimethyl sulphoxide	21100 $\pm$ 1700	28500 $\pm$ 1100	39200 $\pm$ 300

[a] maximum solubility in aqueous medium

Table 8.5 *In vitro* toxicities of 24 chemicals to undifferentiated F9 cells and 3T3-L1 cells

Chemical	F9 ID_{50} (mM)	3T3-L1[a] ID_{50} (mM)
Colchicine	<0.0001	0.02
5-Fluorouracil	<0.0001	<0.0001
Diethylstilboestrol	0.001	0.05
Dexamethasone	0.002	0.64
6-Aminonicotinamide	0.002	0.15
6-Methylcoumarin	0.005	1.1
Cadmium (II) chloride	0.006	0.001
Pentachlorophenol	0.007	0.12
Acetylsalicylic acid	0.02	2.6
Phenytoin	0.03	1.1
Retinoic acid	0.03	0.16
Thalidomide	0.13	>1.9
2,4-Dinitrophenol	0.31	0.39
Isoniazid	0.44	16
Caffeine	0.57	5.7
Chloramphenicol	0.84	0.70
Saccharin	1.6	25
Trichloroacetic acid	4.8	3.2
Cyclophosphamide	6.3	6.5
Sodium benzylpenicillin	7.3	11
Sodium chloride	62	107
Dimethylsulphoxide	365	287
Ethanol	458	783
Methanol	652	1141

[a]Taken from Clothier *et al.* (1986) except for saccharin and thalidomide (Hulme *et al.*, 1990).

gave, in general, very similar figures. There were, however, substantial differences between ID_{50} values from F9 and 3T3-L1 cells for dexamethasone, 6-aminonicotinamide, 6-methylcoumarin, acetylsalicylic acid, pentachlorophenol, phenytoin, isoniazid, caffeine and saccharin. Colchicine and thalidomide also show differences, although the ID_{50} from one of the cell lines is not precise for both chemicals (Table 8.5). All these chemicals showed greater toxicity to F9 cells than to 3T3-L1 cells. There were no chemicals which showed a greater toxicity to 3T3-L1 cells than to F9 cells.

Linear regression analysis of log ID_{50} (mM) for F9 versus log ID_{50} (mM) for 3T3-L1 gave a correlation coefficient of 0.88. A comparison of the unlogged values gave a correlation coefficient of 0.97. Examination of the linear regression plot (Figure 8.5) shows that despite the high correlation coefficients, there are two out-liers, cadmium (II) chloride and 5-fluorouracil.

The toxicity curves (represented by the ID_{20} to ID_{80} dose range) for the majority of the chemicals tested with 3T3-L1 cells were linear, and usually covered no more than 1 order of magnitude (Clothier *et al.*, 1988). Nine of the 24 chemicals tested on the F9 cells gave ID_{20} and ID_{80} concentrations which spanned more than two orders of magnitude, thus giving shallow, angled toxicity curves (for example, 6-methylcoumarin and trichloroacetic acid, Figure 8.6(a); 6-aminonicotinamide and acetyl salicylic acid, Figure 8.6(b)) compared with those for 3T3-L1 cells.

During the cytotoxicity tests on the UD cells, the cultures were examined by phase-contrast microscopy for induction of differentiation. No chemicals induced morphological changes characteristic of differentiation, at the concentrations tested and over the timescale used. Because of this absence of morphological alterations in the cells treated with test chemicals, media from UD F9 cultures, used in these cytotoxicity tests, were not examined for

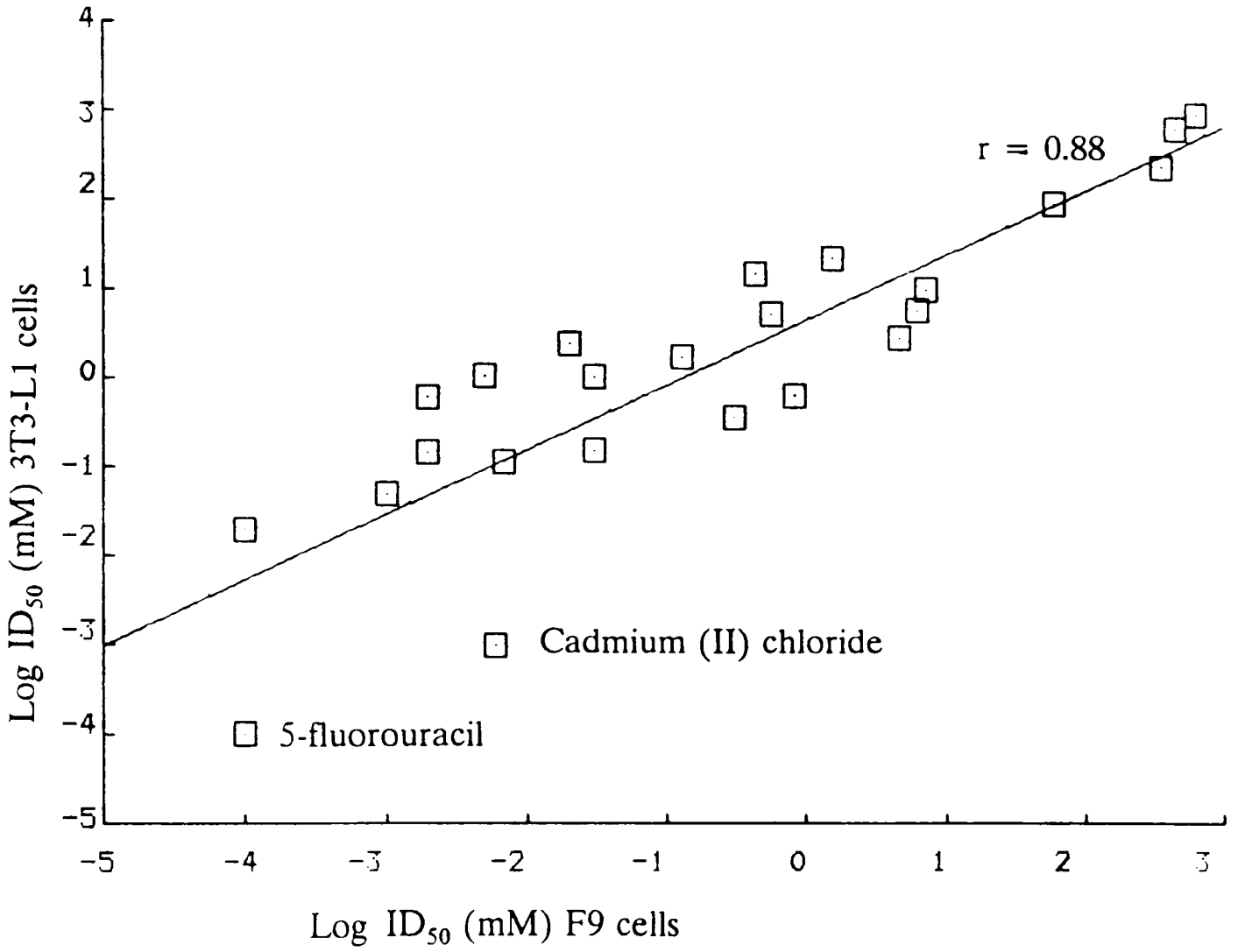


Figure 8.5 Linear regression analysis of comparison of ID_{50} to F9 cells with ID_{50} to 3T3-L1 cells, for 24 chemicals.

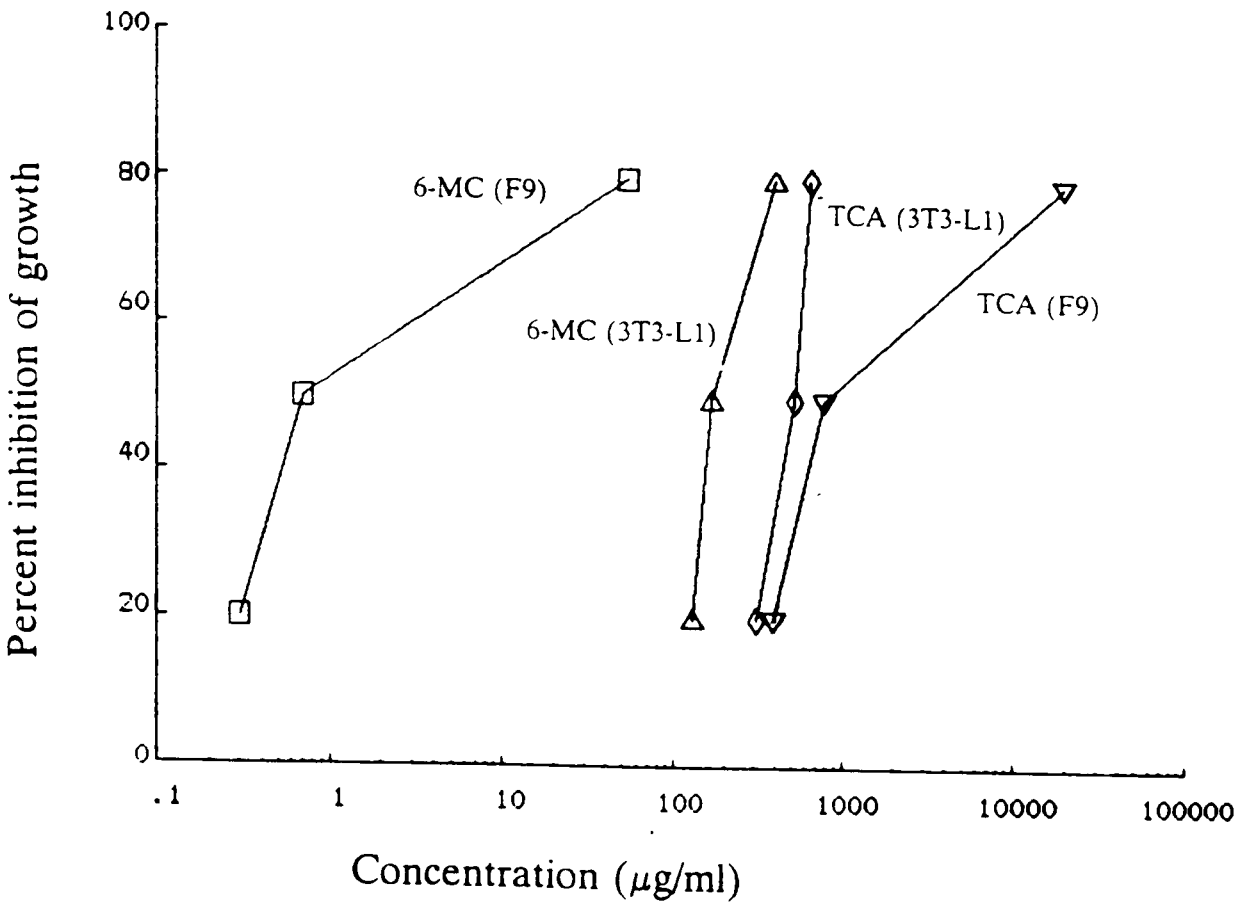


Figure 8.6(a)

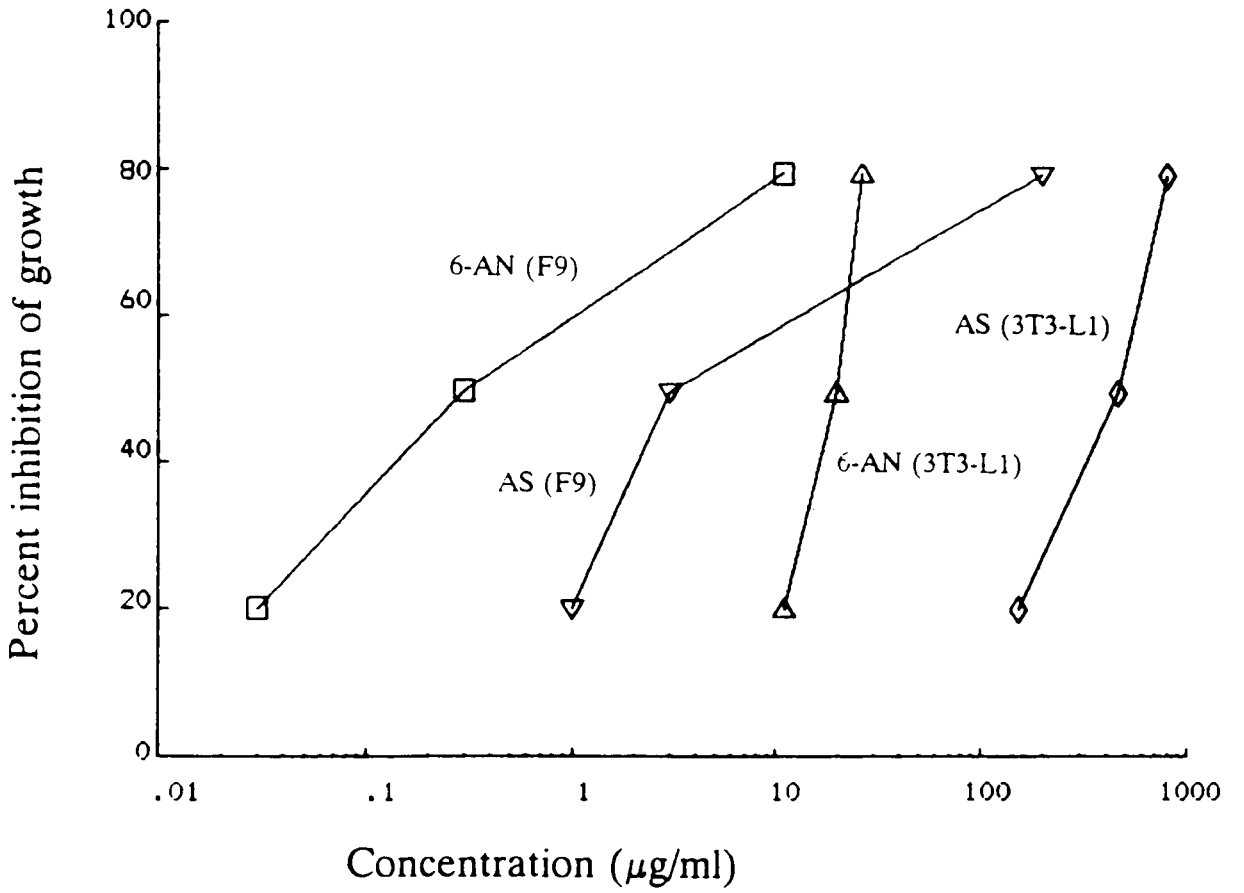


Figure 8.6(b)

Figure 8.6 Toxicity curves to F9 and 3T3-L1 cells, for:
(a) 6-methylcoumarin (6-MC) and trichloroacetic acid (TCA); and
(b) 6-aminonicotinamide (6-AN) and acetylsalicylic acid (AS).

presence of laminin.

8.2.2.2 **Conclusions**

Despite the fact that UD F9 cells were subjected to a shorter test chemical exposure time than the 3T3-L1 cells, the F9 cells showed a greater sensitivity to over half of the chemicals tested (Table 8.5). By contrast, Riddell *et al.* (1986a) and Garle *et al.* (1987) have demonstrated that decreasing the exposure period predominantly decreases the cytotoxic effects of chemicals.

There are some notable discrepancies between the F9 and 3T3-L1 ID_{50} results, and the reason(s) for such differences is (are) not immediately apparent.

The F9 cells exhibited an unusual shape of toxicity curve with some of the 24 chemicals tested, wherein a large increase in concentration of chemical was required to shift the curve from 50% growth inhibition (ID_{50} dose) to 80% growth inhibition (ID_{80} dose) (Figure 8.6). This phenomenon is not apparent with any of the 150 chemicals tested on 3T3-L1 cells in the FRAME KB cytotoxicity assay (Chapter 3; Clothier *et al.*, 1988).

The comparison of ID_{50} values from UD F9 cells and from 3T3-L1 cells showed that, despite large differences with some chemicals, the rank order of toxicity is essentially the same from the two cell lines.

Having established that the UD F9 cells could provide reproducible results in a cytotoxicity test, DG and DD cultures were also investigated for their response to a limited number of chemicals over the same exposure period of 48 hours.

8.2.3 Cytotoxicity tests with DG and DD F9 cells

8.2.3.1 *In vitro* toxicities of 6 chemicals to DG and DD F9 cells

The six chemicals tested on DG and DD F9 cells were 5-fluorouracil, cadmium chloride, caffeine, cyclophosphamide, dimethyl sulphoxide and thalidomide. These were selected from the list of 24, because of their widely varying toxicities, modes of action, and their known teratogenic or non-teratogenic effects *in vivo*.

The cytotoxicity test results (Table 8.6) indicate that F9 cells in the three culture situations (UD, DG and DD) did not give the same responses to each chemical. Cadium chloride, cyclophosphamide and dimethyl sulphoxide each produced essentially similar toxicity curves with UD, DG and DD cells. However, the DG and DD cells were less sensitive to the remaining three chemicals. Thalidomide and 5-fluorouracil gave ID_{50} doses over ten times greater with DG and DD cells than with UD cells. Caffeine gave ID_{50} doses with DG and DD Cells which were two to three times more than the ID_{50} with UD cells. With 5-fluorouracil, thalidomide and caffeine, it is apparent that the whole toxicity curve has shifted, and not just the ID_{50}, when using DG or DD cells. ID values from DG and DD cultures were as reproducible as those obtained with UD cells.

8.2.3.2 Production of laminin by DG and DD F9 cells

To assess whether any of the six chemicals could induce production of detectable amounts of laminin in the DG cultures, medium samples were collected at the 48 hour exposure endpoint and assayed for laminin by the ELISA. No laminin was detected in any of the samples from the treated DG cultures (detection limit of the ELISA was approximately 5ng/ml). However,

Table 8.6 *In vitro* toxicities of six chemicals to undifferentiated, differentiating and differentiated F9 cells

Chemical	Cell State	ID_{20} (mM ± s.e.m.)	ID_{50} (mM ± s.e.m.)	ID_{80} (mM ± s.e.m.)
5-Fluorouracil	UD	0.00004 ± 0.00001	<0.0001	<0.004
	DG	0.0003 ± 0.0002	0.001 ± 0.001	0.001(n=1)
	DD	0.0003 ± 0.0000	0.001 ± 0.000	0.003 ± 0.000
Cadmium (II) chloride	UD	0.003 ± 0.000	0.006 ± 0.002	0.01 ± 0.00
	DG	0.005 ± 0.001	0.01 ± 0.00	0.02 ± 0.00
	DD	0.005 ± 0.001	0.01 ± 0.00	0.02 ± 0.00
Thalidomide	UD	0.02 ± 0.00	0.13 ± 0.03	>1.9[a]
	DG	>1.9[a]	>1.9[a]	>1.9[a]
	DD	>1.9[a]	>1.9[a]	>1.9[a]
Caffeine	UD	0.32 ± 0.15	0.57 ± 0.24	1.7 ± 0.2
	DG	0.93 ± 0.11	1.9 ± 0.1	3.5 ± 0.4
	DD	0.76 (n=2)	1.7 ± 0.2	2.7 ± 0.3
Cyclophosphamide	UD	3.9 ± 0.5	6.3 ± 0.8	9.5 ± 1.1
	DG	1.2 (n=1)	2.8 ± 0.3	6.7 ± 0.3
	DD	1.1 (n=2)	4.9 ± 1.2	12 ± 2

Dimethyl sulphoxide	UD	271 ± 22	365 ± 14	503 ± 4
	DG	178 ± 16	328 ± 87	490 ± 27
	DD	201 ± 19	322 ± 31	467 ± 36

UD = undifferentiated cell cultures
DG = differentiating cell cultures
DD = differentiated cell cultures

[a] = maximum solubility in aqueous medium

cultures of control DD cells at the cytotoxicity test endpoint had produced laminin in detectable amounts.

In three experiments, on separate occasions, a total of 54 control culture wells were used which gave an average of approximately 0.5μg of laminin produced in 48 hours by 1 x 10^6 DD cells (final cell number) or 4μg laminin per mg final cell protein. These values are close to the values of (a) 1-2μg laminin per 10^6 cells, achieved by A. Daly (personal communication), and (b) 13.1μg laminin per mg cellular protein, achieved by Williams *et al.* (1987). Both these latter results were obtained from F9 cells differentiated using RA and dbcAMP, and represent the quantities of laminin released over a 24-hour period only.

Media from test-chemical treated DD cell culture wells were also tested at the end of the 48-hour exposure period. The media samples represented cultures which had shown a range from minimal to maximal toxicity in the cytotoxicity tests. The amounts of laminin produced in these cultures was compared with the amounts of cellular protein in the same wells (Table 8.7). There was a possibility, however, that the chemicals tested could affect the production of laminin at concentrations that were not cytotoxic. Hence, to correct for this, the amount of laminin was plotted as a percentage of the final cellular protein, to take account of the change in cell number with increasing cytotoxicity (Figures 8.7, 8.8 and 8.9).

For cyclophosphamide and dimethyl sulphoxide, there was a direct relationship between increased toxicity and decreased laminin production (Figure 8.7). With 5-fluorouracil and caffeine, while the toxicity increased, the amount of laminin did not decrease to the same extent (Figure 8.8). In marked contrast, with cadmium (II) chloride, no laminin was detectable at the ID_{20} dose (Figure 8.9).

Table 8.7 Production of laminin by differentiated F9 cells during cytotoxicity tests with six chemicals

Concentration of Chemical	Cell Growth (% of control)	ng laminin/ μg protein per well
5-Fluorouracil (mM x 10^{-3})		
SC	100	4.3
0.077	106	3.4
0.38	80	4.0
0.77	45	4.7
3.8	13	11.8
7.7	12	7.9
Cadmium Chloride (mM x 10^{-3})		
MC	100	3.7
0.55	104	2.6
2.7	87	1.1
5.5	76	BDL⊗
10.9	49	BDL
27.3	14	BDL
Thalidomide (mM)		
SC	100	3.0
0.19	102	2.8
0.39	100	3.1
0.97	105	3.1
1.9[a]	92	2.8
Caffeine (mM)		
MC†	100	3.0
0.52	85	3.3
1.03	66	4.0
2.06	36	4.4
3.09	15	10.0
4.12	10	8.0

Table 8.7 (contd.)

Concentration of chemical	Cell Growth (% of control)	ng laminin/ μg protein per well
Cyclophosphamide (mM)		
MC	100	2.5
0.9	83	2.6
1.79	66	3.5
3.58	58	2.6
5.38	44	3.9
7.17	34	3.7⊗
17.9	7	BDL⊗
Dimethyl sulphoxide (mM)		
MC	100	3.3
128	98	3.9
256	64	3.3
385	35	BDL⊗
513	16	BDL⊗

SC = 1% (v/v) DMSO solvent control + 0.1μM RA
MC = Medium control + 0.1μM RA

⊗Laminin was detectable in only one or two out of the three experiments performed with this dose. Result quoted uses the laminin result which was repeated twice out of the three experiments.

BDL = Amount of laminin was below detection limit of ELISA.

[a]maximum solubility in aqueous medium.

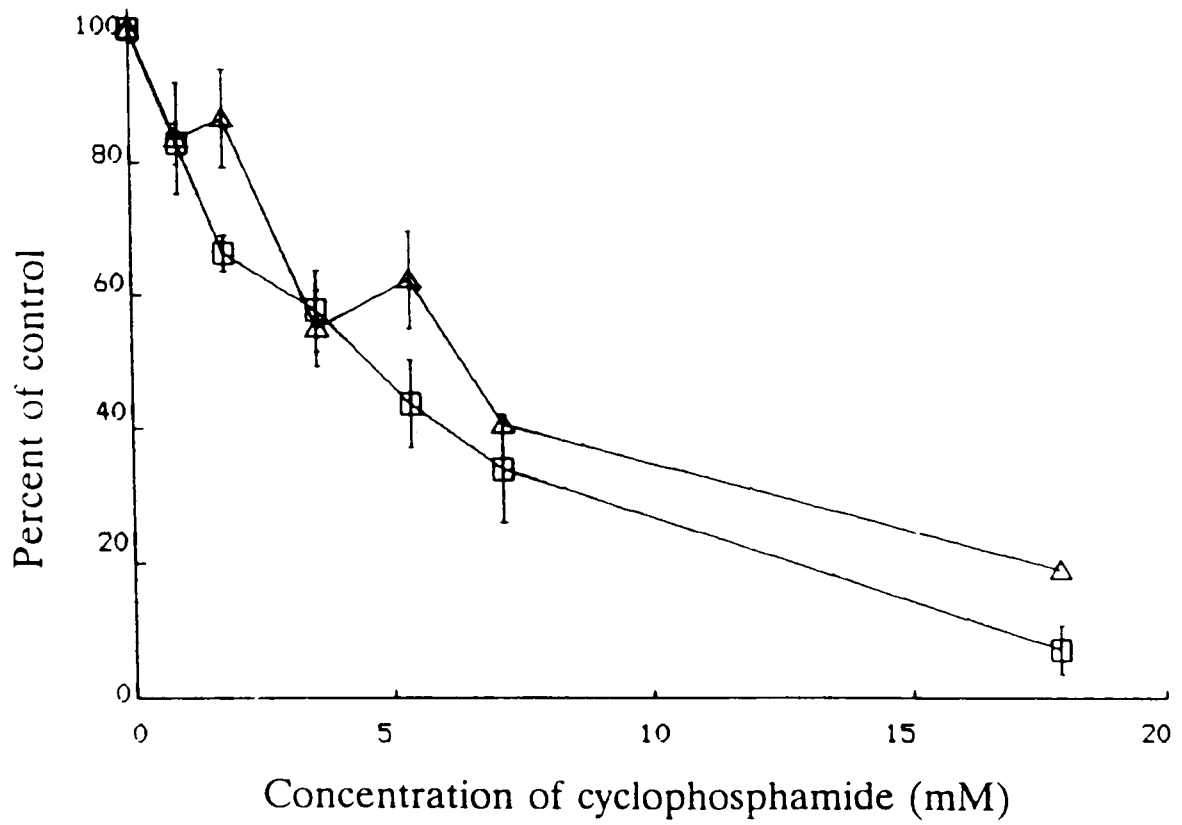


Figure 8.7(a)

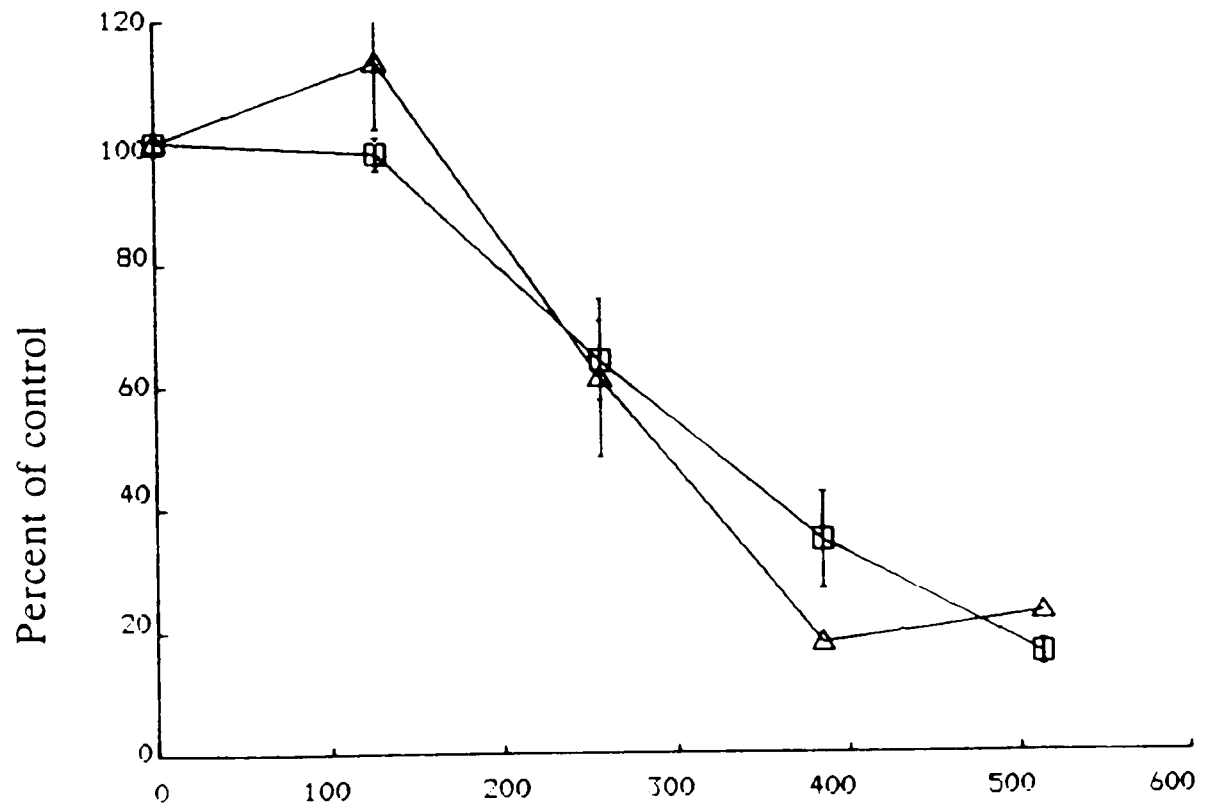


Figure 8.7(b)

Figure 8.7 Effect of (a) cyclophosphamide and (b) dimethyl sulphoxide on protein (□) and laminin (△) levels (as a percentage of control untreated wells) in differentiated F9 cells.

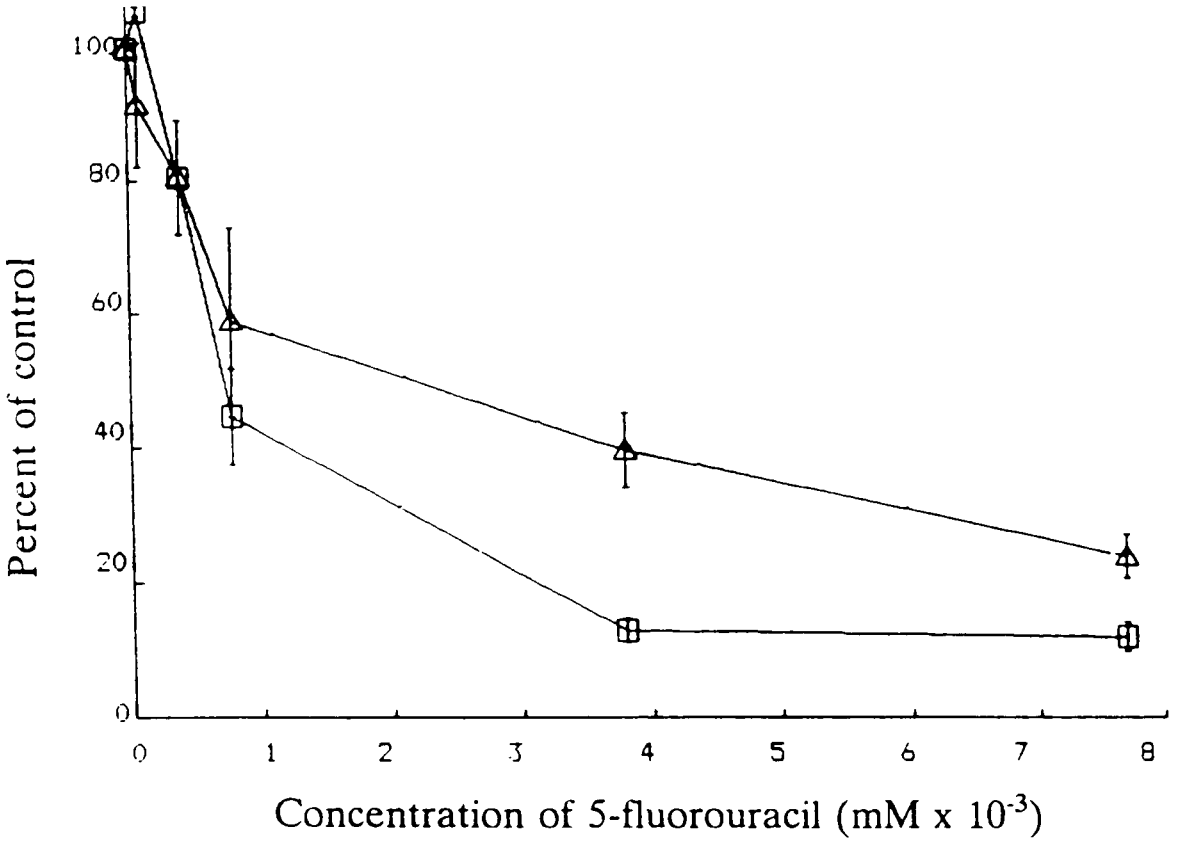


Figure 8.8(a)

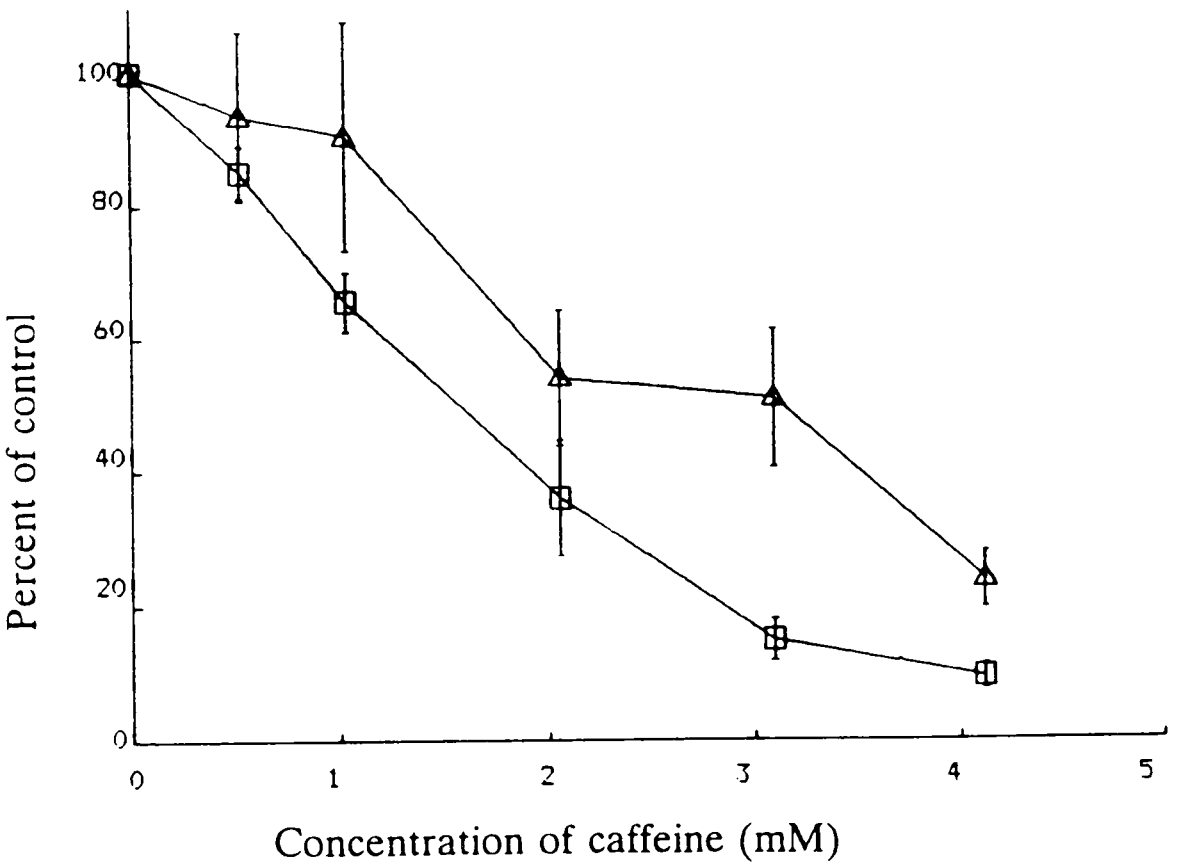


Figure 8.8(b)

Figure 8.8 Effect of (a) 5-fluorouracil and (b) caffeine on protein (□) and laminin (△) levels (as a percentage of control untreated wells) in differentiated F9 cells.

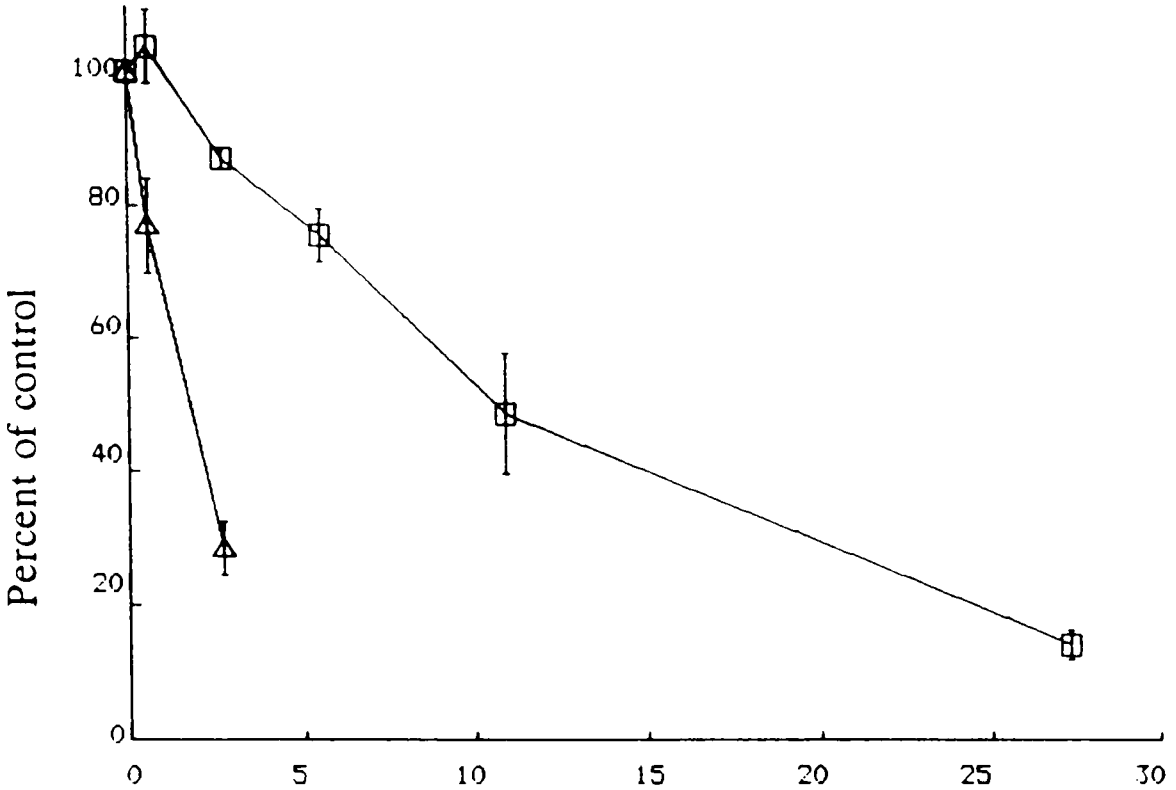


Figure 8.9 Effect of cadmium (II) chloride on protein (□) and laminin (△) levels (as a percentage of control untreated wells) in differentiated F9 cells.

Thalidomide had no effect on the growth of DD cells, up to its solubility limit of 500μg/ml aqueous medium. Also, there was no effect on the amount of laminin produced by those thalidomide-treated cultures (Table 8.7).

Despite the alteration of laminin production by three of the chemicals tested on DD cells (5-fluorouracil, caffeine and cadmium (II) chloride), no overt dedifferentiation (as demonstrated by morphological changes in the cells) was observed over the time period of the cytotoxicity tests.

8.2.3.3 **Conclusions**

5-Fluorouracil, thalidomide and caffeine appeared to be less cytotoxic to DG and DD F9 cell cultures than to UD cultures, over the same exposure period. Cadmium (II) chloride, cyclophosphamide and dimethyl sulphoxide were approximately equally cytotoxic to DG and DD cultures as to UD cultures.

It had been expected that in the cytotoxicity tests with DD cells, the amount of laminin would remain proportional to the cell number. While cyclophosphamide, thalidomide and dimethyl sulphoxide did demonstrate this pattern, cadmium (II) chloride, caffeine and 5-fluorouracil did not. Cultures treated with caffeine and 5-fluorouracil showed laminin levels higher than expected. When these were examined by phase-contrast microscopy, they showed evidence of a slow build-up of toxicity, i.e. wells treated with high concentrations did not exhibit toxic effects after 24 hours of exposure, but effects were very clear at 48 hours. Cadmium (II) chloride, however, caused the cultures to give a laminin level below that expected from the level of protein. Laminin production appeared to have been affected by 2.7μM cadmium (II) chloride, which was only minimally cytotoxic.

Therefore, cyclophosphamide, thalidomide and dimethyl sulphoxide did not

appear to have a direct selective effect on laminin production. 5-Fluorouracil and caffeine were cytotoxic, but did not appear to reduce the metabolic capacity of the cells so greatly, and laminin production was retained. However, cadmium (II) chloride affected the capacity of the cells to produce laminin before being cytotoxic.

8.3 DISCUSSION

Despite the obvious preliminary nature of the experiments with the F9 cell line, some promising results were obtained. Firstly, a reliable method of inducing almost complete morphological differentiation of the cells was established. Secondly, a biochemical marker of the differentiation process was found to be generated at levels which could easily be detected by ELISA.

Although the many different cell lines used in *in vitro* cytotoxicity tests will often provide very similar rank orders of results (Balls and Horner, 1985; Borenfreund and Shopsis, 1985), they may not always exhibit similar susceptibilities, and this was found to be the case with the F9 and 3T3-L1 cell lines. Certain chemicals may give highly discrepant results in different cell lines (Riddell *et al.*, 1986a), and it is not easy to find explanations for this. It may be linked to the fact that F9 cells are a transformed cell line, whereas 3T3-L1 cells are not. Alternatively, the faster growth rate of F9 cells may be a contributory factor. However, V79 cells, which grow more rapidly than 3T3-L1 cells, showed similar sensitivity to 3T3-L1 cells, when tested with 27 chemicals (Garle *et al.*, 1987). Another possible explanation is the difference in culture medium serum supplement for 3T3-L1 (new-born calf serum) and F9 cells (fetal calf serum). However, V79 cells also require 10% fetal calf serum. A combination of these, plus other unknown, factors could probably explain the greater sensitivity of the F9 cells.

The reasons for the F9 cells giving unusually wide toxicity curves may be connected with the protocol for the cytotoxicity assay with UD F9 cells. The cells are plated at 5 x 10^4/well, which by themselves might produce a KB OD of 0.1 or 0.2 (Figure 8.3). Twenty-four hours later, the test chemical is added, by which time the cells may have multiplied to give a KB absorbance of more than 0.2. Thus, for the endpoint absorbance (usually about 1.0) to be reduced by over 80% (to less than 0.2 to calculate the ID_{80}), the endpoint cell number must be less than the number at the time of test chemical addition, i.e. cells need to have become detached. Thus, the assumption is that, although the UD F9 cells were sensitive to many chemicals, in terms of inhibition of growth, it took far greater amounts of those chemicals to actually kill the cells outright. Another, less likely, explanation is that, as the F9 cells were dividing so rapidly, although 50% growth inhibition was achieved by relatively low doses of certain chemicals, it took far higher doses to inhibit division to only 20% of the normal rate. This may have been due to the properties of those particular chemicals. For example, 5-fluorouracil and 6-aminonicotinamide are anti-metabolites, and will inhibit metabolic processes, but would not necessarily kill the cells. Trichloroacetic acid, when at a sufficient concentration to turn the medium acid, will fix the cells *in situ*., i.e. killing them, but not allowing them to detach from the culture well surface.

Although none of the chemicals tested induced any differentiation-related morphological changes in the UD F9 cells, this was not unexpected. Firstly, the chemicals were added to the culture medium at toxic concentrations and hence the cells would have had their capacity to differentiate impaired. Secondly, the chemicals were only allowed to interact with the cells for 48 hours. This is the minimum amount of time required for even the known differentiation-inducer, RA, to produce its initial phenotypic alterations. It is unlikely that any of the other 23 chemicals would be more potent differentiation-inducers than RA. Hence, longer timescales of chemical

treatment at sub-lethal doses would probably be required, before any alterations would become apparent. This could include subculturing and seeding the cells in medium containing the test chemical.

There are several possible reasons why the DG and DD cultures were less sensitive than UD cells to certain chemicals. The DD cells are morphologically and biochemically different from UD cells, and exhibited a slower growth rate. Therefore, they might be expected to respond differently to test chemicals. However, the DG cells were only in the initial stages of RA-induced differentiation during the 48-hour incubation with a test chemical plus 0.1μM RA. The DG cells did not show any clearly detectable morphological alterations until the end of the 48-hour exposure to RA. No detectable laminin was produced, nor any change in the cell growth rate seen compared with UD cells (Hulme *et al.*, 1990). Altered growth rate alone cannot, therefore, be the reason for the decreased sensitivity of DG cells. It appears that the non-toxic concentration of RA in the medium somehow induced changes in the cells, reducing their susceptibility to certain chemicals. There were two main differences between the cytotoxicity test protocol for UD cells and that for DG and DD cells. The UD F9 cell cytotoxicity test used the KB staining to measure relative cellular protein, while tests with DG and DD cells used the Bradford assay to measure actual amounts of protein. However, the difference in the methods of protein assay could not account for these differences since, with 5-fluorouracil, thalidomide and caffeine microscopic examination of cultures, prior to the protein assays, confirmed that both methods of protein determination were a true reflection of relative cell number. Furthermore, if the method of protein assay affected the results obtained with DG and DD cells, then it would be expected that the results for all the chemicals would be similarly affected. This was not the case. It had also been established that the KB and Bradford methods agreed in their determination of cellular protein. However, it is possible that the RA may

have interacted chemically with the 5-fluorouracil, thalidomide and caffeine, rendering them less cytotoxic. This possible explanation could be investigated further by testing chemicals for cytotoxicity in the presence of 0.1μM RA, with another cell line - one which does not respond to RA (such as 3T3-L1). In addition, a series of chemicals (including caffeine, thalidomide and 5-fluorouracil) could be tested for cytotoxicity to UD or DD F9 cells in the presence and absence of 0.1μM RA over different exposure periods. This might offer suggestions as to the time of onset of the altered sensitivity of DG cells, and whether chemicals which had different toxicities in different cell types (i.e. UD, DG or DD) were of a particular chemical group or biological activity.

The higher laminin levels in the near-maximal toxicity DD cultures treated with 5-fluorouracil and caffeine may have been due to the release of intracellular laminin which had been produced before the cells succumbed to cytotoxic effects in the latter half of the 48 hour exposure. Alternatively, 5-fluorouracil, being a specific inhibitor of DNA synthesis, may have prevented cell division without affecting the ability of the cells to produce laminin. Several DNA synthesis inhibitors have been shown to induce plasminogen activator production (another biochemical marker of F9 cell differentiation) in previously undifferentiated F9 cells (Nishimune *et al.*, 1983). It is known that for certain cell lines, inhibition of division is necessary before differentiated characteristics can be demonstrated. For example, EL-4 cell lines, used in the development of an immunocytotoxicity assay (Lasek *et al.*, 1989; Steer *et al.*, 1990), will produce cytokines if cell division is inhibited with phorbol myristyl acetate (McCabe, 1990). Whether highly toxic doses of 5-fluorouracil and caffeine could have actually enhanced laminin production in the already-differentiated cells in this study would have to be investigated by time-course experiments and measurements of both intracellular and extracellular laminin.

The dramatic lack of laminin in DD cultures whose growth was only minimally affected by cadmium (II) chloride, could be explained by the cadmium ions interfering with the process of laminin production, either intracellularly, or at surface sites, without affecting cell proliferation. Cadmium is known to bind to proteins with high affinity (Camner *et al.*, 1986), and could have inhibited specific enzymes or structural proteins involved in laminin production or release. This effect could be investigated further with a wider range of sublethal concentrations of cadmium (II) chloride, and of other chemicals which reveal a similar pattern. The depression of laminin production in the presence of cadmium (II) chloride could have another explanation, namely, that the cadmium interfered with the binding of laminin to anti-laminin antibody in the ELISA (co-incubation of culture medium aliquots with anti-laminin antibody). This could be tested by the addition of cadmium (II) chloride at the appropriate concentration to the standard curve laminin dilutions.

The disruption of other differentiated functions of F9 cells, such as plasminogen activator production, loss of expression of stage-specific embryonic antigen (SSEA-1), or polyoma virus infectivity (Moore *et al.*, 1986) could also be investigated. It would probably be worthwhile to examine at least one other specific function of differentiated F9 cells, when testing any chemical on DD cultures. This additional marker could be one whereby differentiated cells lost (rather than gained) activity or expression, such as loss of SSEA-1 expression, or loss of alkaline phosphatase activity.

It is apparent that the laminin synthetic pathway could be a specific target for certain chemicals at both non-cytotoxic and cytotoxic doses, resulting in raised or decreased laminin production. The cytotoxicity test results suggest that F9 cells in different stages of differentiation do not always respond in the same way to toxic chemicals. In addition, laminin production by differentiated cells

may be altered in different ways by chemicals with different biological activities.

A test using F9 cells to provide information about chemicals that can disrupt cellular differentiation, would need to involve incubation of the cells with the test chemical throughout their differentiation. The effect of the chemical upon cell division, morphological alterations and production of laminin could then be examined. This would make the test comparable to the micromass assay, which measures inhibition of differentiation of the limb bud or neural crest cells. It would still be necessary to measure basal cytotoxicity to the F9 cells over the same exposure period. As in the micromass assay, the chemical concentration which inhibited cell differentiation by 50% would then be related to the cell growth ID_{50}. If 50% inhibition of differentiation was caused at a chemical concentration less than a certain proportion (e.g. half) of the cell growth ID_{50}, then the chemical would be classified as a potential "teratogen" or "developmental toxicant".

Chemicals could also be investigated for their ability to induce morphological alterations and laminin production in F9 cells. Some chemicals are known to be able to induce morphological changes (Moore *et al.*, 1986), but their effects on biochemical parameters were not consistent. This latter proposal has no counterpart in the micromass test, because, in the micromass method, all the mid-brain or limb-bud cells are committed to differentiate. Hence, only the inhibition, and not the induction, of the differentiation process can be measured. F9 cells could therefore be considered more versatile than the primary embryonic cell cultures.

One criticism of the F9 system is that RA must be used to induce differentiation, and RA is itself a potent teratogen (Johnson and Chun, 1989). However, retinoids, such as vitamin A and RA, are highly important for the

health and growth of higher animals (Strickland, 1979) and are thus naturally-occurring essential molecules. In this respect, RA is probably acting as a "natural" chemical messenger to signal F9 cell differentiation, because it is known to be a natural inducer of differentiation *in vivo* (Sporn and Roberts. 1983). Physiological concentrations of RA (3-33nM) have been found to stimulate limb-bud chondrogenesis *in vitro* (Paulsen *et al.*, 1988).

On paper, therefore, the F9 cell line appears to be a highly suitable candidate for a model of cell differentiation for use in *in vitro* toxicity testing. The results presented in this chapter suggest that it is certainly worthy of further investigation. Whether the F9 cells could ultimately be used to detect developmental toxicants accurately could not be predicted from the results obtained to date.

CHAPTER 9 FINAL DISCUSSION

The potential use of basal cytotoxicity tests in the prediction of adverse effects of substances on humans is being evaluated in many countries. The FRAME KB cytotoxicity assay is one such test, and has been shown to be reliable, reproducible and sensitive to a very wide range of toxic potencies (Knox *et al.*, 1986; Clothier *et al.*, 1988). Despite the ultimate aim of predicting human toxicity, the paucity of reliable human data and the voluminous amount of rodent data has led many validation schemes to aim to predict rodent *in vivo* toxicity from *in vitro* assays. This thesis has attempted to discover whether the FRAME KB cytotoxicity assay is a good predictor of rodent acute lethal potency for a group of unrelated pure chemicals, and whether the predictiveness improves when a group of structurally and chemically related substances are selected. The assay was also evaluated for potential use with formulations and mixtures. To endeavour to increase confidence in results for volatile liquids tested in the standard FRAME KB cytotoxicity assay protocol, two methods of sealing the culture plates were investigated. Preliminary studies into the proposed use of embryonal carcinoma cells in an *in vitro* teratogenicity test were also carried out.

Despite the diversity of the 59 chemicals in the first *in vivo/in vitro* comparison (Chapter 3), and the problems associated with testing some of them, linear regression analysis showed good correlations between FRAME KB cytotoxicity assay data and published mouse i.p. or rat oral LD_{50} values (Purchase *et al.*, 1987 Lewis and Tatken, 1982). Although the correlations are good, lending support to the arguments of Ekwall (1983b) and Ekwall *et al.* (1989) that *in vitro* basal cytotoxicity assays *are* able to predict *in vivo* toxicity, the regression line could not be used with confidence to predict the toxic potencies of all chemical types in existence. This is because a limited number of chemical types were represented in the set of 59, and, moreover, only pure chemicals, which were soluble in aqueous medium, were involved. It follows that before an assay can be used as a predictor of *in vivo* acute lethal potency, the data

set used to produce the prediction should contain a number of closely-related chemicals, formulations or substances acting via similar modes of toxicity. If the *in vivo/in vitro* correlation is good for a particular set, then greater confidence can be placed in the ability of the assay to predict the potency of other chemicals of that type. This approach is similar to that taken with QSAR. In general, a small group of structurally-related chemicals is used to establish the QSAR, and this is used to predict the activity of chemicals from the same series (Phillips *et al.*, 1990).

A problem in selecting related chemicals to test *in vitro* is defining what is meant by "related". Chemicals can be related by structure and/or by chemical, biological or pharmacological activity, or by a host of other, less precise, factors such as commercial use, target organ for toxicity and physical form. The prediction of rat oral LD_{50} was not improved for the metal compounds (which are related by simple cation/anion structure and in general by the metal-to-ligand-binding mode of toxicity) compared to the 59 miscellaneous chemicals. However, the FRAME KB cytotoxicity assay results were more closely correlated with mouse i.p. LD_{50} values for the metal compounds than for the 59. With the metal compounds, it was noticeable that the *in vitro* results correlated better with mouse i.p. data than with rat oral data, which is in agreement with the findings of Fry *et al.* (1990). The use of the metal softness parameter on its own as a predictor of metal compound toxicity appears to be rather limited and simplistic. An approach which included more than one physicochemical parameter in the QSAR for metal compounds would offer greater scope (Kaiser, 1985).

The choice of rat oral and mouse i.p. LD_{50} values as the parameters of mammalian acute lethal potency was made partly out of necessity, because of the inavailability of appropriate human toxicity data and the relative abundance of the rat and mouse data. The use of LD_{50} values is not intended

to imply that the FRAME KB cytotoxicity assay was being evaluated solely as a replacement for the LD_{50} test. *In vivo* acute toxicity data was needed in order to make *in vivo/in vitro* comparisons and LD_{50} values were the most applicable under the circumstances. It would not be of scientific merit to develop an alternative toxicity test to the classical LD_{50}, which has been the subject of much criticism in recent years. An *in vitro* test which provided data with 100% correlation with data from an *in vivo* toxicity test would be as limited in its applicability to man as the animal test. Nevertheless, there is a need to validate non-animal techniques which could supply data for the same purposes for which the LD_{50} is performed. The aim is to replace animal testing, but to achieve this, it has been agreed, there will be a period during which the *in vitro* assays will be used as prescreens. For such prescreens, the data generated needs to predict both likely human and animal toxicity. The problem remains as to whether the alternative should have to be validated against results from an out-dated and partly discredited *in vivo* test, such as the classical LD_{50} test.

It must be emphasised that it is unlikely that one alternative test would be used in isolation as a replacement for an *in vivo* test. The battery approach suggested by several groups (Shopsis *et al.*, 1985; Bracher *et al.*, 1989; Balls *et al.*, 1991a) is a more logical option, because it allows several endpoints and mechanisms of action to be assayed. Alternative tests should have reached a satisfactory stage of development and validation before being considered for inclusion in a battery. The predictive performance of a battery should also undergo rigorous evaluation with an appropriate number of reference chemicals (Balls and Clothier, 1992, in press). Alternatives, such as *in vitro* tests, QSAR studies or computer modelling, are often less expensive to run than *in vivo* tests and so the use of several in a battery would not be prohibitively costly.

The FRAME KB cytotoxicity assay showed some promising correlations with *in vivo* toxicity parameters, for groups of pure chemicals, but it must never be assumed that a test that works well with pure chemicals is equally useful with formulations and mixtures. The evaluation of the FRAME KB cytotoxicity assay with certain product formulations, gave results which were found to be difficult to interpret. With the pesticide formulations, some contained "active chemicals" in suspension (i.e. not fully dissolved), which led to difficult questions about whether the active chemical was interacting with the 3T3-L1 cells physically, chemically or neither. With all formulations and mixtures, the possibility of synergistic effects between ingredients can never be ruled out. Hence, the results for formulations should never be viewed in isolation, but in the context of the results of the ingredients. Because the FRAME KB cytotoxicity assay estimates the effects of a substance after 72 hours exposure, it may be more suitable for testing products which are designed for, or may accidentally have, prolonged exposure such as those applied to the skin or hair. The assay may also be useful for ingredients of any product type which may cause toxic effects only after a longer exposure period, rather than immediate effects upon exposure, or chemicals which are rapidly removed, e.g. volatile perfumes.

During the final stages of the preparation of this thesis, the results for Phase II of the Cosmetics, Toiletries and Fragrances Association (CTFA) evaluation scheme (for alternatives to the Draize eye test) became available (S. Gettings, personal communication). The FRAME KB cytotoxicity assay was 1 of 11 (out of an original 30 assays) selected as promising for predicting the eye irritancies of a group of oil-water emulsion type cosmetic and toiletry formulations. In addition, results from the European Community pilot validation study on Draize eye test alternatives became accessible (M. Balls, personal communication). The FRAME KB cytotoxicity assay, the FRAME Neutral Red Release assay and the FRAME Fluoroscein Leakage assay all

performed well. The FRAME KB cytotoxicity assay did not detect acetic acid or sodium hydroxide as potential irritants, presumably because of the buffering system in the culture medium.

Some types of substance will always be problematical for toxicity tests, whether *in vivo* or *in vitro*. To quote Zbinden (1990): "The most interesting compounds are often as soluble as concrete or they are in gaseous form or tend to decom pose rapidly when added to the culture medium". *In vitro* toxicologists must not ignore "difficult" substances, but should make efforts to develop assay protocols to accommodate them. Using sealed culture plates is one solution to the problem of the evaporation of liquid chemicals, but a greater standardisation of methods of preparation of chemical dilutions is also required.

Once basal cytotoxicity has been evaluated, certain specialised tests will be required for the assessment of potential organ- or tissue-specific toxicity. The initial assumption that a continuous cell line could easily replace primary rat embryo cultures in a micromass type teratogenicity test was naive. Embryonic cell differentiation is a highly complex process, and to be able to accurately and reliably model it *in vitro* is a lengthy and complex task - one far beyond the resources of this project. Furthermore, cell differentiation is only one of a wide range of cellular and tissue developmental processes. A model of cell differentiation cannot, therefore, be expected to simulate all of normal or abnormal embryonic development. A considerable amount of work would be required to develop a useful *in vitro* toxicity test based on the differentiation of F9 cells. F9, and other EC cell lines, are the subject of much research world-wide, and could be a useful contribution to research efforts in molecular toxicology, in terms of chemical interference with subcellular mechanisms during differentiation. There is a general belief that for *in vitro* alternatives to be capable of predicting specific forms of toxicity, such as teratogenicity,

these assays must be based on sound mechanistic understanding. For basal cytotoxicity, if the assay is predictive, the mechanisms of action do not need to be similar. When organ- or tissue-specific toxicity is concerned, because of *in vivo/in vivo* variation, the *in vitro* assays should attempt to model the mechanisms of toxicity known to occur in man.

The FRAME KB cytotoxicity assay has proved to be a versatile test for basal cytotoxicity *in vitro*, which satisfies the criteria for an acceptable toxicity test system (namely: quantifiable objective endpoint which provides data for a dose-response relationship; low intralaboratory and interlaboratory variation; rapid and inexpensive; techniques which are simple to learn; low rate of "false" results (Gad, 1989)). There is more research to be done, however, before the assay could be more widely accepted as a useful tool in *in vitro* toxicology and safety assessment studies.

Firstly, with pure chemicals, more groups of related chemicals should be tested. Care needs to be taken over the selection of such groups, particularly with regard to how closely the substances are related, and what *in vivo* data are available for them. In QSAR research, there are three basic requirements for the selection of chemicals (Phillips *et al.*, 1990): (i) there should be a well defined mode of action for the compound considered; (ii) the compounds should form part of a congeneric group; (iii) there should be a common site of action for the biological effect. Perhaps these requirements should be considered when attempting to validate an *in vitro* test with a particular type of chemical.

With formulations and mixtures, the situation is more complex. Because of the possibility of synergistic effects between ingredients, it will be necessary to test some or all of the ingredients individually. This will put the cytotoxicity of the complete formulations into a better perspective. With the continued

collaboration of industrial sponsors, this should be possible. However, the collaboration should be strengthened with increased dialogue between industry and academia. Without industry providing specific objectives of what sort of toxicity data they require (in order to make their safety and risk assessments), academia will not be able to offer relevant solutions. Initially, FRAME has only been able to give results for formulations tested in the existing FRAME *in vitro* toxicity assays (which were developed for pure chemicals). There is now the opportunity to tailor assays to suit the requirements of one industry, or one type of formulation.

Such tailoring of assays need not only be for *chemical* types, but also for *physical* types. Although some success has been achieved with adapting the FRAME KB cytotoxicity assay for volatile liquids, there remain the problems of gases and insoluble solids. The importance of these problems depends on which kind of chemical product is the object of interest, but it is still necessary for these problems to be addressed in *in vitro* toxicology.

The battery approach in *in vitro* toxicology is not new in theory, but there are few examples in practice. Research is required into the usefulness of the FRAME KB cytotoxicity assay in batteries for various categories of toxic effect or product type. Multivariate models, such as those developed by Hellberg *et al.* (1990), offer a way forward, but before the models will be taken up by other toxicologists, these toxicologists will need to gain a greater understanding of the mathematical principles involved. The FRAME Toxicity Committee has recently set new objectives in the search for ways to reduce, refine and replace *in vivo* toxicity tests (FRAME Toxicity Committee, 1991). An important part of these is the development and validation of test batteries, in which scientists are not constrained by a rigid list of required tests, but are able to select an appropriate combination of tests, to suit the substance under test, and the type of adverse effects to be screened for (Balls *et al.*, 1991b). It is possible that

the FRAME KB cytotoxicity assay would be useful as a component in test batteries for several types of toxicity.

The FRAME KB cytotoxicity assay will continue to be developed, adapted and evaluated, as part of the industry-sponsored FRAME Research Programme. Ongoing support of the research by industrial companies will help to promote *in vitro* toxicology and, hopefully, help to validate the role of the FRAME KB cytotoxicity assay, and other FRAME assays, for use in test batteries.

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by Lesley M. Smith (née Hulme)

Clothier, R.H., Hulme, L., Ahmed, A.B., Reeves, H.L., Smith, M. and Balls, M. (1988). *In vitro* cytotoxicity of 150 chemicals to 3T3-L1 cells, assessed by the FRAME Kenacid blue method. *Alternatives to Laboratory Animals* **16**, 84-95.

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APPENDIX A ADDITIONAL BACKGROUND INFORMATION

MECHANISMS OF CELL DEATH

Throughout the life of an animal, some of its cells will die through either physiological or pathological causes. Physiological causes include cell turnover and apoptosis. During cell turnover in epithelia such as the skin, cells die and slough off as new ones replace them from beneath. Apoptosis is the term for a controlled, programmed form of cell death. It is crucial in phenomena such as the development of 5 separate digits in vertebrate limbs and the control of organ size in adults (Young, 1992). It is also a vital part of the functioning of a healthy immune system. The mechanism of apoptosis requires the synthesis of nucleases and other destructive enzymes. Following a stimulus, the enzymes are released internally and the cell rapidly breaks up.

Pathological causes of cell death include loss of blood supply, bacterial toxins and physical and chemical agents (Woolf, 1977). The majority of mechanisms of chemical cytotoxicity begin with binding of the toxic species to cellular molecules. As a result the molecules are altered and hence cannot function efficiently or participate normally in cellular processes. Four main factors determine the form and extent of chemical cytotoxicity (Bridges, 1985):-

(a) the level and persistence of the initiating factor at the molecular target;

(b) the role of the target(s) in cell/tissue function and the extent and irreversibility of their modification;

(c) the effectiveness of cellular defence mechanisms in removing offending agents, and in the repair, replacement, or compensation for, the cellular damage; and

(d) the nature and amount of toxic products released from the injured cell/tissue.

Chemical cytotoxicity can be mediated through the depletion of intracellular glutathione (Orrenius, 1985). Glutathione acts as a cell defence mechnism, by conjugating to toxic, reactive chemical species. Glutathione levels drop when the rate of conjugation exceeds the rate at which the cell can replace it, and, as a consequence, depletion in protein thiols occurs. Certain thiols are critical for cell survival, so, without them, the cell necrotises. Membrane-bound protein thiols are important in modulating calcium ion (Ca^{2+}) fluxes. When these cannot function correctly, a sustained increase in cytosolic Ca^{2+} occurs, and it is postulated that the excess intracellular Ca^{2+} leads to the activation of catabolic processes (e.g. proteolysis) (Orenius and Nicotera, 1987). and hence cell death. There are many manifestations of cytotoxicity (Table A1).

Table A1. Manifestations of cellular toxicity

Loss of structural integrity - death

(a) cell lysis
(b) nuclear/chromosomal fragmentation, micronuclei

Defective reproduction

(a) reproductive sterilization - permanent cycle block
(b) slowed cell cycling - temporary cycle block or permanent slowing
(c) "unregulated" cell cycling - neoplastic transformation

Impaired synthetic and metabolic functions

(a) mutagenesis
(b) defective synthesis, e.g. of RNA, DNA, proteins
(c) defective enzyme function
(d) defective receptor function
(e) defects in many other functions

From Grisham and Smith (1984)

A dead or dying cell (whether caused by apoptosis or necrosis) will be detected by phagocytic cells and engulfed by them.

CRITERIA FOR VALIDATION OF *IN VITRO* TOXICITY TESTS

Part of the validation of an *in vitro* toxicity test involves the evaluation of the test's relevance, by comparing the *in vitro* results with *in vivo* (preferably human) data. As proposed at the CAAT/ERGATT Workshop on the validation of toxicity test procedures (Balls *et al.*, 1990a), tests should attain a certain degree of predictivity, depending on the proposed use of the test. Predictivity may be calculated from specificity and sensitivity values, as defined below.

$$\text{Sensitivity} = \frac{a}{a+b}$$

$$\text{Specificity} = \frac{c}{c+d}$$

$$\text{Positive predictive value} = \frac{a}{a+d}$$

$$\text{Negative predictive value} = \frac{c}{b+c}$$

where a = true positives which the test finds positive
b = false negatives (positives which the test finds negative)
c = true negatives which the test finds negative
d = false positives (negatives which the test finds positive)

The prevalence of true positives in a set of test chemicals in a validation scheme will affect the predictivity of the test. The greater the prevalence of true positives, the greater the sensitivity of the test, but the lesser the specificity. Calculations of specificity and sensitivity are also useful when considering the performance of a battery of tests. It may be thought that

several tests (with the aim of identifying the same toxic property) in a battery would be more accurate than one test alone (Purchase, 1990a). However, although performing more tests causes sensitivity to increase (i.e. less false negatives), specificity drops (i.e. more false positives).

Tests such as the FRAME KB cytotoxicity assay do not provide the type of binary (+ or -) response which is amenable to predictivity calculations (Balls *et al.*, 1990a). The ID_{50} values are continuous, although they could be categorised. It may then be possible to compare the prediction of categories of toxicity, but, as stated in Chapter 3, categorisation of test results must be approached with caution. Balls *et al.*, (1990a) proposed that screening tests should have a predictive value of greater than 0.5 (e.g. better than random classification). The *in vivo/in vitro* comparisons in Chapter 3 demonstrated that the FRAME KB cytotoxicity assay was able to achieve a better than random correlation, and this would suggest that the assay has met this particular criterion of predictivity for a screening test.

HIERARCHICAL TESTING

The hierarchical, or tiered, approach to toxicity testing is envisaged by many workers. It is already in operation through the British Toxicology Society's support of the hierarchical scheme for dermal and ocular irritancy testing (Fielder *et al.*, 1987). The approach involves following a step-wise decision tree, and a general definition has been given by Balls (1986). Five stages are proposed:

1. Non-biological methods (e.g. QSAR).
2. First-order *in vitro* tests (e.g. general cytotoxicity tests).
3. Second-order *in vitro* tests (e.g. target organ tests).
4. Essential tests in animals.
5. Tests in human volunteers.

At each stage, if the data produced is adequate to establish the toxicity of the substance for its intended use, then no further testing need be done. Alternatively, the data could be used to refine the subsequent testing stages.

THE USE OF ACUTE TOXICITY TESTS IN DRUG DEVELOPMENT

Acute toxicity testing is no longer synonymous with the LD_{50} test - there are several versions of the LD_{50} test itself, plus new methods of assessing acute toxicity in animals which require fewer animals than an LD_{50} test and which do not simply look at death as an endpoint. Acute toxicity testing during drug development is carried out for several reasons. At the research stage, screening tests are required in order to make decisions concerning the future of new compounds. The evaluation stage requires more-certain data on which to base further decisions. Finally, the development stage necessitates considerable amounts of toxicity data to be generated, partly to ensure product safety and partly to satisfy certain regulations. Acute toxicity data is required, amongst other reasons, to classify drugs for labelling purposes (transport regulations) and to provide dose ranges for chronic toxicity studies. Clark *et al.*, (1991) support the development of non-mammalian alternatives to animal acute toxicity testing, particularly as pre-screens and for mode of action studies. It is unlikely that one non-mammalian alternative test or battery would be used for all the reasons that the animal acute tests are performed.

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In Vitro Cytotoxicity of 150 Chemicals to 3T3-L1 Cells, Assessed by the FRAME Kenacid Blue Method

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Summary

The cytotoxicities to 3T3-L1 cells of 150 coded chemicals, estimated by the FRAME kenacid blue method, are given as a basis for further studies on the relative *in vitro* toxicities of related and distinct sets of test chemicals, and as a basis for future attempts at the comparison and correlation of estimates of toxicity obtained *in vitro* and *in vivo*.

The expression of the results of *in vitro* cytotoxicity tests is discussed, as are various ways of comparing values obtained for different chemicals. It is suggested that there should be agreement on the inclusion of a standard set of reference chemicals in all cytotoxicity test validation trials.

Key words: cytotoxicity, kenacid blue dye-binding, 3T3-L1 cells, validation, in vitro tests, alternatives to animals.

Introduction

The FRAME cytotoxicity test involves the measurement of total cellular protein by a dye-binding method after a 72-hour period of continuous exposure to the test chemical. The results from a four-centre blind trial with 50 chemicals and human BCL-D1 cells were published in 1986 (1). Other studies have involved comparison of the effects of 50 chemicals on 3T3-L1 cells as assayed by the FRAME kenacid blue and Rockefeller neutral red uptake methods, and the use of 24-hour and 72-hour exposure periods (2,3). Many of the same chemicals have also been tested with V79 cells, using 4-hour and 72-hour exposure periods (4).

The main aim of this paper is to present the results of estimation of the cytotoxicities of 150 coded chemicals tested with murine 3T3-L1 fibroblast-like cells, as a basis for further studies, including *in vitro*/*in vitro* and *in vitro*/*in vivo* comparisons, and studies on particular types of chemicals, e.g. metals, detergents, cosmetics ingredients and pesticide formulations.

In addition, the highest concentration tested in our previous blind trials was 1000μg/ml. We have now re-tested chemicals found not to be toxic at that concentration, up to concentrations at which cytotoxicity could be determined.

Materials and Methods

3T3-L1 cell culture, test chemical addition, and the kenacid blue (KB) assay method were

performed according to previously published procedures (1,2), with the exception that some of the tests were carried out in 96-well plates, rather than in 24-well plates. Control studies indicated that the size of the culture well did not affect the results obtained.

Test chemicals were supplied coded as part of the FRAME Alternative Test Validation Scheme. Chemicals likely to be insufficiently soluble in aqueous media were dissolved initially in ethanol, methanol or dimethyl sulphoxide, according to guidelines issued with them. The final concentration of organic solvents was always 1%, at which concentration the rate of control cell growth was not affected.

Cells were treated 24 hours after plating, and the KB assay was carried out a further 72 hours later. After a range-finding test with a wide range of test chemical concentrations, definitive cytotoxicities were obtained by testing a narrower range of concentrations on three separate occasions, with three culture wells per concentration.

Dose-response curves were calculated from the results obtained, then the results were summarised as ID20, ID50 and ID80 values, i.e. the concentrations of test chemicals (μg/ml) which reduced the final cellular protein content of test wells by 20%, 50% and 80%, respectively, in comparison with that of solvent control wells.

Results and Discussion

Summary of results obtained

The results obtained for the 150 test chemicals are given in Table I, where they are listed and numbered in ID50 value order. They show that the chemicals tested had a wide range of *in vitro* cytotoxicities, with ID50 values ranging from <0.01 to 134,000μg/ml.

The ID50 value is considered to be the best indicator of *in vitro* cytotoxicity, since it is taken from the middle of the dose-response curve. Nevertheless, as we have observed previously (1,2), the ID20 and ID80 values also give useful information about the shape of the dose-response curve. For example, indium nitrate (no. 082, Table I) gave a much less steep dose-response curve than gadolinium chloride (no. 089). Similarly, while bendiocarb (no. 035) and pentachlorophenol (no. 036) gave very similar ID50 values, the dose-response curve for pentachlorophenol was much steeper.

Expression of results

When chemicals are tested as part of the FRAME Alternative Test Validation Scheme, the results are submitted before the identities of the chemicals are revealed. Only then does it become possible for their molecular weights to be taken into account. Although it must be remembered that *in vivo* toxicities tend to be expressed in units of mg/kg body weight, comparison of *in vitro* cytotoxicities based on molar concentration would seem to be preferable, since the toxic effects of most chemicals would be expected to be related to the number of molecules, rather than to the weight of each molecule. The use of molarities also facilitates the comparison of different assay systems (5).

The results obtained for the 150 test chemicals are listed and ranked in Table II in order of ID50 values (mM), as calculated from molecular weights and the estimated ID50 values (μg/ml) taken from the dose-response curves.

The positions of the chemicals do not differ markedly in Table I and Table II, except, as would be expected, in the case of chemicals with relatively high or low molecular weights. For example, Tweens 40, 60, 20, 85 and 80; numbers 067, 073, 075, 093 and 099 in Table I, move up to positions 36, 46, 47.5, 58 and 60 in Table II. Similarly, PEG 1900 and PEG 5000 move up from numbers 149 and 150 in Table I to positions 128 and 122 in Table II. By contrast, sodium fluoride, lithium fluoride and potassium fluoride move down from numbers 046, 052 and 065 in Table I to positions 79, 95 and 91.5 in Table II.

It should be noted that precise molecular weights cannot be given for polymers, such as the Tweens and the polyethylene glycols.

Table I: *In vitro* toxicities of 150 test chemicals, determined by the FRAME 72-hour KB method with 3T3-L1 cells, listed and numbered in order of ID50 values

No.	Test Chemical	Solvent	ID20 µg/ml	ID50 µg/ml	ID80 µg/ml
001	5-Fluorouracil	D	<0.01	<0.01	<0.01
002	6-Mercaptopurine monohydrate	D	<0.01	<0.01	<0.01
003	Acrolein	E	<0.01	<0.01	<0.01
004	Rotenone	D	<0.1	<0.1	<0.1
005	Vincristine sulphate	W	0.01 ± 0	<0.1	>100
006	Cycloheximide	M	<0.1	<0.1	0.3 ± 0.1
007	Cadmium (II) chloride	W	0.1 ± 0	0.1 ± 0	0.2 ± 0.1
008	Tributyltin chloride	E	0.1 ± 0.01	0.3 ± 0.01	0.4 ± 0
009	Silver (I) nitrate	W	1 ± 0	2 ± 0	2 ± 0
010	Vanadium (V) oxide	W	0.2 ± 0.1	2 ± 0	3 ± 1
011	*p*-Chloromercuribenzoic acid	D	<0.1	3 ± 1	8 ± 1
012	Copper (I) chloride	W	2 ± 0	3 ± 0	5 ± 1
013	Copper (II) chloride	W	2 ± 1	3 ± 1	4 ± 1
014	Mercury (II) chloride	E	2 ± 1	4 ± 1	6 ± 0
015	Chlorpromazine hydrochloride	W	3 ± 0	5 ± 1	7 ± 1
016	Benzalkonium chloride	W	4 ± 1	7 ± 0	9 ± 2
017	Chloroquine sulphate	W	4 ± 0	7 ± 0	>10
018	Colchicine	E	2 ± 1	8 ± 1	>10
019	Hexachlorophene	E	3 ± 1	9 ± 2	14 ± 2
020	Zinc (II) chloride	W	6 ± 1	9 ± 0	12 ± 1
021	*p*-Aminophenol	D	4 ± 2	10 ± 1	33 ± 12
022	Selenium (IV) oxide	W	5 ± 1	10 ± 2	15 ± 2
023	Cobalt (II) chloride	W	3 ± 1	11 ± 1	24 ± 6
024	Propanolol hydrochloride	M	8 ± 1	12 ± 0	14 ± 0
025	Diethylstilboestrol	E	4 ± 1	14 ± 3	60 ± 17
026	Triton X-100	W	2 ± 2	14 ± 3	50 ± 12
027	Thallium (I) sulphate	W	7 ± 3	15 ± 3	25 ± 5
028	Manganese (II) sulphate tetrahydrate	W	11 ± 2	15 ± 1	25 ± 3
029	Zinc (II) acetate	W	12 ± 1	15 ± 1	18 ± 1
030	Diethyl maleate	E	12 ± 0	17 ± 2	27 ± 3
031	Thallium (I) acetate	W	9 ± 1	17 ± 2	30 ± 2
032	6-Aminonicotinamide	D	11 ± 1	20 ± 6	27 ± 2
033	Iron (II) sulphate heptahydrate	W	I	20 ± 9	I
034	Tellurium (IV) chloride	D	8 ± 1	22 ± 2	31 ± 2
035	Bendiocarb	D	7 ± 2	30 ± 5	147 ± 3
036	Pentachlorophenol	E	22 ± 1	31 ± 1	38 ± 1
037	Nickel (II) sulphate heptahydrate	W	20 ± 3	33 ± 3	53 ± 3
038	Phenylbutazone	D	29 ± 1	45 ± 1	65 ± 3
039	Versalide	E	30 ± 1	45 ± 1	>50
040	Butylated hydroxyanisole	E	30 ± 1	45 ± 1	>50
041	Ioxynil	D	32 ± 4	47 ± 1	57 ± 1
042	Fungizone	W	8 ± 2	48 ± 3	85
043	Retinoic acid	D	40 ± 10	49 ± 10	57 ± 9
044	Dieldrin	D	26 ± 2	50 ± 3	123 ± 25
045	Tin (II) chloride	W	21 ± 6	53 ± 10	124 ± 31
046	Sodium (I) fluoride	W	35 ± 6	53 ± 4	73 ± 4
047	Rhodium (III) chloride trihydrate	W	26 ± 3	55 ± 2	100 ± 1
048	Tin (IV) chloride pentahydrate	W	34	64 ± 3	98
049	2.4-Dinitrophenol	D	34 ± 10	72 ± 9	87 ± 10
050	Sodium tetrathionate dihydrate	W	33 ± 4	74 ± 5	128 ± 22

No.	Test Chemical	Solvent	ID20 µg/ml	ID50 µg/ml	ID80 µg/ml
051	Nickel (II) chloride hexahydrate	W	43 ± 3	77 ± 13	98 ± 18
052	Lithium (I) fluoride	W	51 ± 12	77 ± 25	142 ± 24
053	Paracetamol	M	44 ± 4	90 ± 10	136 ± 18
054	Sodium dodecyl sulphate	W	66 ± 11	94 ± 12	162 ± 17
055	Phenacetin	D	84 ± 1	95 ± 1	>100
056	Palladium (II) chloride	W	62 ± 14	103 ± 2	115
057	Methylparaben	E	64 ± 4	105 ± 16	>300
058	Lead (II) nitrate	W	53 ± 21	119 ± 45	345 ± 19
059	Lead (IV) tetra-acetate	D	82 ± 22	119 ± 5	286 ± 34
060	1,2,4-Trichlorobenzene	E	89 ± 11	122 ± 14	>200
061	Acrylamide	W	<100	123 ± 8	182 ± 8
062	Iron (III) sulphate	W	37 ± 7	123 ± 24	309 ± 69
063	Naphthalene	D	44 ± 13	123 ± 1	189 ± 9
064	Dodecylbenzene sulphonic acid, sodium salt	W	120 ± 6	137 ± 1	150 ± 2
065	Potassium (I) fluoride	W	70 ± 16	150 ± 6	260 ± 25
066	Beryllium (II) sulphate tetrahydrate	W	66 ± 9	157 ± 17	387 ± 48
067	Tween 40	E	53 ± 5	159 ± 49	295 ± 50
068	2,4-Dichlorophenoxyacetic acid	M	70 ± 15	162 ± 19	>500
069	6-Methylcoumarin	E	130 ± 7	169 ± 16	403 ± 140
070	Diethyl phthalate	E	93 ± 22	198 ± 8	281 ± 8
071	Chloramphenicol	M	96 ± 28	226 ± 41	397 ± 42
072	2-Chloroquinoline	E	145 ± 25	233 ± 17	331 ± 42
073	Tween 60	E	146 ± 31	244 ± 27	345 ± 9
074	Dexamethasone	D	74	252 ± 43	>400
075	Tween 20	W	123 ± 5	262 ± 43	396 ± 42
076	Yttrium (III) nitrate hexahydrate	W	227 ± 20	262 ± 7	286 ± 3
077	Phenytoin	D	57 ± 3	265 ± 44	>1000
078	Acrylonitrile	E	147 ± 28	266 ± 40	290 ± 16
079	Thioacetamide	E	167 ± 11	274 ± 8	>500
080	Warfarin	D	187 ± 39	285 ± 26	398 ± 25
081	1-Octanol	E	236 ± 6	298 ± 4	360 ± 2
082	Indium (III) nitrate hydrate	W	26 ± 3	303 ± 57	546 ± 97
083	3-Chloroaniline	E	97 ± 11	308 ± 68	460 ± 138
084	L-Ascorbic acid	W	295 ± 60	323 ± 58	351 ± 55
085	Ammonium chloride	D	168 ± 47	337 ± 63	>500
086	Allyl alcohol	W	191 ± 23	355 ± 52	521 ± 34
087	Phorone	E	188 ± 16	378 ± 27	600 ± 68
088	Bromobenzene	E	284 ± 23	389 ± 29	486 ± 38
089	Gadolinium (III) chloride hexahydrate	W	360 ± 3	389 ± 6	419 ± 12
090	Iproniazid phosphate	W	117 ± 20	393 ± 37	420 ± 76
091	Lanthanum (III) chloride heptahydrate	W	373	441 ± 34	567 ± 19
092	Acetylsalicylic acid	E	153 ± 28	465 ± 86	821 ± 52
093	Tween 85	E	253 ± 149	507 ± 50	821 ± 7
094	Trichloroacetic acid	W	311 ± 69	524 ± 109	658 ± 126
095	2,4,6-Trinitrobenzene sulphonic acid	W	269 ± 42	536 ± 46	772 ± 11
096	Phenobarbital	M	<500	543 ± 15	917 ± 34
097	Lithium (I) chloride	W	343 ± 67	554 ± 95	810 ± 73
098	Carbon tetrachloride	E	506 ± 32	600 ± 28	665 ± 32
099	Tween 80	E	135 ± 25	619 ± 69	836 ± 9
100	Isoxepac	D	292 ± 90	644 ± 38	>1000

No.	Test Chemical	Solvent	ID20 µg/ml	ID50 µg/ml	ID80 µg/ml
101	1.2-Dibromoethane	D	589	645 ± 240	924 ± 250
102	Imidazole	D	371 ± 44	688 ± 12	>750
103	Frusemide	D	628 ± 70	756 ± 52	873 ± 45
104	Aluminium (III) chloride hexahydrate	W	738 ± 120	830 ± 60	1050 ± 82
105	Acetonylacetone	W	798 ± 34	937 ± 23	>1000
106	Barium (II) acetate	W	566 ± 280	982 ± 62	2470
107	1-Heptanol	E	887 ± 26	1010 ± 86	1150 ± 74
108	Caffeine	W	330 ± 30	1110 ± 57	3240 ± 310
109	Aluminium (III) nitrate nonahydrate	W	953 ± 75	1190 ± 37	1280
110	L-Cysteine hydrochloride monohydrate	W	498 ± 120	1190 ± 160	1650
111	Aniline	E	1320 ± 170	1480 ± 330	2540 ± 190
112	Chromium (III) chloride hexahydrate	W	857 ± 160	1500 ± 24	2170 ± 84
113	Thiophene	E	1240 ± 31	1680 ± 130	>2000
114	Cyclophosphamide	W	1320 ± 150	1800 ± 490	2470 ± 100
115	Isoniazid	W	1320 ± 78	2170 ± 330	3970 ± 730
116	Lithium (I) sulphate	W	953 ± 210	2460 ± 600	6460 ± 2000
117	Lithium (I) bromide	W	1790 ± 390	3290 ± 130	7050 ± 630
118	1-Pentanol	W	2880 ± 490	3540 ± 550	4160 ± 610
119	1-Hexanol	W	2580 ± 530	3600 ± 540	4320 ± 960
120	Calcium (II) chloride dihydrate	W	2230	3860	8900
121	Sodium benzylpenicillin	W	1730	3970 ± 78	6280 ± 48
122	Strontium (II) chloride hexahydrate	W	1940 ± 290	4060 ± 220	10.900 ± 940
123	Streptomycin sulphate	W	1450	4160 ± 430	6990 ± 240
124	Potassium (I) chloride	W	3060 ± 300	4360 ± 200	5560 ± 75
125	Lithium (I) iodide	W	2470 ± 810	4690 ± 460	7710 ± 1100
126	1-Butanol	W	3320 ± 970	4780 ± 490	6320 ± 570
127	Magnesium (II) chloride hexahydrate	W	3260 ± 420	5600 ± 750	7910 ± 960
128	Sodium chloride	W	1790 ± 700	6230 ± 720	8390 ± 660
129	Pyrrole	W	5740 ± 230	6570 ± 310	7430 ± 380
130	Potassium iodide	W	3280 ± 860	7260 ± 440	8790 ± 440
131	Potassium (I) bromide	W	5900 ± 140	7780 ± 390	9540 ± 360
132	Potassium (I) sulphate	W	6270 ± 990	7860 ± 690	9230 ± 330
133	Sodium (I) sulphate	W	6090 ± 890	8930 ± 560	10.000 ± 390
134	Sodium (I) bromide	W	7250 ± 450	9840 ± 910	11.300 ± 1000
135	Kanamycin sulphate	W	2960 ± 970	11.000 ± 1200	19.200
136	1-Propanol	W	7580 ± 480	11.200 ± 480	17.000
137	Sodium (I) iodide	W	8730 ± 1700	11.800 ± 1500	16.400 ± 1300
138	1.2-Dichloroethane	W	8020 ± 1600	13.600 ± 2800	15.900 ± 4000
139	2-Propanol	W	7750 ± 1000	14.200 ± 1400	25.200
140	Dimethyl sulphoxide	W	11.800 ± 2100	22.400 ± 1700	38.300 ± 5000
141	Acetonitrile	W	14.300 ± 710	23.100 ± 2200	31.400 ± 4000
142	1,2-Propanediol	W	19.100 ± 1900	26.000 ± 2600	46.200 ± 8900
143	Acetone	W	26.900 ± 7400	35.900 ± 8500	43.700 ± 6700
144	Ethanol	W	23.200 ± 1700	36.100 ± 870	38.700 ± 1300
145	D-Fructose	W	15.500 ± 2100	39.200 ± 1200	55.900 ± 340

No.	Test Chemical	Solvent	ID20 µg/ml	ID50 µg/ml	ID80 µg/ml
146	Ethyl acetate	W	24,600 ± 4900	**43,300 ± 5800**	60,000 ± 7100
147	Methanol	W	36,500 ± 9800	**46,100 ± 10,000**	47,500 ± 5300
148	D-Glucose	W	36,400 ± 7600	**48,500 ± 3900**	57,900 ± 5200
149	PEG 1900	W	21,000 ± 6300	**77,100 ± 5500**	115,000 ± 2200
150	PEG 5000	W	22,500 ± 5100	**134,000 ± 6400**	>160,000

Values given are the mean ± standard error of the mean for the concentrations of test chemicals that reduced the final cellular protein content of test cells by 20% (ID20), 50% (ID50) and 80% (ID80) in comparison with solvent control wells. I = indeterminate; the points around the ID20 and ID80 points of the dose-response curve were too variable for values to be recorded with confidence. Solvent code: D = dimethyl sulphoxide; E = ethanol; M = methanol; W = water. Some values are taken from the published (2,3) and unpublished work of Rosemary J. Riddell.

Comparison of in vitro cytotoxicities

If the measurement of *in vitro* cytotoxicity is to be useful, then ways of comparing values obtained with one *in vitro* method, with other *in vitro* methods, and, ultimately, with *in vivo* test procedures, must be devised. This is not straightforward, since the values themselves have no absolute significance and the various methods which could be used for comparing them all have drawbacks.

Rank order can be a useful basis for comparison of tests carried out by different methods within an experiment, but this is only valid when the same set of chemicals has been used. Also, ranking tends to imply that differences between positions in the ranking are equivalent. However, this is not necessarily the case. In Table II, for example, rank positions 22–42 cover a much narrower range of molarities than do rank positions 110–130.

Categorisation is the basis of classification of *in vivo* toxicities, such as acute lethal toxicity, long-term toxicity and irritancy, and EEC regulatory guidelines name the categories as "very potent", "potent", "marginal" and "no significant effect" (6,7). The chemicals listed in Table II could similarly be placed in four categories, according to their ID50 values, e.g. ≤0.05, 0.06–1.0, 1.1–10.0 and ≥10.1mM. However, this drastically reduces the significance of the values obtained in the *in vitro* cytotoxicity test, because it does not reflect the precise position of a chemical within a category which contains a wide range of values. Also, as in all categorisation schemes, values at the end of one category and at the beginning of the next category tend to be closer than to some others within the same category. For example, the value for gadolinium chloride (1.0mM, Table II) would be closer to that for phenytoin (1.1mm), in the next category, than that for zinc chloride (0.07mM), in the same category. This is also reflected in their rank order positions, being 74.5, 76.5 and 26.5, respectively.

The use of *standard reference chemicals* could provide the best basis for comparison of results obtained by different methods. For example, the following ten chemicals selected from Table II would have a wide range of ID50 values and positions in the rank order:

Chemical	ID50 (mM)	Rank
cadmium chloride	0.001	7.5
colchicine	0.02	16
pentachlorophenol	0.12	36
dinitrophenol	0.39	58
sodium fluoride	1.3	79
trichloroacetic acid	3.2	96.5
ammonium chloride	6.3	107
isoniazid	16	116.5
pentanol	40	127
dimethyl sulphoxide	287	144

Table II: ***In vitro*** **toxicities of the 150 chemicals tested by the FRAME 72-hour KB method with 3T3-L1 cells, listed in order of ID50 values (mM), together with number of listing in order of ID50 value (μg/ml)**

Rank	No.	Test Chemical	MW	ID50 μg/ml	ID50 mM
2.5	001	5-Fluorouracil	130	<0.01	<0.0001
2.5	002	6-Mercaptopurine monohydrate	170	<0.01	<0.0001
2.5	004	Rotenone	394	<0.1	<0.0001
2.5	005	Vincristine sulphate	923	<0.1	<0.0001
5	003	Acrolein	56	<0.01	<0.0002
6	006	Cycloheximide	281	<0.1	<0.0004
7.5	007	Cadmium (II) chloride	183	0.1	0.001
7.5	008	Tributyltin chloride	326	0.3	0.001
10.5	011	*p*-Chloromercuribenzoic acid	357	3	0.01
10.5	010	Vanadium (IV) oxide	182	2	0.01
10.5	009	Silver (I) nitrate	170	2	0.01
10.5	015	Chlorpromazine hydrochloride	355	5	0.01
16	014	Mercury (II) chloride	272	4	0.02
16	017	Chloroquine sulphate	418	7	0.02
16	018	Colchicine	399	8	0.02
16	016	Benzalkonium chloride	353ave	7	0.02
16	013	Copper (II) chloride	134	3	0.02
16	019	Hexachlorophene	407	9	0.02
16	026	Triton X-100	647ave	14	0.02
20.5	012	Copper (I) chloride	99	3	0.03
20.5	027	Thallium (I) sulphate	505	15	0.03
22	024	Propranolol hydrochloride	296	12	0.04
23.5	025	Diethylstilboestrol	268	14	0.05
23.5	042	Fungizone	924	48	0.05
26.5	031	Thallium (I) acetate	263	17	0.07
26.5	020	Zinc (II) chloride	136	9	0.07
26.5	028	Manganese (II) sulphate tetrahydrate	223	15	0.07
26.5	033	Iron (II) sulphate heptahydrate	278	20	0.07
29.5	029	Zinc (II) acetate	184	15	0.08
29.5	034	Tellurium (IV) chloride	269	22	0.08
32	023	Cobalt (II) chloride	130	11	0.09
32	022	Selenium (IV) oxide	111	10	0.09
32	021	*p*-Aminophenol	109	10	0.09
34	030	Diethyl maleate	172	17	0.10
36	036	Pentachlorophenol	266	31	0.12
36	037	Nickel (II) sulphate heptahydrate	281	33	0.12
36	067	Tween 40	1284	159	0.12
39	041	Ioxynil	371	47	0.13
39	044	Dieldrin	381	50	0.13
39	035	Bendiocarb	223	30	0.13

Rank	No.	Test Chemical	MW	ID50 µg/ml	ID50 mM
42	032	6-Aminonicotinamide	137	20	0.15
42	038	Phenylbutazone	308	45	0.15
42	043	Retinoic acid	300	49	0.16
44	039	Versalide	258	45	0.17
45	048	Tin (IV) chloride pentahydrate	351	64	0.18
46	073	Tween 60	≈1300	244	0.19
47.5	047	Rhodium (III) chloride trihydrate	263	55	0.21
47.5	075	Tween 20	1228	262	0.21
49	050	Sodium tetrathionate dihydrate	306	74	0.24
50	040	Butylated hydroxyanisole	180	45	0.25
51	059	Lead (IV) tetraacetate	443	119	0.27
52	045	Tin (II) chloride	190	53	0.28
53	062	Iron (III) sulphate	400	123	0.31
54	051	Nickel (II) chloride hexahydrate	238	77	0.32
55	054	Sodium dodecyl sulphate	288	94	0.33
56	058	Lead (II) nitrate	331	119	0.36
58	093	Tween 85	≈1300	507	0.39
58	049	2,4-Dinitrophenol	184	72	0.39
58	064	Dodecylbenzene sulphonic acid, sodium salt	349	137	0.39
60	099	Tween 80	1310	619	0.47
61	055	Phenacetin	179	95	0.53
62	056	Palladium (II) chloride	177	103	0.58
63	053	Paracetamol	151	90	0.60
64	074	Dexamethasone	393	252	0.64
65	060	1,2,4-Trichlorobenzene	182	122	0.67
66	076	Yttrium (III) nitrate hexahydrate	383	262	0.68
67	057	Methylparaben	152	105	0.69
68	071	Chloramphenicol	323	226	0.70
69	068	2,4-Dichlorophenoxyacetic acid	221	162	0.73
70.5	066	Beryllium (II) sulphate tetrahydrate	177	157	0.89
70.5	070	Diethyl phthalate	222	198	0.89
72	080	Warfarin	308	285	0.92
73	063	Naphthalene	128	123	0.96
74.5	082	Indium (III) nitrate hydrate	301	303	1.0
74.5	089	Gadolinium (III) chloride hexahydrate	372	389	1.0
76.5	077	Phenytoin	252	265	1.1
76.5	069	6-Methylcoumarin	160	169	1.1
78	091	Lanthanum (III) chloride heptahydrate	371	441	1.2
79	046	Sodium (I) fluoride	42	53	1.3
80.5	090	Iproniazid phosphate	277	393	1.4

Rank	No.	Test Chemical	MW	ID50 µg/ml	ID50 mM
80.5	072	2-Chloroquinoline	164	233	1.4
82	061	Acrylamide	71	123	1.7
83.5	095	2,4,6-Trinitrobenzenesulphonic acid	293	536	1.8
83.5	084	L-Ascorbic acid	176	323	1.8
86	103	Frusemide	331	756	2.3
86	081	1-Octanol	130	298	2.3
86	096	Phenobarbital	232	543	2.3
88.5	100	Isoxepac	268	644	2.4
88.5	083	3-Chloroaniline	128	308	2.4
90	088	Bromobenzene	157	389	2.5
91.5	092	Acetylsalicylic acid	180	465	2.6
91.5	065	Potassium (I) fluoride	58	150	2.6
93	087	Phorone	138	378	2.7
94	123	Streptomycin sulphate	1458	4160	2.9
95	052	Lithium (I) fluoride	26	77	3.0
96.5	109	Aluminium (III) nitrate nonahydrate	375	1190	3.2
96.5	094	Trichloroacetic acid	163	524	3.2
98.5	101	1,2-Dibromoethane	188	645	3.4
98.5	104	Aluminium (III) chloride hexahydrate	242	830	3.4
100	079	Thioacetamide	75	274	3.6
101	106	Barium (II) acetate	255	982	3.8
102	098	Carbon tetrachloride	154	600	3.9
103	078	Acrylonitrile	53	266	5.0
104	112	Chromium (III) chloride hexahydrate	266	1500	5.6
105	108	Caffeine	194	1110	5.7
106	086	Allyl alcohol	58	355	6.1
107	085	Ammonium chloride	54	337	6.3
108	114	Cyclophosphamide	279	1800	6.5
109	110	L-Cysteine hydrochloride monohydrate	176	1190	6.8
110	105	Acetonyl acetone	114	937	8.2
111	107	1-Heptanol	116	1010	8.7
112	102	Imidazole	68	688	10
113	121	Sodium benzylpenicillin	356	3970	11
114	097	Lithium (I) chloride	42	554	13
115	122	Strontium (II) chloride hexahydrate	267	4060	15
116.5	115	Isoniazid	137	2170	16
116.5	111	Aniline	93	1480	16
118	135	Kanamycin sulphate	581	11,000	19
119	113	Thiophene	84	1680	20
120	116	Lithium (I) sulphate	110	2460	22

Rank	No.	Test Chemical	MW	ID50 µg/ml	ID50 mM
121	120	Calcium (II) chloride dihydrate	147	3860	26
122	150	PEG 5000	≈5000	134,000	27
123	127	Magnesium (II) chloride hexahydrate	203	5600	28
124.5	125	Lithium (I) iodide	134	4690	35
124.5	119	1-Hexanol	102	3600	35
126	117	Lithium (I) bromide	87	3290	38
127	118	1-Pentanol	88	3540	40
128	149	PEG 1900	≈1900	77,100	41
129	130	Potassium (I) iodide	166	7260	44
130	132	Potassium (I) sulphate	174	7860	45
131	124	Potassium (I) chloride	75	4360	58
132	133	Sodium (I) sulphate	142	8930	63
133	126	1-Butanol	74	4780	64
134	131	Potassium (I) bromide	119	7780	65
135	137	Sodium (I) iodide	150	11,800	79
136	134	Sodium (I) bromide	103	9840	96
137	129	Pyrrole	67	6570	98
138	128	Sodium (I) chloride	58	6230	107
139	138	1,2-Dichloroethane	99	13,600	138
140	136	1-Propanol	60	11,200	186
141	145	D-Fructose	180	39,200	218
142	139	2-Propanol	60	14,200	236
143	148	D-Glucose	180	48,500	269
144	140	Dimethyl sulphoxide	78	22,400	287
145	142	1,2-Propanediol	76	26,000	342
146	146	Ethyl acetate	88	43,300	491
147	141	Acetonitrile	41	23,100	562
148	143	Acetone	58	35,900	617
149	144	Ethanol	46	36,100	783
150	147	Methanol	32	46,100	1441

ID50 value (mM) is calculated using the ID50 concentration (µg/ml), as measured, and the molecular weight (MW).

Thus, silver nitrate would be classed as less toxic to 3T3-L1 cells than cadmium chloride, but more toxic than colchicine; chloramphenicol would be classed as less toxic than dinitrophenol, but more toxic than sodium fluoride; pyrrole would be classed as less toxic than pentanol, but more toxic than dimethyl sulphoxide; and ethanol would be classed as less toxic than dimethyl sulphoxide.

Difficult chemicals

The FRAME set of test chemicals for use in validation studies was deliberately chosen to include not only chemicals of known and different degrees and mechanisms of toxicity, but also chemicals likely to cause difficulties when applied to continuous cell lines in culture.

For example, the *in vivo* toxicity of a number of chemicals is mediated through the active production of reactive metabolites, which often results in organ-selective toxicity (8). The enzyme systems involved are not likely to be sufficiently active in most cell lines to permit the identification of such metabolism-mediated toxicity. This is reflected in the low cytotoxicity to 3T3-L1 cells of chemicals such as bromobenzene, carbon tetrachloride, allyl alcohol, cyclosphosphamide and aniline, which are known to be metabolised to toxic reactive metabolites *in vivo*.

Some of the other chemicals in the set are particularly volatile at 37°C, including acetonitrile, dichloroethane, thiophene and acrylonitrile, so the values obtained may not be a true reflection of the effects of their interactions with 3T3-L1 cells. Particular care was taken with cultures treated with these chemicals, because if high concentrations of the test substance were close to the control wells, contamination could possibly occur. The plating was arranged to minimise this possibility, and on occasion two or more sets of controls were used.

Other chemicals, such as acetone and carbon tetrachloride, reacted visibly with the plastic at higher range-finder concentrations. However, such high doses were not found to be close to the ID50 doses.

Some chemicals in the set are only sparingly soluble in any of the four solvents used, including lead tetraacetate, iron (III) sulphate, aluminium chloride, aluminium nitrate and barium acetate. Thus, the cytotoxic concentration given may not accurately reflect the actual concentration to which the cells were exposed, if precipitation occurs.

Finally, some of the test chemicals are already present in the culture medium in significant amounts, including sodium chloride, potassium chloride, calcium chloride and glucose. These amounts are not included in the calculation of toxicity of the chemicals concerned, as we prefer to determine the toxicity of *added* chemical, just as LD50 determinations do not take into account the natural presence of a chemical within the bodies of test animals.

All of these factors would be expected to affect *in vitro* cytotoxicity, and they must be recognised when one is interpreting and determining the value of the information provided by tests such as the FRAME KB test.

Conclusions

Rigorous validation and evaluation will be essential, if the potential value of *in vitro* tests in toxicology is to be realised and usefully exploited (9). This will necessitate the establishment of an extensive amount of *in vitro* toxicity data and critical comparison and assessment of the various methods which are in the course of development.

As a result of our experience both in establishing the FRAME Alternative Test Validation Scheme and as participants in it, we suggest that comparison between different *in vitro* tests will only be possible if an agreed set of standard reference chemicals are tested in all of them. The choice of such a set should reflect types of chemicals, as well as mechanisms of toxicity and also the availability and reliability of full *in vivo* toxicity profiles.

The ten chemicals we have listed could be included in such a set, but it should also include chemicals which have been shown to have different cytotoxicities in different cell lines, such as phenacetin, paracetamol and aniline (3,4).

Acknowledgement

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In Vitro Cytotoxicity of 24 Chemicals to Mouse Teratocarcinoma Cells

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Introduction

In vitro cytotoxicity assays involving undifferentiated permanent cell lines are now in widespread use. However, since differentiation is such a pivotal event in the maintenance of many adult tissues *in vivo*, and is of particular importance during embryonic, fetal and postnatal growth and development, it was decided to investigate the effects of chemicals on cells capable of differentiation *in vitro*. F9 was chosen as a suitable cell line, since F9 cells are derived from a mouse testicular teratocarcinoma and can be stimulated to differentiate in culture on exposure to retinoic acid (1), an event which can be monitored according to both morphological and biochemical criteria. F9 cells have also been used extensively as an *in vitro* model in studies on early events in embryogenesis (2).

The chemicals chosen included some of those recommended for use in the validation of *in vitro* tests for teratogenicity (3). In the initial phase of this study, the basic cytotoxicity of 24 chemicals to undifferentiated F9 cells was assessed, and the results were compared with values obtained by the standard FRAME kenacid blue method with 3T3-L1 undifferentiated mouse fibroblast-like cells (4).

Materials and Methods

Reagents

The test compounds were supplied coded by FRAME (Fund for the Replacement of Animals in Medical Experiments), as part of the FRAME Alternative Test Validation Scheme (5).

Cells

F9 cells (obtained from Dr Ann Daly, Department of Dermatology, The Royal Victoria Infirmary, Newcastle-upon-Tyne, UK), were cultured according to the method of Daly & Redfern (6).

Prior to use for cell culture, all culture surfaces were pre-rinsed with a 0.1% gelatin solution (Sigma Chemical Co., Poole, Dorset, UK). After aspiration of excess gelatin, tissue culture plasticware was left to dry overnight before cells were plated. Undifferentiated F9 cells were routinely maintained in 80cm^2 flasks (Costar, Northumbria Biologicals Ltd, Cramlington, Northumbria, UK) in a humidified 5% CO_2 atmosphere at 37°C. The culture medium used was Dulbecco's modified Eagle's medium (DMEM: Gibco Ltd, Paisley, UK), supplemented with: 10% fetal calf serum (Gibco), 100IU/mL sodium benzylpenicillin (Glaxo Laboratories Ltd, Greenford, Middlesex, UK) and 100µg/ml streptomycin sulphate (Evans Medical Ltd, Liverpool, UK). Before plating, the F9 cells were washed twice with pre-warmed phosphate buffered saline (PBS), and dissociated with 1ml trypsin/EDTA.

Basic cytotoxicity testing

F9 cells were plated at a seeding density of 5×10^4 cells/well, in 23 wells of a gelatin-coated 24-well plate (Costar), and incubated overnight. The last well contained no cells and was used as a reference blank. Test chemicals were dissolved in medium or in medium containing an organic solvent (ethanol or dimethyl sulphoxide), as recommended by FRAME. Organic solvents were kept at 1% v/v, a concentration which did not adversely affect the rate of growth of control cells. 24 hours after plating, medium was aspirated from the wells and replaced

with a range of doses of test chemical. Three wells were treated with medium alone (medium control) or medium containing 1% solvent (solvent control). The cells were left exposed to the test chemical for a 48-hour period. The cells were then washed twice with pre-warmed PBS before being fixed, stained and assayed for total cellular protein by the FRAME kenacid blue method (5). During the exposure period, the cells were regularly examined for any changes in morphology.

Initially, a range-finding test was carried out using a wide range of test chemical concentrations. Definitive cytotoxicities were then obtained by testing a narrower range of concentrations on at least three separate occasions, with three culture wells per concentration. Dose-response curves were calculated from the results obtained, and ID_{20}, ID_{50} and ID_{80} values estimated, i.e. the concentrations of test chemical (μg/ml) which reduced the final cellular protein content of test wells by 20%, 50% and 80%, respectively, in comparison with that of solvent control wells.

Table I: *In vitro* cytotoxicities of 24 chemicals to F9 cells

Chemical	ID_{20}μg/ml ± S.E.M.	ID_{50}μg/ml ± S.E.M.	ID_{80}μg/ml ± S.E.M.
5-Fluorouracil	0.005 ± 0.001	0.007 ± 0.002	>0.5
Colchicine	0.009 ± 0.007	0.02 ± 0.01	0.03 ± 0.02
6-Aminonicotinamide	0.03 ± 0.01	0.3 ± 0.1	11 ± 2
Diethylstilboestrol	0.04 ± 0.02	0.3 ± 0.2	8 ± 2
Dexamethasone	0.2 ± 0.1	0.7 ± 0.2	>100
6-Methylcoumarin	0.3 ± 0.1	0.7 ± 0.1	54 ± 10[c]
Cadmium(II) chloride	0.5 ± 0.02	1 ± 0.4	2 ± 0.1
Acetylsalicylic acid	1 ± 0.3	3 ± 0.6	203 ± 92
Phenytoin	0.5 ± 0.1	7 ± 3	>200
Retinoic acid	3 ± 1	9 ± 1	15 ± 1
Pentachlorophenol	14 ± 1	20 ± 3	30 ± 3
Thalidomide	5 ± 0.3	33 ± 9	>500[a]
2,4-Dinitrophenol	28 ± 3	57 ± 6	86
Isoniazid	30 ± 7	60 ± 13	136 ± 33
Caffeine	62 ± 29	111 ± 46	333 ± 42
Chloramphenicol	57 ± 19	272 ± 26	614 ± 172
Saccharin	130 ± 20	330 ± 70	7800[b]
Trichloroacetic acid	390 ± 60	790 ± 80	>20000
Cyclophosphamide	1100 ± 150	1750 ± 210	2650 ± 310
Sodium benzylpenicillin	1300 ± 260	2600 ± 390	5900 ± 340[c]
Sodium(I) chloride	2400 ± 350	3600 ± 540	5300 ± 610
Methanol	10000 ± 1500	20900 ± 1400	70300 ± 3400
Ethanol	12000 ± 2000	21000 ± 3400	38800 ± 5700
Dimethyl sulphoxide	21100 ± 1700	28500 ± 1100	39200 ± 300

[a] *maximum solubility in aqueous medium*
[b] *n = 1*
[c] *n = 2*
The ID_{20}, ID_{50} and ID_{80} are the concentrations of test chemical (μg/ml) which reduces the final cellular protein content of test wells by 20%, 50% and 80% respectively in comparison with that of solvent control wells.

Results and Discussion

The ID_{20}, ID_{50} and ID_{80} values obtained for F9 cells following exposure for 48 hours to 24 test chemicals ranged from 0.005 to 70,300µg/ml (Table I). The toxicity profiles were not all linear or exponential, i.e. the ID_{80} value was difficult to estimate. This was particularly true for phenytoin and thalidomide, where problems of solubility were encountered.

When the chemicals were ranked in order of ID_{50} (mM) values for F9 cells alongside the values obtained for 3T3-L1 cells after 72 hours (4), there appeared to be substantial differences between the cytotoxicities for 3T3-L1 cells and undifferentiated F9 cells for some of the chemicals tested, e.g. dexamethasone, 6-methylcoumarin, caffeine and thalidomide (Table II).

As is the case with F9 and 3T3-L1 cells, it is not always possible to maintain different cell lines under the same conditions. The standard kenacid blue test uses an exposure period of 72 hours, during which time control 3T3-L1 cells would be expected to remain in the exponential growth phase. However, F9 cells grow more rapidly, and reach the plateau phase before 72 hours.

Table II: *In vitro* cytotoxicities of 24 chemicals to F9 and 3T3-L1 cells

Chemical	F9 ID_{50} (mM)	3T3-L1[a] ID_{50} (mM)
Colchicine	<0.0001	0.02
5-Fluorouracil	<0.0001	<0.0001
Diethylstilboestrol	0.001	0.05
Dexamethasone	0.002	0.64
6-Aminonicotinamide	0.002	0.15
6-Methylcoumarin	0.005	1.1
Cadmium (II) chloride	0.006	0.001
Pentachlorophenol	0.007	0.12
Acetylsalicylic acid	0.02	2.6
Phenytoin	0.03	1.1
Retinoic acid	0.03	
	0.16	
Thalidomide	0.13	<1.9[b]
2,4-Dinitrophenol	0.31	0.39
Isoniazid	0.44	16
Caffeine	0.57	5.7
Chlorophenicol	0.84	0.70
Saccharin	1.6	25
Trichloroacetic acid	4.8	3.2
Cyclophosphamide	6.3	6.5
Sodium benzylpenicillin	7.3	11
Sodium chloride	62	107
Dimethylsulphoxide	365	287
Ethanol	458	783
Methanol	652	1141

[a]taken from Clothier et al. (1988), except for saccharin and thalidomide (previously unpublished)

[b]maximum solubility in aqueous medium

The ID_{50} is the concentration of test chemical which reduced the final cellular protein content of test wells by 50% in comparison with that of appropriate solvent control wells

In the presence of 0.1×10^{6}M retinoic acid, F9 cells can be induced to differentiate. Undifferentiated F9 cells in culture are relatively small and rounded in appearance, and are densely packed; however, after a 48-hour exposure to this concentration of retinoic acid, the cells became larger and paler, and several developed long processes. Since such changes in cell appearance are thought to give an indication of cellular differentiation, F9 cells were observed at regular intervals during the determination of the basic cytotoxicities of all the test compounds, in an effort to establish whether any of the chemicals were capable of inducing differentiation. With the exception of retinoic acid, microscopical observation indicated little change in the appearance of the cells.

When linear regression analysis was performed on Log ID_{50}mM (F9) versus Log ID_{50}mM (3T3-L1) values, a correlation coefficient of 0.88 was obtained. Differences between the ID_{50} values of the two cell lines with the chemicals tested support the conclusion of Riddell *et al.* (7) that different cell lines may not always exhibit similar susceptibilities to the cytotoxic effects of chemicals.

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COMPARISON OF THE IN VITRO CYTOTOXICITIES AND ACUTE IN VIVO TOXICITIES OF 59 CHEMICALS

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The in vitro cytotoxicities of 59 chemicals, expressed as ID50 values (i.e., concentrations of test chemicals that reduced the final cellular protein content of test cultures by 50% in comparison with appropriate solvent control cultures) and obtained using murine 3T3-L1 cells and the FRAME kenacid blue method, have been compared with rat oral and mouse intraperitoneal (ip) LD50 values. A better in vivo/in vitro correlation was obtained for the 59 chemicals with mouse ip LD50 values (r = .80) than with rat oral LD50 values (r = .76), but the best in vivo/in vitro correlation was found when the most toxic of the rat and mouse values were used in the comparison (r = .81).

INTRODUCTION

Much attention has recently been focused on the potential value of in vitro tests for identifying chemicals likely to cause ocular and dermal irritation (e.g., Reinhardt et al., 1985; Frazier et al., 1987), but less consideration is being given to their use in predicting other expressions of acute toxicity resulting from short-term exposure.

The FRAME cytotoxicity test for assessing the relative acute in vitro toxicities of chemicals was developed through the collaboration of four tissue culture laboratories, with the support of a number of industrial

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companies (Balls and Horner, 1985; Knox et al., 1986). The method involves determination of the concentration of test chemical that inhibits cell proliferation, calculated from measurements of total cellular protein by a dye-binding method after a 72-h exposure period. The results of a blind trial with 50 coded chemicals and human BCL-D1 fibroblast like cells indicated that the test procedure gave results with a high degree of interlaboratory reproducibility (Knox et al., 1986). Riddell et al. (1986) compared the cytotoxicities of these 50 chemicals to BCL-D1 and murine 3T3-L1 fibroblastlike cells and found that there were substantial differences with eight test chemicals. Agreement between the results obtained with different cell lines, with some notable exceptions, was also found by Garle et al. (1987), who studied the effects of 27 of the FRAME chemicals on hamster V79 cells.

A set of more than 150 blind trial chemicals has now been collected with the advice and assistance of the FRAME Research Advisory Committee on Cytotoxicology. The set of chemicals comprises chemicals with known and different degrees and mechanisms of toxicity, some of which are unstable, volatile, or insoluble in water, or are metabolically activated or detoxified in vivo. These chemicals form the basis of the FRAME Alternative Test Validation Scheme (Balls and Horner, 1985).

In vivo/in vitro validation studies are complicated and difficult to organize, carry out, and evaluate (Balls and Clothier, 1989). One of the biggest problems is the informed but unbiased selection of appropriate in vivo toxicity data of sufficiently high quality, since data are available from various laboratories, obtained with a variety of species, strains, and treatment routes. The Central Toxicology Laboratory of Imperial Chemical Industries (ICI) plc is supporting FRAME through the provision of in vivo toxicity profiles for chemicals included in the FRAME validation set (Purchase et al., 1986). Summaries of profiles for 50 of the FRAME blind trial chemicals have been published (Purchase et al., 1987). An alternative and comprehensive source of information is the *Registry of Toxic Effects of Chemical Substances* (RTECS) of the U.S. National Institute for Occupational Safety and Health (Lewis and Tatken, 1980).

Fry et al. (1988) have recently drawn attention to the wide range of routes and species used in assessing the in vivo acute toxicities of chemicals. They have recommended the use of mouse intraperitoneal (ip) or intravenous (iv) LD50 values in preference to rat oral LD50 values suggested by Balls and Horner (1985), mainly on the grounds that the cells used in in vitro cytotoxicity tests are directly exposed to test chemicals, as are cells receiving chemicals by the ip or iv route in vivo, whereas only a fraction of an oral dose may become available in the systemic circulation.

We have recently published the results of a blind trial with 150 coded chemicals and murine 3T3-L1 fibroblastlike cells (Clothier et al., 1988). In this paper, as a contribution to consideration of how in vivo/in

vitro comparisons should be carried out, we have compared the in vitro toxicities of some of these chemicals with published in vivo acute toxicity data.

IN VIVO/IN VITRO COMPARISONS

In the ICI toxicology profiles, toxicity is classified and tabulated as very potent, potent, marginal, or without significant effects, broadly according to guidelines given for acute and long-term toxicity in Annex (VII)D to the VIth Amendment of the EEC Directive on the Classification of Dangerous Substances (67/548/EEC). However, although comparison of in vitro numerical values and in vivo toxicity categories *is* possible, where numerical expressions of in vivo toxicity can reasonably be obtained from the literature, the comparison of two sets of quantitative data is more straightforward. We have therefore calculated rat oral and mouse ip LD50 values from the data given in the ICI toxicology profiles (Purchase et al., 1986), expressed in Table 1 as the mean of the range of values given (mg/kg) ± the standard error of the mean.

These rat and mouse LD50 values were used to calculate the in vivo toxicities (mmol/kg) in Table 2. For chemicals for which no ICI profiles were available, values were taken from RTECS (Lewis and Tatken, 1980). Table 2 also contains the in vitro cytotoxicity results taken from Clothier et al. (1988), given as ID50 values, the ID50 value being the concentration of test chemical (m*M*) that reduces the final total cellular protein content of test wells to 50% of that of appropriate solvent control wells.

Although rat oral LD50 values were available for 94 of the 150 FRAME test chemicals, and mouse ip LD50 values were available for 86 of them, both rat oral *and* mouse ip data were found for only 59 of the chemicals. These 59 chemicals are included in Table 2, and this was the only reason for their selection from the 150 in the full set.

When linear regression analysis was used to compare the in vitro (log ID50) values and the rat and mouse in vivo (log LD50) acute toxicities, the following correlation coefficients (r values) were obtained:

Rat oral/in vitro	$r = .76$
Mouse ip/in vitro	$r = .80$
Most toxic (rat or mouse)/in vitro	$r = .81$
Least toxic (rat or mouse)/in vitro	$r = .78$

DISCUSSION

It will not be possible to fully evaluate the relevance of in vitro cytotoxicity measurements to acute in vivo toxicity, or their potential value in replacement alternative tests, until a large number of chemicals have been tested in a variety of test systems. We have been able to

TABLE 1. Rat Oral and Mouse ip LD50 Values Calculated from the ICI Toxicology Profiles[a]

Chemical	Rat oral LD50 (mg/kg)	Mouse ip LD50 (mg/kg)
Acetonitrile	3730 ± 500 (12)	336 ± 77 (4)
Acetonyl acetone	2150 (2)	1600 (1)
Acetylsalicylic acid	1780 ± 180 (13)	340 ± 32 (3)
Acrolein	36 ± 8 (4)	7 (1)
Acrylamide	197 ± 15 (10)	170 (1)
Acrylonitrile	109 ± 12 (9)	39 ± 8 (4)
AETT (versalide)	316 (1)	n.a.
p-Aminophenol	519 ± 86 (3)	n.a.
Aminophylline	384 ± 43 (3)	244 ± 10 (4)
Aniline	486 ± 74 (5)	492 (1)
L-Ascorbic acid	n.a.	1760 ± 590 (3)
Bendiocarb	139 ± 26 (5)	n.a.
Benzalkonium chloride	405 ± 49 (9)	10 (1)
Bromobenzene	2570 ± 410 (4)	1000 (1)
Butylated hydroxyanisole	3320 ± 420 (8)	n.a.
Caffeine	350 ± 89 (9)	268 ± 9 (3)
Chloramphenicol	2500 (1)	1270 ± 20 (4)
p-Chloromercuribenzoic acid	n.a.	38 (2)
Chloroquine	832 ± 114 (4)	73 (2)
Chloropromazine	329 ± 116 (4)	114 ± 13 (3)
Colchicine	n.a.	3.9 ± 1.7 (4)
Cycloheximide	2.1 ± 0.2 (3)	138 (1)
Cyclophosphamide	219 ± 73 (8)	461 ± 86 (6)
2,4-Dichlorophenoxyacetic acid	1490 ± 400 (11)	263 (2)
Diethylstilbestrol	n.a.	67 ± 28 (1+)
Dimethyl sulfoxide	16,900 ± 3200 (9)	13,100 ± 1500 (8)
2,4-Dinitrophenol	37 ± 4 (3)	26 (1)
Ethanol	13,200 ± 1800	6000 ± 890 (5)
D-Glucose	25,000 (1)	n.a.
Ioxynil	161 ± 31 (8)	n.a.
Iproniazid	312 ± 62 (3)	596 ± 49 (7)
Isoniazid	2350 ± 400 (4)	138 ± 7 (3)
Lithium chloride	764 ± 202 (3)	n.a.
Methanol	10,000 ± 800 (9)	8,400 ± 2310 (3)
6-Mercaptopurine	382 (1)	251 ± 22 (7)
6-Methylcoumarin	1680 (1)	n.a.
Methylparaben	n.a.	860 (2)
Phenacetin	2570 ± 410 (10	1040 ± 200 (6)
Phenobarbital	349 ± 77 (6)	231 ± 43 (4)
Phenylbutazone	742 ± 92 (8)	259 ± 47 (3)
Phenytoin	2140 ± 310 (3)	220 ± 30 (4)
Potassium iodide	n.a.	1120 ± 30 (1+)
Retinoic acid	2000 (1)	425 (2)
Sodium chloride	4300 ± 620 (4)	3100 (1)
Sodium dodecyl sulfate	1860 ± 400 (3)	250 (1)
Thioacetamide	n.a.	300 (1)
Warfarin	74 ± 29 (11)	n.a.

[a]Values given represent the mean ± SEM of (*n*) individual rat oral or mouse ip LD50 values given in the full ICI toxicology profiles (Purchase et al., 1986). Data from rats under 3 mo old and "average lethal dose" values were excluded from the calculation. The value for AETT was kindly provided by Stephen Pennisi (see Purchase et al., 1987). n.a., Not available; (1+) = mean of an unspecified number of values.

TABLE 2. In Vitro Toxicities of 59 Chemicals, Tested by the FRAME 72-h KB Method with 3T3-L1 Cells, and In Vivo Toxicities Based on Rat Oral and Mouse ip LD50 Data

Chemical	ID50 (m*M*)[a]	LD50 (mmol/kg)	
		Rat oral	Mouse ip
6-Mercaptopurine monohydrate	0.0001	2.5	1.7
Rotenone	0.0001	0.34	0.01
Acrolein	0.0002	0.64	0.13
Cycloheximide	0.0004	0.01	0.49
Cadmium(II) chloride	0.001	0.48	0.05
Chlorpromazine hydrochloride	0.01	0.93	0.32
Mercury(II) chloride	0.02	0.004	0.02
Chloroquine sulfate	0.02	2.6	0.23
Benzalkonium chloride	0.02	1.1	0.03
Copper(II) chloride	0.02	1.0	0.06
Zinc(II) chloride	0.07	2.6	0.23
Iron(II) sulfate heptahydrate	0.07	2.1	0.42
Zinc(II) acetate	0.08	14	0.31
Cobalt(II) chloride	0.09	0.62	0.38
Phenylbutazone	0.15	2.4	0.84
Retinoic acid	0.16	6.7	1.2
Tin(II) chloride	0.28	3.7	0.35
Nickel(II) chloride hexahydrate	0.32	0.81	0.20
Sodium dodecyl sulfate	0.33	6.5	0.87
2,4-Dinitrophenol	0.39	0.20	0.14
Phenacetin	0.53	14	5.8
Palladium(II) chloride	0.58	1.1	0.59
Paracetamol	0.60	16	2.4
Chloramphenicol	0.70	7.7	3.9
2,4-Dichlorophenoxyacetic acid	0.73	6.7	1.2
Naphthalene	0.96	14	1.2
Phenytoin	1.1	8.5	0.87
Lanthanum(III) chloride heptahydrate	1.2	17	0.49
Sodium(I) fluoride	1.3	4.3	1.2
Iproniazid	1.4	1.7	3.3
Acrylamide	1.7	2.8	2.4
Phenobarbital	2.3	1.5	1.0
Bromobenzene	2.5	16	6.4
Acetylsalicylic acid	2.6	9.9	1.9
1,2-Dibromoethane	3.4	0.57	1.2
Carbon tetrachloride	3.9	18	30
Acrylonitrile	5.0	2.1	0.74

[a]The ID50 value is the concentration of test chemical (m*M*) that reduced the final cellular protein content of test wells by 50% in comparison with that of appropriate solvent control wells.

TABLE 2. In Vitro Toxicities of 59 Chemicals, Tested by the FRAME 72-h KB Method with 3T3-L1 Cells, and In Vivo Toxicities Based on Rat Oral and Mouse ip LD50 Data

Chemical	ID50 (m*M*)[a]	LD50 (mmol/kg)	
		Rat oral	Mouse ip
Chromium(III) chloride hexahydrate	5.6	6.7	2.0
Caffeine	5.7	1.8	1.4
Allyl alcohol	6.1	1.1	0.72
Ammonium chloride	6.3	31	9.0
Cyclophosphamide	6.5	0.79	1.7
Acetonyl acetone	8.2	19	14
Lithium(I) chloride	13	18	14
Strontium(II) chloride hexahydrate	15	14	5.7
Isoniazid	16	17	1.0
Aniline	16	5.2	5.3
Calcium(II) chloride dihydrate	26	9.0	2.5
Magnesium(II) chloride hexahydrate	28	40	1.0
Sodium(I) iodide	79	29	5.8
Sodium(I) chloride	107	74	53
2-Propanol	236	97	16
Dimethyl sulfoxide	287	217	168
1,2-Propanediol	342	263	150
Ethyl acetate	491	125	8.1
Acetonitrile	562	91	8.2
Acetone	617	168	22
Ethanol	783	287	130
Methanol	1441	313	263

[a]The ID50 value is the concentration of test chemical (m*M*) that reduced the final cellular protein content of test wells by 50% in comparison with that of appropriate solvent control wells.

compare the in vivo and in vitro toxicities of 59 chemicals, including organic solvents, metal salts, and pharmaceuticals. Since no allowance was made for the chemicals deliberately included in the FRAME set because they would cause difficulties for in vitro cytotoxic test systems, the good correlations found between the in vitro ID50 values and either rat oral or mouse ip LD50 values were encouraging. The correlation with mouse ip values was better than that with rat oral values, which supports the suggestion of Fry et al. (1988).

The best in vivo/in vitro correlations were obtained when the most toxic of the rat oral or mouse ip values or the mouse ip values alone were used in the comparison. Fry et al. (1988) suggested that LD50 values determined in the most sensitive species of those for which a reasonable database exists should be used for in vivo/in vitro comparisons.

They were referring to ip or iv LD50 values, but the use of ip, iv, *or* oral LD50 values at this stage of development of in vivo/in vitro comparative studies might also be considered more reasonable.

Another approach to in vitro test validation is to consider groups of related compounds, such as surfactants, metals, or alcohols. This depends on having sufficient data for a meaningful comparison to be made. Our experience with metallic compounds (Hulme et al., 1989), however, indicates that the in vivo/in vitro correlation coefficients obtained with mouse ip are better than for the 59 unrelated chemicals considered in this paper.

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ASSESSMENT OF TWO ALTERNATIVE METHODS FOR PREDICTING THE IN VIVO TOXICITIES OF METALLIC COMPOUNDS

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The FRAME in vitro cytotoxicity assay and a physicochemical parameter for metal ions (i.e., "softness," σ_p) were assessed for their ability to predict the in vivo acute toxicities of 52 metallic compounds. The in vitro assay was found to be more useful, since it measures the toxicity of the whole compound, as does the in vivo method. The softness parameter applies to the metal ion only, so it cannot be used to predict the toxicity of compounds containing relatively nontoxic metal ions and toxic anions (e.g., potassium fluoride). The in vitro toxicity values (expressed as ID50 values, i.e., concentrations of test chemicals that reduced the final cellular protein content of test cultures by 50% in comparison with appropriate solvent control cultures) correlated better with mouse ip LD50 values than with rat oral LD50 values.

INTRODUCTION

The exposure of living organisms to metallic compounds is increasing, but the potential toxic hazard of most metals remains poorly understood (Friberg and Nordberg, 1986). At present, the vast majority of toxicity tests are conducted on laboratory animals. However, relatively

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simple and rapid in vitro assays are now being developed that could act as preliminary screens and possibly replace the need for animal tests altogether (Balls and Horner, 1985). The use of quantitative structure-activity relationship models (involving parameters such as metal "softness") is also increasingly a part of the initial phase of toxicity assessment (Balls, 1986).

The FRAME test is a straightforward and reproducible method for assessing acute basal cytotoxicity (Knox et al., 1986). The results of a blind trial with 150 chemicals and mouse 3T3-L1 fibroblastlike cells have recently been published (Clothier et al., 1988). In this paper, the cytotoxicities of 52 metallic compounds (expressed in terms of the ID50 value, i.e., the concentration of test chemical required to reduce final total cellular protein to 50% of appropriate solvent control levels after a 72-h exposure period) are compared with published in vivo acute toxicities.

Rat oral LD50 values are widely used as indices of acute toxicity. However, Fry et al. (1988) have recently suggested that data obtained following exposure by the intraperitoneal (ip) or intravenous (iv) route might be more appropriate for comparison with in vitro cytotoxicity test results. Both rat oral and mouse ip LD50 values were therefore used in our analysis.

A physicochemical parameter, namely, the "softness" of the metal ion, has been compared with in vivo and in vitro metal ion acute toxicity (Williams et al., 1982; Cox and Harrison, 1983; Tan et al., 1984; Babich et al., 1986). The "softness" of a metal ion governs its metal-ligand interactions, but the biological significance of this (in terms of metal-ligand binding as the toxic mechanism) is questionable. There is some controversy concerning the capacity of the softness parameter to predict the in vivo acute toxicities of certain groups of metals (Williams et al., 1982; Kaiser, 1985). We therefore carried out in vitro/softness and in vivo/softness comparisons, in addition to the in vivo/in vitro comparisons.

MATERIALS AND METHODS

3T3-L1 cells were cultured, exposed for 72 h to the test chemicals, and then final cellular protein content was determined by the kenacid blue assay method, as previously described (Knox et al., 1986; Riddell et al., 1986). $Pb(CH_3COO)_4$ and $CrCl_3 \cdot 6H_2O$ were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK, and KCl, $CaCl_2 \cdot 2H_2O$, $Ba(CH_3COO)_2$, $AlCl_3 \cdot 6H_2O$, $SnCl_4 \cdot 5H_2O$, $CuCl_2$, $Zn(CH_3COO)_2$, $MnSO_4 \cdot 4H_2O$, $NiSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $Fe_2(SO_4)_3$, and $BeSO_4 \cdot 4H_2O$ were obtained from BDH Ltd., Dagenham, Essex, UK. The remaining compounds were obtained from Aldrich Chemical Co. Ltd., Gillingham, Kent, UK. The test compounds were dissolved directly into culture me-

$Pb(CH_3COO)_4$ and $TeCl_4$, which were dissolved in dimethyl sulfoxide (DMSO). The DMSO was diluted to 1% final concentration in the test solution according to a previously published protocol (Riddell et al., 1986).

Metal salts present as components of the cell culture medium were 1.8 m*M* calcium(II) chloride, 0.3×10^{-3} m*M* iron(III) nitrate, 5.4 m*M* potassium(I) chloride, 0.81 m*M* magnesium(II) sulfate, 110 m*M* sodium(I) chloride, 45 m*M* sodium(I) hydrogen carbonate, and 0.9 m*M* sodium(I) dihydrogen phosphate.

Acute in vivo toxicity data (rat oral and mouse ip LD50 values) were taken from the ICI Toxicology Profiles (Clothier et al., 1989) or, if not available from this source, from the NIOSH *Registry of Toxic Effects of Chemical Substances* (Lewis and Tatken, 1980).

The "softness" (σ_p) of a metal is defined in terms of the coordinate bond energies (CBE) of the iodide (I) and fluoride (F) of the metal:

$$\sigma_p = \frac{\text{CBE(F)} - \text{CBE(I)}}{\text{CBE(F)}}$$

Softness values were taken from Williams et al. (1982), except those for Li^+, Na^+, K^+, Cu^+, Ca^{2+}, Fe^{2+}, Sn^{2+}, Al^{3+}, Y^{3+}, and La^{3+}, which were taken from Pearson and Mawby (1967).

Linear regression analysis was performed on in vivo versus in vitro values, in vivo versus softness values, and in vitro versus softness values, using log ID50, log LD50, and log σ_p values.

RESULTS

The ID50 values (m*M*) for the 52 compounds tested are shown in Table 1, and range from 0.001 m*M* to more than 100 m*M*. Selected in vivo data (namely, rat oral and mouse ip LD50 values) are also given, where available, along with softness values.

The results for different salts of the same metals (e.g., lithium, sodium, and potassium, Table 1) indicate that the toxicity of the anion of the metallic compound is also of great importance. Of the halides, fluorides are clearly the most toxic in vitro. Sulfates also appear to be slightly more toxic than the chlorides, bromides, and iodides. Since 22 chlorides were tested, direct comparison between the toxicities of the metal ions is possible.

Comparison of in vitro and in vivo toxicities gave good correlations (Table 2a), with in vitro results correlating better with mouse ip values ($r = .86$) than with rat oral values ($r = .73$). The correlation coefficients were slightly higher for the chlorides than for all the metal compounds ($r = .90$ and .75 for mouse ip and rat oral values, respectively).

TABLE 1. In Vitro Cytotoxicities of 52 Metallic Compounds to 3T3-L1 Cells, with Rat Oral LD50, Mouse ip LD50, and Softness (σ_p) Values

	ID50[a] (mM)	LD50[b] rat oral (mmol/kg)	LD50[b] mouse ip (mmol/kg)	Softness[c] (σ_p)
Cadmium(II) chloride	0.001	0.48	0.05	0.081
Vanadium(V) oxide	0.01	0.05	—	—
Silver(I) nitrate	0.01	—	0.13	0.073
Mercury(II) chloride	0.02	0.004	0.02	0.064
Copper(II) chloride	0.02	1.0	0.06	0.104
Copper(I) chloride	0.03	2.7	—	0.112
Thallium(I) sulfate	0.03	—	—	0.215
Thallium(I) acetate	0.07	—	0.14	0.215
Zinc(II) chloride	0.07	2.6	0.23	0.115
Manganese(II) sulfate·$4H_2O$	0.07	—	2.4	0.124
Iron(II) sulfate·$7H_2O$	0.07	2.1	0.42	0.129
Zinc(II) acetate	0.08	14	0.31	0.115
Tellurium(IV) chloride	0.08	—	—	—
Cobalt(II) chloride	0.09	0.62	0.38	0.130
Selenium(IV) oxide	0.09	—	—	—
Nickel(II) sulfate·$7H_2O$	0.12	—	0.14	0.126
Tin(IV) chloride·$5H_2O$	0.18	—	0.18	0.075
Rhodium(III) chloride·$3H_2O$	0.21	—	—	0.055
Sodium(I) tetrathionate·$2H_2O$	0.24	—	—	0.211
Lead(IV) tetraacetate	0.27	—	—	—
Tin(II) chloride	0.28	3.7	0.35	0.148
Iron(III) sulfate	0.31	—	—	0.097
Nickel(II) chloride·$6H_2O$	0.32	0.81	0.20	0.126
Lead(II) nitrate	0.36	—	—	0.131
Palladium(II) chloride	0.58	1.1	0.59	0.069
Yttrium(III) nitrate·$6H_2O$	0.68	—	6.2	0.147
Beryllium(II) sulfate·$4H_2O$	0.89	0.78	—	0.172
Indium(III) nitrate·xH_2O	1.0	—	—	0.100
Gadolinium(III) chloride·$6H_2O$	1.0	—	1.4	0.157
Lanthanum(III) chloride·$7H_2O$	1.2	17	0.49	0.171
Sodium(I) fluoride	1.3	4.3	1.2	0.211
Potassium(I) fluoride	2.6	4.2	—	0.232
Lithium(I) fluoride	3.0	—	—	0.247
Aluminum(III) nitrate·$9H_2O$	3.2	0.7	—	0.136
Aluminum(III) chloride·$6H_2O$	3.4	28	—	0.136
Barium(II) acetate	3.8	3.6	—	0.184
Chromium(III) chloride·$6H_2O$	5.6	6.7	2.0	0.107

[a]The ID50 values were taken from Clothier et al. (1988). The ID50 is the concentration of test chemical which reduces the final cellular protein content of test wells by 50% in comparison with that of appropriate solvent control wells.

[b]LD50 values were taken from the *Registry of Toxic Effects of Chemical Substances* (Lewis and Tatken, 1980), except those for lithium chloride, potassium iodide, and sodium chloride, which were taken from *ICI Toxicology Profiles* (Clothier et al., 1989).

[c]Softness (σ_p) values were taken from Williams et al. (1982), except for those for Li^+, Na^+, K^+ Cu^+, Ca^{2+}, Fe^{2+}, Sn^{2+}, Al^{3+}, Y^{3+}, and La^{3+}, which were taken from Pearson and Mawby (1967).

TABLE 1. In Vitro Cytotoxicities of 52 Metallic Compounds to 3T3-L1 Cells, with Rat Oral LD50, Mouse ip LD50, and Softness (σ_p) Values (*Continued*)

	ID50[a] (m*M*)	LD50[b] rat oral (mmol/kg)	LD50[b] mouse ip (mmol/kg)	Softness[c] (σ_p)
Lithium(I) chloride	13	18	14	0.247
Strontium(II) chloride·$6H_2O$	15	14	5.7	0.172
Lithium(I) sulfate	22	—	—	0.247
Calcium(II) chloride·$2H_2O$	26	9.0	2.5	0.181
Magnesium(II) chloride·$6H_2O$	28	40	1.0	0.167
Lithium(I) iodide	35	—	—	0.247
Lithium(I) bromide	38	—	—	0.247
Potassium(I) iodide	44	—	6.7	0.232
Potassium(I) sulfate	45	—	—	0.232
Potassium(I) chloride	58	—	7.4	0.232
Sodium(I) sulfate	63	—	—	0.211
Potassium(I) bromide	65	—	—	0.232
Sodium(I) iodide	79	29	5.8	0.211
Sodium(I) bromide	96	34	—	0.211
Sodium(I) chloride	107	74	53	0.211

[a]The ID50 values were taken from Clothier et al. (1988). The ID50 is the concentration of test chemical which reduces the final cellular protein content of test wells by 50% in comparison with that of appropriate solvent control wells.

[b]LD50 values were taken from the *Registry of Toxic Effects of Chemical Substances* (Lewis and Tatken, 1980), except those for lithium chloride, potassium iodide, and sodium chloride, which were taken from *ICI Toxicology Profiles* (Clothier et al., 1989).

[c]Softness (σ_p) values were taken from Williams et al. (1982), except for those for Li^+, Na^+, K^+, Cu^+, Ca^{2+}, Fe^{2+}, Sn^{2+}, Al^{3+}, Y^{3+}, and La^{3+}, which were taken from Pearson and Mawby (1967).

Softness values can only be used on a comparative scale among ions of the same valency (Williams et al., 1982). The compounds tested were therefore divided into metal valency groupings before their in vitro and in vivo toxicities were compared with their σ_p values. However, the numbers of compounds containing monovalent, trivalent, and tetravalent metals were inadequate for valid correlations to be made. Hence, correlation coefficients were calculated for all the divalent compounds tested and for divalent chlorides only (Table 2b).

For all divalent compounds, the softness parameter had a similar correlation with in vivo toxicities ($r = .71$ and $r = .69$) to that for the in vitro assay ($r = .71$). The softness/in vivo correlations improved when divalent chlorides only were used in the comparison (Table 2b). Nevertheless, the highest correlation coefficient ($r = .89$) was obtained when the in vitro ID50 values were compared with mouse ip LD50 values.

TABLE 2. Comparison of In Vitro and In Vivo Acute Toxicities and Softness Parameter Values for Metallic Compounds

(a) Correlation of In Vivo (log ID50) with In Vitro (log LD50) Toxicities

Comparison	Compounds tested	Correlation coefficient (r)	n
Rat oral/in vitro	All compounds	0.73	27
	Chlorides only	0.75	17
Mouse ip/in vitro	All compounds	0.86	28
	Chlorides only	0.90	18

(b) Correlation In Vivo (log LD50), Softness (log σ_p), and In Vitro (log ID50) Values

Comparison	Compounds tested	Correlation coefficient (r)	n
Rat oral/σ_p	All divalents	0.69	15
	Divalent chlorides	0.81	11
Mouse ip/σ_p	All divalents	0.71	15
	Divalent chlorides	0.76	11
In vitro/σ_p	All divalents	0.71	18
	Divalent chlorides	0.73	11
Rat oral/in vitro	All divalents	0.61	15
	Divalent chlorides	0.70	11
Mouse ip/in vitro	All divalents	0.77	15
	Divalent chlorides	0.89	11

DISCUSSION

Of the two alternatives assessed, the in vitro cytotoxicity assay was found to be the better predictor of in vivo toxicity for the metallic compounds tested.

The in vitro toxicity data gave a better correlation with mouse ip values than with rat oral values (Table 2a). Absorption of metallic compounds from the gut is known to vary widely according to the metal and anion concerned, the solubility of the compound, and the state of the subject animal (Camner et al., 1986; Nordberg et al., 1986). Continuous treatment of cells in culture with a compound possibly mimics better ip dosage, where there are fewer absorption barriers to tissue exposure (Fry et al., 1988).

In order to compare toxicities of the metal ions, one should select compounds with the same anion, bearing in mind the predominant anion in the culture medium is chloride. The contribution of the anion to the toxicity of a compound is unimportant when the more toxic compounds are tested [e.g., tin(II) chloride, ID50 0.28 m*M*]. However, when compounds of low toxicity [e.g., sodium(I) chloride, ID50 107

m*M*] are dissolved, a significant increase in the total anion concentration in the culture medium occurs, giving the possibility of anion effects.

Our in vivo/softness correlations (Table 2b) show how softness, being a property of the metallic ion, becomes more useful in comparisons between compounds with the same anion. The softness parameter is incapable of predicting the toxicities of compounds consisting of "toxic" anions and "nontoxic" cations [such as sodium(I) tetrathionate and potassium(I) fluoride]. In addition, in vivo/softness comparisons among compounds with the same anion are only meaningful where there are sufficient metal ions in the valency groupings. This limits comparisons involving metal ions with unusual valencies. A further limitation of the softness alternative is the variability and availability of published σ_p values for metals in all their valency states. Thus, there is no positive indication that σ_p has any real value as a means of predicting metal toxicity, least of all metal compound toxicity.

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THE POTENTIAL USEFULNESS OF A DIFFERENTIATING TERATOCARCINOMA CELL LINE IN *IN VITRO* TOXICITY TESTING

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Abstract—A number of *in vitro* systems have been put forward as potential alternative methods for testing chemicals for teratogenic potential. The most promising of these systems, for example mammalian whole embryo culture and the micromass technique, are currently undergoing further interlaboratory validation. However, such tests involve the use of a considerable number of animals. It was therefore decided to investigate the possible use of a permanent cell line that possessed many of the properties of embryonic cells, that is a differentiating cell line, F9 (derived from a mouse teratocarcinoma), in the development of an *in vitro* teratogenicity test. In a preliminary study, six chemicals were tested for their modulating effects on differentiation in undifferentiated, differentiating and differentiated F9 cells. These effects were assessed morphologically and by measuring the production of laminin (a biochemial marker of F9 differentiation). The use of the F9 cell line in *in vitro* teratogenicity testing shows promise, but further work is necessary before its potential can be fully evaluated.

Introduction

Over the past decade a variety of *in vitro* systems have been suggested as possible alternatives for use in the prediction and evaluation of the teratogenic potential of chemicals (for reviews, see Brown and Freeman, 1984; Neubert, 1989). Of these, mammalian whole embryo culture (Schmid, 1983) and the micromass technique (Flint, 1987) appear to be among the most promising. Already established as prescreens, these methods are currently undergoing further interlaboratory validation to assess their potential as genuine replacement alternatives. However, both these methods require the sacrifice of a significant number of animals, and the whole embryo method is legally classified as an animal experiment in Britain. It was therefore decided to investigate the possible use of a permanent cell line that would grow and differentiate in culture in a manner related to that which occurs during embryonic development, thus eliminating the need for animals. Since cell differentiation and growth are pivotal events during embryogenesis, their modulation *in vitro* by a chemical might be indicative of teratogenic potential of the latter. A literature survey revealed that a number of cell lines are commonly used as *in vitro* models of differentiation. For example, Mummery *et al.* (1984) used a neuroblastoma cell line in a test for teratogenicity but the endpoint of differentiation was only assessed morphologically; it is desirable to use a quantifiable endpoint wherever possible (Kimmel *et al.*, 1982).

F9 cells, originally derived from a mouse testicular teratocarcinoma, can be stimulated by retinoic acid (RA) to differentiate biochemically as well as morphologically (Strickland and Mahdavi, 1978), hence allowing differentiation to be both observed and quantified. The biochemical markers of the differentiation include increased synthesis of laminin and plasminogen activator, increased infectivity by SV40 virus and decreased alkaline phosphatase activity (Moore *et al.*, 1986; Strickland and Mahdavi, 1978). The differentiated phenotype is thought to resemble that of parietal endoderm cells in the mouse embryonic ectoderm (Hogan *et al.*, 1983; Strickland *et al.*, 1980), and the cells have been widely used as an *in vitro* model of early events in embryogenesis (Strickland, 1981).

In a preliminary study, the general cytotoxicities and the effects on cellular morphology of six chemicals were determined in undifferentiated, differentiating and differentiated F9 cells. The amount of laminin produced by differentiated cells exposed to chemicals was measured and compared with final total cellular protein levels. The potential use of F9 cells in an *in vitro* test for teratogenicity is discussed.

Materials and Methods

Cells. F9 cells, a gift from Dr A. K. Daly of the University of Newcastle-upon-Tyne, UK, were cultured according to the method of Daly and Redfern (1987).

Reagents. Cell culture reagents, laminin and anti-laminin antibody were obtained from Gibco, Paisley, UK; anti-rabbit IgG alkaline phosphatase conjugate, all-trans retinoic acid, caffeine, cyclophosphamide, Tween 20 and *p*-nitrophenol phosphate were from Sigma Chemical Co. Ltd, Poole, Dorset, UK; cadmium (II) chloride and 5-fluorouracil were from Aldrich Chemical Co. Ltd, Gillingham, UK; and dimethylsulphoxide was from BDH, Poole, Dorset, UK. Thalidomide was a gift from Rhône-Poulenc Ltd, Dagenham, UK.

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Abbreviations: ELISA = enzyme-linked immunosorbent assay; PBST = phosphate buffered saline containing 0.05% v/v Tween 20; RA = retinoic acid.

Table 1. *In vitro* toxicities of six chemicals to undifferentiated, differentiating and differentiated F9 cells

Chemical	ID_{50} (mM)*		
	Undifferentiated cells	Differentiating cells	Differentiated cells
5-Fluorouracil	<0.0001	0.001	0.001
Cadmium (II) chloride	0.006	0.01	0.01
Thalidomide	0.13	>1.9†	>1.9†
Caffeine	0.57	1.9	1.7
Cyclophosphamide	6.3	2.8	4.9
Dimethylsulphoxide	365	328	322

*The ID_{50} is the concentration of test chemical that reduced the final cellular protein content of test wells by 50% in comparison with that of appropriate solvent control wells.
†Maximum solubility in aqueous medium.

Cytotoxicity tests.

(a) On undifferentiated cells. 5×10^4 cells/well were plated in 24-well tissue culture plates and incubated overnight. The medium was replaced with medium containing test chemical dissolved in solvent, with solvent alone (final concentration of solvent being maintained at 1%) or with medium alone. After exposure to test chemicals for 48 hr, the cells were assayed for total cellular protein by the kenacid blue method (Knox *et al.*, 1986).

(b) On differentiating cells. Cells were plated and treated as for differentiated cells, but test chemical and control media also contained 0.1×10^{-6} M-RA. After the 48-hr exposure, cells were solubilized in 1 M-NaOH for 2 hr and the extracts were assayed for total cellular protein by the method of Bradford (1976). Standards were prepared from a protein standard solution (Sigma).

(c) On differentiated cells. Differentiated cells were prepared by growing F9 cells for 5 or 6 days (including one subculture) in the presence of 0.1×10^{-6} M-RA. Chemical treatments and the Bradford assay were conducted as for differentiating cells.

Each chemical concentration was tested in triplicate on at least three separate occasions. The ID_{50} values, that is concentrations that reduced the final total cellular protein content of test wells by 50% in comparison with the appropriate solvent control wells, were calculated from the dose-response curves.

Enzyme-linked immunosorbent assay (ELISA) for laminin. This method was modified from that of Williams *et al.* (1987). Medium samples were taken at the end of the 48-hr test period, and were stored at −20°C until required. Samples were centrifuged to remove dead cells, diluted 1 in 5 in phosphate buffered saline (PBST; containing 0.05%, v/v, Tween 20) and co-incubated overnight at 22°C with rabbit anti-laminin antibody. Co-incubations were then transferred to a laminin-coated ELISA microplate (50 ng laminin/well) for 30 min at room temperature. After washing with PBST, anti-rabbit IgG alkaline phosphatase conjugate was left on for 60 min. After further washing with PBST, the enzyme substrate, *p*-nitrophenol phosphate, was added in a pH 9.8 buffer. The production of yellow *p*-nitrophenol was measured at 404 nm in a Kontron plate reader. A standard curve was constructed using purified mouse laminin in the co-incubation.

Results

Six chemicals were tested for cytotoxicity to undifferentiated, differentiating and differentiated F9 cells. They were chosen because of their widely varying toxicities, modes of action and effects *in vivo*. The results (Table 1) indicate that the cells in the three culture situations were equally sensitive to cadmium (II) chloride, cyclophosphamide and

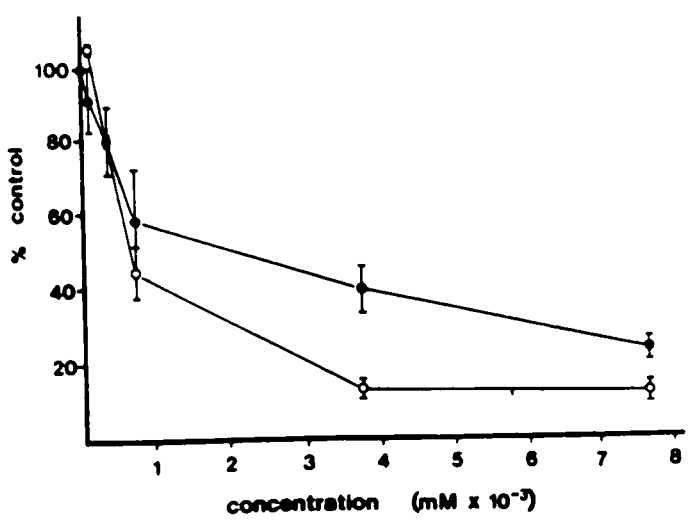

Fig. 1. The effects of 5-fluorouracil on laminin and protein levels from differentiated F9 cell cultures. (—●—) laminin in culture medium (—○—) cellular protein.

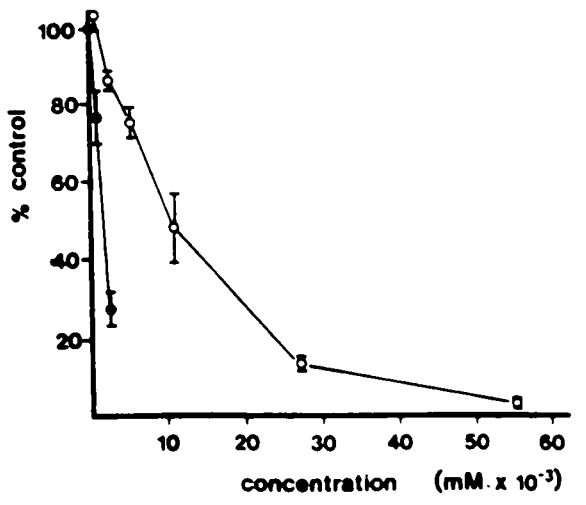

Fig. 2. The effects of cadmium (II) chloride on laminin and protein levels from differentiated F9 cell cultures. (—●—) laminin in culture medium, (—○—) cellular protein.

dimethylsulphoxide, and that the differentiating and differentiated cells were less sensitive to 5-fluorouracil, thalidomide and caffeine.

Treatment medium samples from cytotoxicity tests with differentiating and differentiated F9 cells were assayed for the presence of laminin. Medium samples from differentiating cells did not show any detectable laminin (lower limit of detection is approximately 5 ng/ml), while differentiated cells produced laminin in readily detectable amounts.

Three categories of relationship between protein and laminin levels following exposure to the chemicals were observed: (a) where the protein level fell more than the laminin level, with 5-fluorouracil (Fig. 1) and caffeine (data not shown); (b) where the laminin level fell more than the protein level, with cadmium (II) chloride (Fig. 2); (c) where the protein and laminin levels followed each other, with dimethylsulphoxide (Fig. 3), cyclophosphamide and thalidomide (data not shown).

Apart from RA, none of the chemicals tested induced morphological changes in normal F9 cells at the concentrations and over the time scales tested (except for the expected shrinkage of dying cells). Morphological changes induced by RA in the differentiating and differentiated cell test were not affected by the chemicals tested.

Discussion

F9 cells possess several characteristics that make them a useful model of cellular differentiation in toxicity studies: (1) they differentiate morphologically and biochemically in response to RA and other chemicals (Moore *et al.*, 1986; Nishimure *et al.*, 1983). (2) They are easy to maintain in culture, and, being a continuous cell line, offer considerable advantages over primary cultures in terms of cost, technical expertise required and reproducibility. (3) The biochemical marker of differentiation, that is laminin, is readily quantifiable by ELISA without needing to fix or disrupt the cells.

These preliminary observations suggest that further investigations in the potential use of F9 cells in *in vitro* teratogenicity testing would be worthwhile. It appears that different chemicals can affect laminin production by differentiated F9 cells in several ways, and that the differentiated function as well as cell division can be the target for chemical action.

There appears to be no simple explanation as to why differentiating and differentiated F9 cells are less sensitive to 5-fluorouracil, thalidomide and caffeine than normal cells. Differentiated cells have a slower growth rate than the undifferentiated cells, but this is not true for the differentiating cells, so altered growth rate cannot be the only reason why there is differential sensitivity.

The biochemical marker of differentiation, that is the laminin level in the medium, was higher than expected from the protein level in 5-fluorouracil (Fig. 1) and caffeine-treated culture wells. Microscopical observation showed a slow build-up of toxicity over the 48 hr-period for both chemicals, hence laminin may have been released by cells before they were killed. Alternatively, 5-fluorouracil may have affected cell division without affecting cell viability or its ability to produce laminin. Cadmium (II) chloride caused the laminin level to drop below that expected from the protein level (Fig. 2). This points to cadmium interfering with the process of laminin production either intracellularly or at surface sites, without affecting cell proliferation.

In addition to the effect of chemicals on laminin production by differentiated cells, the possibility of chemicals inducing differentiation or inhibiting the action of retinoic acid over longer time scales needs to be examined.

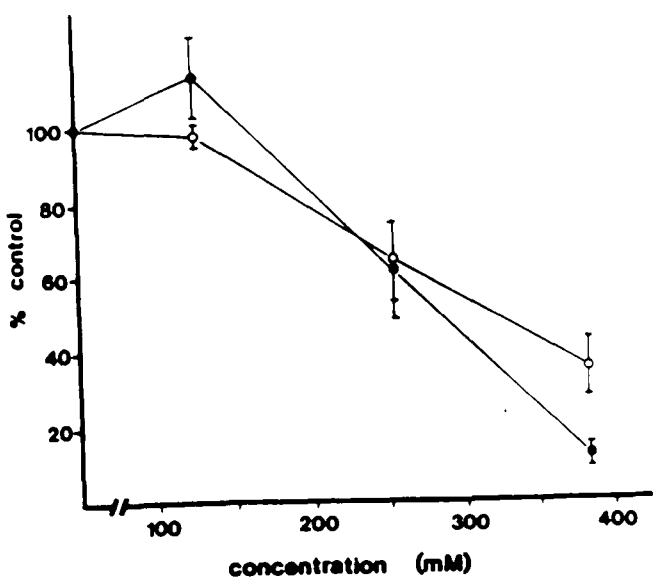

Fig. 3. The effects of dimethylsulphoxide on laminin and protein levels from differentiated F9 cell cultures. (—●—) laminin in culture medium, (—○—) cellular protein.

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