MONOGRAPH No. 12

ALTERNATIVE APPROACHES FOR THE ASSESSMENT OF REPRODUCTIVE TOXICITY

(with emphasis on embryotoxicity/teratogenicity)

FOREWORD

In 1983 the European Chemical Industry and Toxicology Centre (ECETOC) published Monograph No 5 on the Identification and Assessment of the Effects of Chemicals on Reproduction and Development (Reproductive Toxicology). This Monograph recommended that more effort should be devoted to develop and validate alternative tests for predicting toxic effects on reproduction.

The current Monograph assesses the present status of the alternative *in vivo* and *in vitro* tests which were developed for evaluating the effects of chemicals on reproduction in relation to a possible substitution of the classical *in vivo* assays, to their relevance to man and to the more humane use of experimental animals.

It is with pleasure that I present this Monograph to all who are concerned with both human and animal welfare.

R.R. Knowland

Chairman of the
ECETOC Board

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SUMMARY AND CONCLUSIONS

Much progress has been made over the past decades in the development of *in vivo* and *in vitro* techniques for the assessment of the reproductive toxicity of chemicals. This monograph reviews the present position of alternatives to the conventional techniques and assesses their relevance and validity. Most of the new tests detect teratogenic activity rather than other reproductive toxic effects.

At present no alternative test can totally replace the existing reproductive toxicity tests which use live animals. In many instances the alternative methods can be used to screen chemicals for further technical development or for further testing. Additionally they can be used to provide information on the reproductive toxicity of those chemicals which do not warrant full scale testing. Some of the alternative tests provide valuable information on the mechanisms of reproductive toxicity and its relevance to man.

Application of the results from the alternative test approaches to human hazard assessment is also discussed. Without additional knowledge from standard *in vivo* studies, neither alternative *in vivo* nor *in vitro* tests are generally suitable for defining the abnormalities which may occur in human reproduction. Information from which to judge the probability of chemicals effecting human reproduction can come only from conventional experimental reproductive toxicity studies or from observations in man himself.

A. INTRODUCTION

A variety of *in vivo* and *in vitro* techniques have been developed for evaluating the effects of chemicals on reproduction. The alternative tests described by ECETOC (1983) suffered from a number of shortcomings and it was stressed that efforts should be devoted to develop and validate them. This monograph reviews progress.

In vitro assays have generally been developed as screening tests and to provide information on the mechanisms of reproductive toxicity. They have not been developed primarily to replace the present conventional reproductive toxicity tests.

Debate has recently focussed on the ethics of animal experimentation and the possible replacement or reduction in the use of animals. This may be achieved by refining existing *in vivo* techniques or their replacement by new test systems. An examination of such approaches in the evaluation of risk to the reproductive system is of importance to those responsible for the safety of chemicals. A Task Force was set up to examine this area with terms of reference to:

1. review and assess the most relevant alternative test approaches for the prediction of reproductive toxic effects of chemicals and compare the results obtained from such assays with those obtained in *in vivo* assays;

2. assess the reliability of results obtained with these test approaches in predicting hazard to man and whether they are likely to bring about a reduction in animal usage;

3. state, if possible, which are the most promising new assays and what, if any, further development or validation is necessary to improve their usefulness.

The emphasis in this monograph is on the detection of the embryotoxic potential of chemicals since techniques for this are the most fully developed. Definitions of some important terms used are given in Appendix 1.

B. BACKGROUND

Much progress has been made over the last two decades in the development of methods to determine toxicity of chemicals to reproduction.

Test methods using animals have been developed which demonstrate the toxic effect of chemicals on all aspects of the reproductive cycle. Some methods were first adopted by the US-FDA in 1966 and are now required by all developed countries (OECD, 1981, 1983; Baeder et al., 1985; ECETOC, 1985).

In vitro techniques have long been used to study the mechanism of normal and abnormal reproductive phenomena (Ebert and Marois, 1975). Interest in their development for toxicological screening has arisen more recently. Wilson (1978) reviewed the suitability of *in vitro* tests in screening for teratogenic activity; since then a substantial amount of work has been carried out to validate their use (Kimmel et al., 1982).

The value of new tests for reproductive toxicity depends on their intended use and on the aspect of reproduction measured. As most new tests measure a smaller spectrum of reproductive end-points than conventional *in vivo* reproduction tests, several of them would be needed to cover all aspects of reproduction.

In product development where only small quantities of a substance are available it may not be possible to carry out animal studies. In such cases *in vitro* screens can be of value to determine whether the chemical presents a reproductive hazard.

There is also a possibility that screening tests could be used to rank a family of chemicals in order of their reproductive toxic potential. If closely related chemicals act by the same mechanism an *in vitro* screening test need not cover all aspects comprehensively to place them in rank order; the test needs only to be sensitive to the reproductive toxic mechanism they have in common. Thus an *in vitro* screen has been used to classify synthetic retinoids according to their developmental toxicity (Kistler, 1987, Cicurel and Schmid, 1988-a).

This monograph addresses the criteria which determine whether any new test approach is useful for the previously discussed purposes. The following approaches have been considered: new *in vivo* mammalian tests such as the Chernoff-Kavlock test, *in vitro* systems which use established cell lines, primary cultures of embryonic cells, organ cultures, intact rodent embryos and free living embryos of non-mammalian species. The more effective use of animal data from conventional range finding studies and limit tests is also discussed as a possible alternative to full scale conventional evaluation of reproductive toxicity. The manner in which data from these new tests may be interpreted is discussed, particularly in relation to the acceptability of the data in fulfilling the requirements of present regulations. Lastly, the monograph describes how the data from the alternative tests may be applied to human hazard assessment.

C. REVIEW OF AVAILABLE APPROACHES

1. *INTRODUCTION*

One- or two-generation studies are commonly used for evaluating fertility and general reproductive performance of animals exposed to substances. Specific phases of the reproductive cycle such as gametogenesis, fertilisation, blastogenesis and implantation, which are included in these studies, can also be investigated by in vivo fertility studies and the dominant lethal assay. Only a few in vitro techniques are available which permit an investigation of the effects of chemicals on gametogenesis, fertilisation, blastogenesis and implantation. Sperm have been exposed during the capacitation phase and sperm and ova during in vitro fertilisation (Brackett, 1978; Seyler et al., 1984). Pre-implantation embryo culture methods have been used to test for toxic effects induced by chemicals in gametes and expressed during development from the zygote to the blastula (Binkert and Schmid, 1977; Schmid et al., 1983-a).

Effects on embryogenesis and foetogenesis can be usually investigated in embryotoxicity and peri- and postnatal studies; numerous in vivo alternatives are available. Parturition and postnatal development are also covered by peri- and postnatal studies; some new tests are available which give information on these phases of reproduction.

2. *CRITERIA FOR THE SUITABILITY OF ALTERNATIVE TESTS*

To be of any value an alternative test should satisfy several criteria: it should be predictive of toxicity to the human reproductive system; it should be sensitive, specific and quantitative (i.e. the response should increase with increasing dose or concentration of the toxicant). In addition several other factors will increase the general usefulness of these tests, e.g. compatibility with various types of chemicals and inclusion of a metabolic activating capacity.

2.1. Prediction of Human Reproductive Toxicity

There are about twenty confirmed human teratogens whereas several hundred animal teratogens have been identified. Consequently the number of substances available for the validation of conventional *in vivo* tests and any new tests as predictors of reproductive toxicity to man is small and validation can often be carried out only by making comparisons with results from conventional animal studies. In addition, in most cases, the dose levels required to cause reproductive toxic effects in man are not firmly established; thus only qualitative comparisons are possible between effects seen in man and effects occurring in test systems. Quantitative comparisons can in general be made only by comparing results of *in vivo* tests with data generated in controlled animal experiments. The endpoints of the alternative tests should be relevant and it has been shown that those tests which give information on more than one element of reproduction hazard mimic more closely the human situation (e.g. mammalian systems such as mouse limb bud assay for retinoids; the micromass assay for triazoles; whole embryo culture for imidazoles; Chernoff Kavlock test for glycol ethers; limit test for chemicals with low general toxicity).

2.2. Sensitivity and Specificity

For a test to be useful, it must detect reproductive toxicants and distinguish them from harmless substances. The ideal test would generate no false positive or false negative findings. To protect human health it is preferable for screening tests to generate false positive, rather than false negative results; compounds which give positive results will tend to be discarded, whereas negative findings will tend to be checked by standard *in vivo* tests. Nevertheless detection of too many false positives during early screening means that promising compounds could be discarded unnnecessarily. For a fuller discussion of attitudes of researchers to acceptance of false positive or false negative results see Johnson (1986).

2.3. Dose or Concentration - Effect Relationships

It is a basic rule of toxicology that increasing concentrations or dose levels of a material elicit corresponding increases in the severity, incidence and variety of the toxic effects they produce. Another important aim of establishing the dose/concentration - effect relationship is to determine the "no-effect level", i.e. that exposure level at which no morphological, physiological or functional modification at any reproductive stage in a given species is detectable. The no-effect level and the pattern of the dose/concentration-effect relationships are used to extrapolate and predict the likely effects in man at levels to which he is exposed.

2.4. Influence of Physical-Chemical Properties

To be widely applicable a test should give reliable results with substances of varying physical and chemical properties (e.g. gas, liquid or solution, water soluble or insoluble, light sensitive or insensitive).

2.5. Metabolic Activating Capacity

In non-mammalian test systems a metabolic activating capacity should be present which, ideally, produces a spectrum of metabolites similar to that encountered by the human embryo. Some *in vitro* teratogenicity tests incorporate metabolic activating systems of varying complexity. The most common is the post-mitochondrial fraction (S-9) from a male rat liver homogenate, typically with the monooxygenase system induced by a xenobiotic (e.g. Aroclor) (Kitchin et al., 1981; Shepard et al., 1983). Hepatic S-9 from other species has also been added to *in vitro* teratogenicity screening tests e.g. male mouse S-9 to rat and mouse embryo culture (Daston et al., 1987). Intact cells have been used with some success in some systems (Brown and Kram, 1982, Oglesby et al., 1986).

Another possible source of xenobiotic metabolism are the cells, tissues or organs of the *in vitro* system itself; a few have been shown to have

some capacity to metabolise exogenous compounds. (Juchau, 1987; Bechter and Brouillard, 1988).

2.6. Validation

Before new tests are used they should be validated i.e. the results obtained with them should be compared with human evidence or results obtained with experimental animals. Alternative tests for reproductive toxicity may be validated using known mammalian teratogens and substances known to be free from teratogenic potential. The reproductive toxic properties of substances used for validation should be unequivocal and confirmed in two mammalian species when possible. When validation studies are performed care should be taken that the substances with and without reproductive toxicity belong to a range of chemical classes and that those with reproductive toxicity produce different effects on the reproductive process.

3. *ALTERNATIVE TECHNIQUES*

3.1. In vivo Assays

3.1.1. Mammalian Tests. Tests commonly used are described in most guidelines (OECD, 1981-1983; EPA/TSCA, 1982, 1983, 1984; EPA/FIFRA, 1982; EEC, 1983; Japan/MAFF, 1985). The range of alternative techniques available are listed in Appendix 2.

3.1.1.1. Use of range finding studies. The main use for range finding tests is the selection of appropriate dose levels for full scale studies. In general a small number of animals is used (3 - 10 pregnant rats/mice or 2 - 5 pregnant rabbits per dose compared to 20 pregnant rats/mice and 12 pregnant rabbits in a full scale study). The doses cover a wide range in order to determine the dose inducing slight maternal toxicity. Generally 3 - 5 dose groups and a sham treated control are used. Body weight gain, feed and water consumption and clinical signs are recorded in the dams. They are killed, caesarean sectioned and subjected to gross necropsy and the number of live and dead foetuses, conceptuses under resorption, foetal weight and

externally visible abnormalities recorded. In some cases, clinical chemistry, haematology and histological, skeletal or postnatal investigations are carried out and the weights of certain organs determined. Other species may also be tested.

This type of study can be used to screen several test compounds in parallel. If the compounds tested belong to the same chemical class, a reduction in the number of dose groups may be possible and one common control may be sufficient. The findings may indicate a need for further studies, in which case the results obtained from the range finding study will allow a choice of appropriate doses.

The advantages of this study design are: small numbers of animals are used, small amounts of test compound are needed, maternal and foetal metabolising capacities are taken into consideration, embryonic and foetal development are determined and species differences can be examined.

Range finding tests are more time consuming than in vitro studies and weak teratogens may not be detected because of the small group size. Although widely used on a variety of chemicals, only a limited amount of information relevant to its validation has been published (Christian et al., 1987).

3.1.1.2. Limit-Test. Guidelines on toxicological evaluation advise that chemicals of low toxicity need not be investigated at low dose levels if a dose of at least 1,000 mg/kgbw (oral studies) or 5mg/l (inhalation studies) produce no evidence of embryotoxicity. Moreover, "*if a preliminary study at the high dose level with a definite evidence of maternal toxicity shows no adverse effects on the embryo or foetus, studies at other dose levels may not be considered necessary*" (OECD, 1981).

The limit test procedure follows the guidelines with respect to animal and strain, number of animals per group and observation to be made on the dams and foetuses. However, only one treated and one control group of 20 pregnant rats or mice or 12 pregnant rabbits are

needed. This test is recommended for those chemicals for which a low toxicity can be assumed. The advantages and disadvantages of the limit test are similar to those of the range finding study, except that group size is larger, a detailed foetal evaluation is performed and larger amounts of test compound are needed. With positive test results additional studies are necessary.

3.1.1.3. Chernoff-Kavlock-Assay. The test is based on the assumption that most prenatal insults manifest themselves as reduced viability and/or impaired growth of the progeny.

Pregnant mice are dosed daily on the 8th - 12th day of gestation with test substance at a dose level predicted to induce a mild degree of maternal toxicity. Control and test groups consist of 30 - 40 animals. The change in maternal weight during the treatment period is determined. Dams are allowed to give birth and the litters are counted and weighed on postnatal days 1 and 3. Dead pups recovered from the nest are necropsied and abnormalities noted. Dams that do not give birth are killed and their uteri examined for the presence of implantation sites.

Those chemicals that induce perinatal death or produce obvious malformations or a reduced litter size would merit the highest priority for further testing. Those which induce perinatal weight change only would merit a lower priority and those producing no effect the lowest priority for further testing (Chernoff and Kavlock, 1982).

This assay has been simplified and applied to the rat, using at least 10 animals/group, and its evaluation modified (Wickramaratne, 1987). Test animals are exposed on days 7 to 16 of gestation. Maternal observations are limited to body weight measurements on days 1, 7 - 17 and 22 of gestation. Observations in offspring are restricted to the number of dead and the number and weight of live pups on days 1 and 5 after birth. If no effect on litter size, survival, or postnatal weight gain is observed, the chemical is considered to be "negative". If litters contain less than 8 pups at

birth or the postnatal survival is reduced to 80 % or less, the chemical is considered a "potential teratogen". If the postnatal weight gain is reduced by more than 30 % with no reduction in percentage survival the chemical is considered a "potential foetotoxin".

Other protocols for Chernoff - Kavlock preliminary developmental toxicity tests and a weighted scoring system from which priorities for further testing can be determined were described by Hardin et al. (1987).

The advantages of this test are that maternal and foetal toxicity can be investigated, the whole of embryonic and foetal development and part of postnatal development can be assessed. No precise assessment of the teratogenic potential is possible. Other disadvantages are that only rodents are used, the study takes four weeks and it uses a large number of animals.

3.1.2. Non-Mammalian Tests using Free Living Embryos

Whole embryos of non-mammalian species, such as chick and amphibia, have been extensively used in basic research studies. The advantages of these tests are the need for small amounts of test compound and short study duration. All of the tests suffer from some major disadvantages. They use embryos of a lower class as a model of the mammalian embryo and maternal influences are therefore excluded and they produce too many false positive results. The tests considered below are representative of tests of this type.

3.1.2.1. Chick embryotoxicity test. Jelinek (1977) developed the chick embryotoxicity screening test (CHEST), which is subdivided into CHEST I, in which the growth of the caudal trunk of young embryos is monitored over a period of 24 hours and CHEST II, in which morphological defects in more advanced stages of development (days 2, 3 and 4) are monitored in the chick embryo.

Fertilised chick eggs (60 for CHEST I or 120 for CHEST II) are incubated for a period of 40 hours and opened by the "window technique". The diluted test substance (vehicles used are ethanol, dimethylsulfoxide, sunflower oil or aqueous carboxymethylcellulose solution) is injected sub-germinally and after covering the window of the egg-shell with paraffin the egg is incubated for a further 24 hours (CHEST I) or 2 - 4 days (CHEST II). The eggs are then opened and the distance between vitelline arteries and the tip of the tail is measured (CHEST I) or the number and ratio of live/dead embryos, their weight and embryonic morphology are determined (CHEST II).

Both CHEST systems require only small amounts of test substance, the duration of the study is short and there is adequate information on normal chick embryo development. This test system has been used for screening chemicals but while methodological problems have largely been overcome by improved techniques, the problem of too many false positives remains. Virtually all substances tested produce an effect in both tests and results gathered have not been systematically compared with mammalian data (Summerbell and Hornbruch, 1981; Fisher and Schoenwolf, 1983).

A test that uses chick embryos cultured _in vitro_ was proposed by Kucera and Burnand (1987). Chick embryos are dissected out at the gastrula stage and transferred to a silicon chamber where they are treated and continue to develop for 3 additional days. Six chemical agents were tested in this system and the authors claim they could discriminate between teratogenic and non-specific toxic effects. The metabolising capacity, the mode of delivery of non-soluble compounds to the test system as well as the response of the system to additional chemicals have to be investigated to assess the usefulness of this test.

3.1.2.2. _Frog embryo (Xenopus) - Fetax assay_. This assay was originally developed by Dumont et al. (1983) and modified by Courchesne and Bantle (1985). For each substance to be tested two groups of 20 mid to late blastula stage embryos are placed into Petri dishes containing the test substance in aqueous solutions for 96 hours.

After 24, 48, 72 and 96 hours the number of dead embryos and their stage of development are determined. Death at 24 and 48 hours is ascertained by the absence of embryonic skin pigmentation or structural integrity and loss of simple reflexes, while at 72 and 96 hours the lack of heartbeat in the transparent embryo is a sign of death. At 96 hours survivors are rated on their ability to swim and the dorsal pigmentation of each embryo is observed. The head-tail length of surviving embryos is measured and morphological effects are assessed. Concentrations which are lethal, induce structural defects or have no effects are calculated.

A "teratogenic index", defined as LC 50/EC 50 (LC = lethal concentration, EC = effective concentration producing structural defect), is proposed by the authors as an estimate of a "developmental hazard". With 34 known teratogens and 6 non-teratogens a false negative rate less than 9 % was found; the false positive rate was higher (Dumont et al., 1983).

3.1.2.3. Fish embryo. Embryos of a number of freshwater fish species including fathead minnows, trout, largemouth bass and channel catfish have been used to test chemicals for their ability to cause reproductive toxic effects (Birge et al., 1983). The early life stage (ELS) tests focus on effects on hatchability, survival, embryonic development and growth as indices of reproductive toxicity (ASTM, 1986). Most efforts have been applied to toxicological screening of chemically uncharacterised industrial effluents.

The Japanese medaka, a small oviparous fish which is easily cultivated and bred, appears to be a good candidate for reproductive toxicity screening. Its developmental biology has been well studied, presumably because medaka embryos are remarkably transparent, making repeated non-invasive observation possible. The medaka has been used as a teratogen screen by Cameron et al. (1985) but these authors have only reported on the effects of lead on medaka embryos. The zebra fish *Brachydanio rerio* embryo has also been used as a model system in developmental biology (Eisen et al., 1986) for similar reasons.

Although embryos of all of these species exhibit classic reproductive toxic responses, no rigorous validation programme has been performed to determine whether they can predict human reproductive toxic effects.

3.1.2.4. Drosophila embryo. The fruitfly Drosophila melanogaster is an easily obtainable and breedable animal whose genetics are probably the best characterised of any eukaryotic species. The Drosophila embryo test was first advanced as a developmental toxicant screen by Schuler et al. (1982). Test agents are mixed with the semi-solid paste which serves as the diet for Drosophila larvae and adults and as the substrate for egg laying by adult females. The technique involves placing five adult male and five adult female flies into a vial containing test substance; breeding proceeds for the next six days. The parents are then removed and their offspring examined for viability and indications of abnormal development as they reached adulthood (3-4 days later).

Schuler et al. (1982, 1985) and Ranganathan et al. (1987) tested 25 substances using this system. Initial results were promising and further validation of this test is being conducted by the National Toxicology Programme (NTP-US).

3.1.2.5. Hydra. Hydra attenuata is a freshwater coelenterate which has been promoted as an alternative for investigating developmental toxicity. Johnson and Gabel (1983) described a system in which an artificial "embryo" is created by dissociating adult Hydra into single cells, then centrifuging them into a solid pellet. The ability of this solid mass of randomly aggregated cells to form an adult Hydra in the presence of test compounds is assessed.

Johnson and Gabel (1983) tested 24 chemicals using the system and reported the findings as A/D ratios, rather than as absolute concentrations causing developmental toxicity. A/D is the ratio of the minimum toxic concentration to the adult (A) to the minimum toxic concentration affecting development (D). Hydra A/Ds compared favourably to mammalian A/Ds derived from the literature. It should

be noted that the sources of the mammalian A/Ds are not given in the paper and Brown (1986, 1987) has been unable to confirm the values after a comprehensive literature search.

3.1.2.6. Other invertebrates. Theoretically, any species could serve as an alternative for determining developmental toxicity and many invertebrate species have been promoted for this purpose. These include planaria, sea urchins, crickets, brine shrimp, and others. For example Sabourin et al. (1985) tested the ability of decapitated planaria to regenerate a head in the presence of teratogenic agents. They found that regeneration was affected by three of the four developmental toxicants tested.

Hose (1985) described an assay using sea urchin embryos for evaluating cytotoxic, genotoxic and teratogenic effects of environmental chemicals. She reported results with only one compound, benzo (a) pyrene, a potent mutagen but not a strong developmental toxicant. Thus, it is not possible to assess the utility of this assay for developmental toxicity screening.

The cricket has been used as a model to test the teratogenic effects of a series of polycyclic aromatic compounds (Walton, 1981; Walton et al., 1983). Cricket development appears to be sensitive to these compounds. No comparisons were made to mammalian reproductive toxicity.

Development of the embryos of brine shrimp (Artemia nauplii) is sensitive to the action of cadmium, mercury and sodium azide (Sleet and Brendel, 1985). This species is attractive because its embryos can exist for indefinite periods in a state of anhydrobiosis. However, as with other assays described in this section, there have been no further efforts to validate this system as an alternative for examining for reproductive toxicity .

3.2. In vitro Assays

The range of alternative techniques available are listed in Appendix 3.

All tests suffer from some major disadvantages and maternal influences are excluded.

3.2.1. Assays using whole embryo cultures. Cultivation of mammalian animal and human embryos in vitro has been successfully achieved.

3.2.1.1. Preimplantation embryos. Development of embryos from the two-cell stage to the blastocyst stage have been investigated in vitro with mouse embryos (McLaren and Biggers, 1958, Brinster, 1963; Whittingham, 1968; Spielmann and Eibs, 1977). The influence of heavy metals has been examined on 2-cell, 4-cell or inner cell mass development (Wide, 1978; Storeng and Jonson, 1980) and of cyclophosphamide on blastocysts (Spielmann and Eibs, 1977).

It appears that preimplantation embryos are not sensitive to the induction of gross structural abnormalities (Austin, 1973) and are therefore not commonly employed in teratology studies in vitro. Preimplantation embryos can, on the other hand, be used to study the embryolethal action of chemicals, especially those which exert effects on cell proliferation and induce chromosomal aberrations (Giavini et al., 1984).

3.2.1.2. Postimplantation embryo culture. Rodent embryos can be maintained in culture for limited time periods during the early stages of organ formation. The available techniques make it possible to support embryonic development from a few hours to four days (New et al., 1976). Rat serum successfully supports embryonic development, as does cow (Neubert et al., 1986), monkey and human serum (Chatot et al., 1980; Klein et al., 1982).

Generally, three to five somite stage embryos are cultivated within the intact visceral yolk sac and amnion in the presence or absence of a test compound at 37°C for 48 hours. Then the diameter of the yolk-sac, its vascularisation, the embryonic heart beat and rotation of the heart to the concave position are normally examined under a dissecting microscope. Crown-rump and head lengths and/or protein contents are taken as indicators of embryonic growth. The degrees

of embryonic differentiation can be evaluated using a scoring system (Brown and Fabro, 1981) and/or by counting somites. Embryonic features, including curvature, heart, fore-, mid- and hindbrain, otic, optic and olfactory systems, branchial bars, mandibular and maxillary processes, fore- and hindlimbs and somite formation are then examined and anomalies noted.

A study of the effect of 39 chemicals (pharmaceuticals, dyes, solvents) has shown a good correlation between in vitro and in vivo findings: all 18 human and/or animal teratogens tested were found positive and of 21 non-teratogens 20 were negative and 1 positive (Cicurel and Schmid, 1988-b). A separate study of 25 compounds (Cicurel and Schmid, 1988-a) has confirmed these results: 16 human and/or known animal teratogens induced specific malformations in embryos grown in culture; none of the 9 non-teratogens provoked specific dysmorphogenic effects in vitro. The activity of 5 structurally related retinoids were also assessed with this test procedure (Cicurel and Schmid, 1988-a). The ranking found was similar to the one obtained in rat studies in vivo. Teratogenic effects can so far only be detected up to day 12 since the embryo cannot be cultivated at later stages.

Liver microsomal activating systems (Kitchin et al., 1981), hepatocytes (Oglesby et al., 1986) and conjugating systems e.g. glutathione (Slott and Hales, 1987) can be incorporated into the in vitro test on whole mammalian embryos after implantation. On the other hand the organism used in the in vitro screen has itself a metabolising capacity; Juchau (1987) and Bechter and Brouillard (1988) showed that monooxygenase enzymes can be induced transplacently in rat embryos which can then be explanted for whole embryo culture. Embryos can also be exposed in vitro to serum taken from animals or humans who have been exposed to the chemicals under investigation, making it possible to include whole-organism metabolism in the test (Chatot et al., 1980; Schmid et al., 1983-b).

3.2.2. Assays using whole organ cultures; Limb Bud Cultures. A wide range of embryonic and foetal organs can be explanted and maintained in culture, for example palatal shelfs (Pratt, 1983), primordia (tooth anlagen) (Thesleff and Pratt, 1980), embryonic lenses (Karkinen - -Jeaskelainen et al., 1975), embryonic kidney (Saxen and Ekblom, 1981), gonads and other reproductive organs (Lasnitzki and Mizuno, 1981) and the thyroid (Shepard, 1974). Such cultures have been used particularly to elucidate development mechanisms in vitro by means of histological and biochemical techniques. Detection of the possible reproductive toxic effects of chemicals has rarely been the prime objective.

Of all the embryonic organs, limb buds have been most investigated using mouse embryos on the 12th day of gestation. The forelimb and hindlimb buds are cut off from the body along a line immediately lateral to the row of somites. All four limbs from each embryo can be used; thus each litter yields an average of about 40 limbs.

The most commonly employed culture medium is BGJ6 supplemented with ascorbic acid and 25% foetal bovine serum. Neubert et al. (1976) and Kwasigroch et al. (1981) have tried to eliminate the need for serum entirely so that the medium could be clearly defined chemically. The limb buds are normally cultivated over a 6-day period, the limb buds withdrawn, immersed in Bouin's fixative and then stained either with alcian blue or with toluidine blue to visualize the cartilage skeleton.

Despite considerable progress in the culture conditions of limb buds, they do not develop at the same rate as in vivo. Nevertheless, differentiation is reproducible in vitro and chemically induced effects can be clearly distinguished. It has further been shown (Manson and Simons, 1979) that microsomes and postmitochondrial liver activating systems are both capable of metabolising cyclophosphamide to alkylating metabolites in a limb bud system.

The teratogenic effects of individual compounds and their analogs (particularly pharmaceuticals) have been studied using this system (Neubert et al., 1977) but further validation is needed if this system is to be used for general testing purposes.

3.2.3. Assays using primary cultures of embryonal cells. These assays use cells derived from embryos which continue to undergo a number of developmental processes while in culture including growth, cell differentiation, appearance of specific gene products, etc. Although these assays are more complex than those using established cell lines, they typically measure a single endpoint of development. An exception is chick embryo neural retina culture for which several endpoints may be evaluated as a means of gaining information about teratogenic mechanisms. These assays appear to be more powerful predictors of developmental toxicity than tests on established cell lines. The disadvantages are that they require cells from living embryos and are more time consuming and labour intensive.

3.2.3.1. Drosophila neuroblast-myoblast culture. The histological differentiation of *Drosophila* embryo neuroblasts and myoblasts in the presence of teratogens has been assessed (Bournias-Vardiabasis and Teplitz, 1982; Bournias-Vardiabasis et al., 1983). Neuroblasts and myoblasts are isolated from embryos and cultured in the presence of the test agent. The ability to affect histological differentiation, outgrowth of neurites from neuroblasts and formation of myotubes from myoblasts are evaluated microscopically. Bournias-Vardiabasis et al. (1983) tested 100 chemicals of different classes and structure using this system and reported that 43 of 47 teratogens were positive and 51 of 53 non-teratogens were negative.

3.2.3.2. Chick embryo neural crest and limb bud cells. The ability of various developing chick embryo tissues to differentiate in culture in the presence of teratogens has been evaluated as a potential *in vitro* screening test. Wilk et al. (1980) determined the sensitivity of chick embryo limb bud and neural crest cells to 16 compounds, 12 known to be teratogenic *in vivo* and four non- teratogenic. They assessed the histological differentiation of neural crest cells into

neurons and the ability of limb bud mesenchyme to express macromolecules associated with cartilage formation (proteoglycans). Ten of 12 teratogens gave positive and all 4 non-teratogens negative results. Greenberg (1982), from the same laboratory using chick embryo neural crest cells confirmed the earlier work of Wilk et al. (1980).

3.2.3.3. Chick embryo neural retina cell culture. This assay was originally developed as a model for studying tissue-specific recognition processes during development (Moscona, 1961), but has been adapted as a teratogen screen (Daston and Yonker, 1987). Neural retinas from chick embryos on day 6 of incubation are dissociated into single cells and placed in a rotating culture system in the presence of the test agent for 24 hours. Under normal conditions the cells form spherical aggregates of a specific diameter during the first hours of culture. The cells within the aggregates form tissue layers which are comparable to those in the intact retina. Over several days in culture, cells continue to grow, divide and differentiate. Several endpoints can be quantitatively assessed, including the number and size of aggregates (a measure of cell-cell recognition and adhesion), protein content at the end of culture (an index of growth), histology of the aggregates at the end of culture (to assess histological organisation and differentiation) and expression of specific proteins (a more quantitative estimate of differentiation). Analysis of individual endpoints may provide mechanistic information which may occasionally be of value in screening. Seventeen substances have been tested in this system, with an accuracy of 94 % when compared to mammalian in vivo data (Daston et al., 1988).

3.2.3.4 Rat and mouse limb bud cells and CNS cells in micromass cultures. Several authors have described in vitro developmental toxicity screening tests on mouse embryo limb bud cells (Hassell and Horigan, 1982; Guntakatta et al., 1984; Kistler, 1987). The cells are obtained from mouse embryo limb buds generally between the tenth and fourteenth day of gestation and grown in culture in the presence of the test agent. These investigators measure the alcian blue

staining of the proteoglycans associated with cartilage matrix. Some investigators also evaluate cell growth, for example Kistler (1987) did this by measuring tritiated thymidine incorporation as an index of DNA synthesis.

Guntakatta et al. (1984) reported the results of testing 22 known teratogens and 5 known non-teratogens in this system; 19 of 22 teratogens gave positive and all 5 non-teratogens negative results.

Kistler (1987) used this assay to screen a series of 25 retinoids for teratogenic activity. The author was able to rank the teratogenic potency of these retinoids except those requiring metabolism to the biologically active form (e.g. etretinate).

Flint and Orton (1984) described an _in vitro_ teratogen screening test with micromass cultures of rat embryo midbrain and limb buds. These structures were dissected from rat embryos on day 12 of gestation and grown in micromass culture, a procedure which concentrates cells into a small area. The test agent is added after two hours of culture; differentiation is assessed after 5 days by staining the limb bud cultures with alcian blue and the midbrain cultures with hematoxylin and eosin. Alcian blue stains the cartilage matrix whilst hematoxylin and eosin distinguishes between differentiated and undifferentiated areas of the midbrain cultures. The authors tested 46 chemicals in this system; 25 of 27 teratogens were found positive and 17 of 19 non-teratogens were negative.

The _in vitro_ system may in itself be a source of xenobiotic metabolism. Brown et al. (1986) reported that monooxygenase inhibitors can alter the response of cells derived from rat embryo limb bud and midbrain cells to phenytoin, and monooxygenase inducers can stimulate limb bud cells to activate cyclophosphamide, providing indirect evidence that these cells have some xenobiotic metabolising activity.

3.2.3.5 Aggregating rat brain cell cultures. Relatively simple techniques are now available which make it possible to prepare large quantities of aggregate cultures from foetal rat brain cells and to grow them in a chemically defined medium.

Honegger and Werffeli (1988) have developed a technique in which tissue is taken from the brain of 15 day old rat embryos. This tissue is then mechanically dissociated and maintained in culture for up to 16 days, the cells giving rise to three-dimensional, organotypic structures. These cell cultures were exposed at different developmental stages to eight drugs and their responsiveness determined by measuring a set of biochemical parameters such as total protein, DNA content and specific cell enzyme activities. It was found that each test compound elicited a distinct concentration-dependent response pattern. According to the authors foetal liver cells can be incorporated as metabolic activating agents in the nerve cell aggregate making it possible to metabolise, for example, cyclophosphamide.

3.2.4. Assays using established cell lines. Assays using established cell lines all measure a single endpoint which has some relevance to cellular developmental processes such as growth, cellular adhesive properties, cell-cell communication via gap junctions and gene expression. These assays are easy to perform, require only small amounts of test substance and use no animals as the cell lines are already established. Since these assays are rapid many have been used to screen a large number of chemicals. On the other hand, because they are so simple they are not comprehensive screens for developmental toxicity. All of the assays reviewed in this section have been validated using known human and/or animal teratogens and non-teratogens but there is less evidence for the latter. For the separate tests, many of which have been used to test a large number of chemicals, the accuracy was reported by the authors of the individual papers as a percentage concordance of the results with the teratogenic and non-teratogenic effects predicted by *in vivo* mammalian data. It

should, however, be noted that these comparisons were made without regard to the concentration at which the effect was observed.

3.2.4.1. Mouse ovarian tumour cells (MOT) assay. This assay measures the attachment of ascitic MOT cells to a lectin (concanavalin A) - coated surface. Cellular adhesiveness is a general property of embryonic cells and the test was based on the assumption that inhibition of adhesion in the presence of a test substance is an indicator of a teratogenic potential. The test agent is added to a suspension of MOT cells which have been exposed to radiolabelled thymidine and a plastic sheet coated with concanavalin A is placed in the culture vessel. Over a period of hours normal MOT cells adhere to the lectin-coated surface. Inhibition of adhesion is assessed by counting the radioactivity associated with the plastic sheets (Braun et al., 1982-a). Rodent liver S-9 has successfully been used as a metabolic activating system in the MOT assay.

This assay has been used to screen more than 100 substances (pharmaceuticals, pesticides), including known animal and/or human teratogens and non-teratogens. Overall 79 % of teratogenicity and non-teratogenicity results were as predicted from in vivo results (Braun et al., 1979; Braun et al., 1982-b; Braun and Horowicz, 1983; NTP, 1986; Steele et al., 1987).

3.2.4.2. Human embryonic palatal mesenchyme (HEPM) assay. This assay measures the growth of HEPM cells. As growth and division of cells is one fundamental process in developing tissues, inhibition is assumed to be indicative of developmental toxicity. HEPM cells are plated at a low density in tissue culture dishes. After 24 hours the test agent is added and the cultures are maintained for an additional 72 hours without a media change. At the end of this period the number of cells present is counted using a Coulter counter (Pratt and Willis, 1985).

Approximately 100 chemicals (including pharmaceuticals and the chemicals listed by Schmid et al., 1983) have been tested in this assay with and without rat liver S-9 as a metabolic activating

system (Pratt and Willis, 1985; NTP, 1986; Steele et al., 1987). Pratt and Willis (1985) reported a 64 % agreement with in vivo animal results and suggested that this test could be used as part of a battery with another test which measures a different endpoint (MOT was suggested by the authors). The NTP ran the HEPM/MOT battery and reported a 72% agreement with in vivo results (NTP, 1986; Steele et al., 1987). The NTP concluded that the accuracy of the HEPM test results alone was comparable to that of the battery.

3.2.4.3. Poxvirus proliferation assay. This assay assesses the ability of a cell monolayer to support the growth of poxviruses. Since growth of the virus particles requires expression of the virus genome and morphogenesis of the virion, this endpoint may be relevant for teratogenicity testing. BSC 40 cells infected with vaccinia WR are exposed to known teratogens and non-teratogens and the inhibition of growth of the virus is used as an index of teratogenic potential of the test substance. Growth of the virus is assessed by counting the number of plaques (spaces on the culture plate with no cells) in each culture vessel. Activation systems have not been added to the test. Fifty-one chemicals of different families were screened in this system, and an 86 % agreement with in vivo results was reported (Keller and Smith, 1982). It should be noted that the poxvirus used in the test represents a human health hazard.

3.2.4.4. Metabolic cooperation assay. This assay detects intercellular communication via gap junctions. As with cellular adhesion, this type of cell-cell interaction is undoubtedly important in the development of all tissues and interference with this could ultimately lead to a teratogenic event. In the most commonly applied system two populations of standard V79 cells are co-cultured, one with the ability to metabolise 6-thioguanine into a cytotoxin, the other without that ability. Under control conditions the toxic metabolite is passed between the different cell types through gap junctions and all cells in the monolayer are killed. Under test conditions, the cells are cultured in the presence of thioguanine and the test substance. If the test substance interferes with gap junctions the passage of the toxic metabolite

through them will be inhibited and the metabolically incompetent cells will be spared (Loch-Caruso and Trosko, 1985). No systematic validation of this test as a teratogen screen has been carried out although it has been used as a screen for tumour promoters. Welsch et al. (1987) have been experimenting with a modification of this assay, using mouse limb bud cells as a model for studying the role of intercellular communication in teratogenesis.

3.2.4.5. Neuroblastoma cell differentiation assay. A teratogen screening test has been described which assesses the effects of test chemicals on terminal differentiation of mouse neuroblastoma cells which are derived from malignant tumours arising originally from neural crest. Under certain culture conditions these cells will differentiate into neurons. The ability of chemicals to inhibit or enhance this differentiation is evaluated. Of 42 teratogens tested, 32 were positive, 7 negative and 3 unclassifiable; of 19 non-teratogens, 14 were negative, 4 positive and 1 unclassifiable (Mummery et al., 1984).

4. *APPLICABILITY OF RESULTS FROM ALTERNATIVE TEST APPROACHES FOR EXISTING LEGISLATION*

Existing guidelines for testing chemicals and pesticides (OECD, 1981-1983; EPA/TSCA, 1982, 1983, 1984; EPA/FIFRA, 1982; EEC, 1983; Japan/MAFF, 1985) for reproductive toxicity mention only the classical in vivo tests methods, the Limit Test and the Range Finding Test.

For classification or labelling of chemicals, which is mandatory in some cases, results obtained from alternative test approaches may be of importance. The 6th Amendment of the European Communities' Council Directive 67/548/EEC (EEC, 1979), for example, states that a category 2 teratogen classification can be based on relevant information not resulting from standard animal studies, i.e. that results from other tests can be used for classification of a chemical. In practice, the use of such results will depend on the relevance of the test to man, its predictability and its state of validation.

There is no specific mention of new tests in the U.S. legislation regulating pesticides, industrial chemicals, drugs or food additives. Nevertheless use of these tests is not excluded. For example, the Hazard Communication Regulations of the Occupational Safety and Health Administration (OSHA) (EPA, 1986) states that workers must be notified of health hazards of a substance if a single study, conducted in accordance with good scientific principles, has found a hazardous effect with that chemical. Sufficiently validated alternative test methods may fall in this category.

Alternative developmental toxicity screening tests are specifically mentioned in the latest EPA Guidelines for the Health Assessment of Suspected Developmental Toxicants (Francis and Farland, 1987). The guidelines specifically cite the Chernoff-Kavlock protocol and also mention several in vitro screening tests. They state that none are sufficiently developed to replace existing tests but may be used to establish priorities for future testing. Although the EEC and Japan/MAFF do not mention specifically alternative test approaches, results obtained from alternative test approaches should, in well defined and justified cases, be accepted as supporting evidence by these legislatory bodies.

5. *CONCLUSIONS*

At present none of the alternative in vitro and in vivo approaches as described above (with exception of the limit test) can be used alone for reproductive toxicity testing of chemicals. Nevertheless some of the new test approaches are sufficiently developed and validated to allow their use for the identification of a reproductive hazard of a chemical compound during its development.

Alternative approaches can be used as screening tests at an early stage of development of a chemical to allow the selection of compounds with no reproductive toxic hazards from among structural analogues. This procedure may help to reduce the number of larger scale animal tests needed to select a compound and thus reduce the overall number of animals used during a compound's development, the amount of test compound needed and the work load. These alternative approaches can also be used to

supplement the standard *in vivo* tests, e.g. to elucidate the mode of action where this needs further clarification or to verify embryotoxic potential. This is especially true for *in vitro* mammalian tests (limb bud cells, whole embryo, organ cultures) which measure more specific developmental endpoints. These tests can lead to a better understanding of the mechanisms of reproductive toxic effects and their relevance for man. These new tests may also be used for screening classes of compounds in research and development programmes.

A further use of the alternative tests is the reproductive hazard assessment of chemical mixtures, the components of which have already been tested in the standard *in vivo* reproduction tests, (Bechter and Schoen, 1988). This may allow the detection of synergistic effects.

Most of the *in vitro* techniques are static, closed systems in which both biotransformation and excretion are negligible and where exposure is constant. They may or may not include metabolic activating systems. Information on human blood plasma levels for the compound derived from exposed individuals allows a comparison with *in vitro* concentrations and enhances the value of any conclusions drawn from test results. This information is essential in the case of pharmaceuticals and is also helpful for all other chemicals. In the case of valproic acid and acyclovir, for example, it was shown that low but constant plasma levels were of higher biological relevance than short-lasting high plasma peak levels. Thus *in vitro* techniques could more accurately model the human situation than the classical *in vivo* animal models (Nau, 1987; Stahlmann et al., 1988).

Decisive factors in the use of the alternative approaches to reproductive toxicity testing are the endpoints measured. This has to be taken into consideration in drawing conclusions from results of such tests. As many of the *in vitro* models cover fewer endpoints than the standard *in vivo* test, several of the former might be needed to give the same amount of information as the classical animal test.

D. INTERPRETATION OF RESULTS FROM ALTERNATIVE TESTS

1. *INTRODUCTION*

The final aim of any toxicological investigations is the prediction of the possible hazard when man is exposed to the chemical. Direct observations in man provide the most reliable information for assessing human hazard. This information is not available for new chemicals and may often be difficult to interpret with existing chemicals. For this reason animal tests have to be used to detect any toxic potential. Care must be taken in assessing whether results of animal tests are relevant to man. In many cases prediction of possible effects in man cannot be deduced solely from a consideration of animal data because of the morphological and physiological differences between the animal models and man. In assessing human hazard all tests should be interpreted in the light of the use and exposure conditions of the chemical.

2. *PRESENT PROCEDURES WITH STANDARD IN VIVO TESTS*

When a chemical has shown no reproductive toxic effects in the most appropriate animal models and under conditions relevant to human exposure, it is assumed that the chemical should not pose a hazard to man. As, in general, there is no definitive proof that the animal species used in a test is a good model for man, a decision that the chemical is not reproductive toxic to man should only be taken when negative responses are observed with several species (Baeder et al., 1985).

When reproductive toxic effects are observed in animals exposed to a chemical, the relevance to man should be assessed by taking into consideration the circumstances under which the effects occur, the types and frequency of the anomalies, the dose-effect relationship (including the no-effect level) and the number of species in which the effects are found. Information is also required, where possible, on the pharmacokinetics (e.g. absorption, excretion) and biotransformation (metabolism) of the chemical in the animal model as well as in man.

3. *PROCEDURES WITH ALTERNATIVE IN VIVO APPROACHES*

3.1. Mammalian Tests

By their design all mammalian tests take into account embryo/foeto - maternal interactions as well as kinetics, metabolism and the effects of the placenta. It is emphasised that these tests detect the final effects only and that no information is obtained on the influence of the individual factors. The limit test and the range-finding studies permit a comprehensive evaluation of embryonic and foetal development with respect to general embryotoxicity including teratogenicity. The limit test results should be interpreted as in the case of the standard reproductive toxicity tests.

Positive results in range finding studies indicate that the chemical is a reproductive toxin but the converse does not allow a conclusion that the chemical is not a reproductive toxin. Standard in vivo studies are then needed to assess the hazard to man.

The Chernoff-Kavlock assay provides information about effects on the dam and its progeny during the perinatal period. The intention of this screening test is to draw conclusions from a limited number of parameters (e.g. maternal body weight, number of dead and number and weight of live pups) about the reproductive toxic potential of unknown test compounds and the priority for further testing. When no effects are found in this test a more definitive testing may need to be performed. This procedure is already recognised by the Office of Toxic Substances of the EPA (Francis and Farland, 1987) but when this assay gives an indication of a reproductive effect, further standard in vivo studies should be performed (cf. above).

3.2. Non-mammalian Tests

These tests do not allow an assessment of maternal or placental influences on foetal development. The large phylogenetic difference between these species and man limit their predictiveness. Although these tests consider more endpoints than most of the in vitro

approaches, the results obtained with them must be assessed in the same way as the in vitro test results (cf. below). In mammalian assays, the upper limit dose to the embryos is limited by maternal toxicity while in non-mammalian in vivo and in vitro assays such an upper limit of concentration of test agent is not automatically set. Thus due note must be made of exposure levels when interpretating data from non-mammalian tests.

4. *PROCEDURES WITH IN VITRO TESTS*

Most in vitro tests were originally used to investigate the mechanism of reproductive toxicity observed in animals. However, a comparison of in vitro with in vivo findings has still to be performed. Thus with the present state of knowledge and experience, in vitro tests alone cannot be used directly for a human hazard assessment. In most cases the in vitro results will need to be followed by conventional in vivo assays.

There are some cases where in vitro test results might be used to indicate the human hazard. For example :

i. some compounds, e.g. DNA damaging agents, can be expected to be toxic to the developing organism. For these agents an appropriate in vitro test may be directly used to confirm the high toxic potential of such a chemical to reproduction (e.g. anticancer or immunosuppressant agents (Schmid, 1984));

ii. when all members of a class of compounds are known to be devoid of teratogenic hazard and an appropriate in vitro test performed on a new chemical of that class also provides a negative result, it may be reasonable to assume for that chemical that the probability of it being a human teratogen is small. Similarly a positive finding could indicate the risk from that particular compound is high. Nevertheless, legislatory authorities may still require a further confirmation by in vivo test methods;.

iii. convincing results in one or more in vitro tests conducted on classes

of chemicals for which an extensive in vivo data base exists may allow direct hazard assessment to man. For example micromass culture and the whole embryo culture have been used in the assessment of a class of antifungal agents (Flint and Boyle, 1985, Bechter and Schmid, 1987) and retinoids (Kistler, 1987; Bechter and Hall, 1987; Cicurel and Schmid, 1988-a).

iv. preparations containing components that have already been tested in vivo may be reassessed with in vitro assays (Bechter and Schoen,1988).

Where there is good concordance of in vitro and in vivo findings, and as more validation studies are performed, it may be possible to use tests in a tier testing approach for hazard assessment. In vitro data do allow a ranking of compounds tested according to their reproductive toxic potency but rankings are valid only for groups of closely related compounds (congeneric series) which can be presumed to act through a common mechanism of reproductive toxicity. For example Rawlings et al. (1985) used rodent whole embryo culture to generate a ranking of teratogenic potencies of alkoxy acids (metabolites of the glycol ethers).

Ranking systems are easy to use but their value will be limited since it is not possible to determine exactly the relationship between the effective concentrations in the in vitro test and the expected concentrations in exposed human beings. It may be possible in some cases to solve this problem by comparing in vitro test results on a chemical with those for a positive and/or negative control for which the in vivo reproductive toxicity is known and which have similar chemical properties to the test substance (e.g. retinoids, Kistler, 1987; Cicurel and Schmidt, 1988-a; antimycotic drugs, Bechter and Schmidt, 1987).

Pharmacokinetic information is essential for final human hazard assessment. Most in vitro as well as in vivo tests lack this pharmacokinetic component. A general disadvantage of in vitro systems is the fact that they are usually static closed systems in which exposure is constant and excretion is neglected. More efforts should therefore be undertaken to obtain more information on the exact nature of the exposure of the embryo to a given chemical or its metabolites (Nau, 1987).

5. *CONCLUSIONS*

Hazard assessments from standard in vivo tests are possible as they include a knowledge of the dose which has a maternal toxic effect. In the absence of this knowledge, which is generally the case where in vitro screening tests might be used, it is not generally possible to extrapolate results directly for assessment of reproductive toxic hazard. There are some exceptions to this, such as when in vivo pharmacokinetic data are available for setting biologically relevant concentrations for use in in vitro tests, or when the tests are used to verify a predicted effect based on a prior knowledge of the biological activity of related chemicals.

E. GENERAL CONCLUSIONS

There are a number of important applications for alternative tests in reproductive toxicity testing. The value of any test depends strongly on its appropriate use. There is at present no single test which can be universally applied to identify compounds interfering with the reproductive cycle either in total or in part. There are many instances in which alternative methods can be used. Examples are the selection of materials for further development and selecting among existing chemicals for testing, detecting highly teratogenic materials at an early stage of product development, and providing information on reproductive toxicity of chemicals which may not otherwise be examined.

Some of the *in vivo* alternative tests are already used in place of the standard animal tests. The range finding test is the most routinely used. The limit test is an abbreviated embryotoxicity/teratogenicity protocol and is recommended for all materials whose reproductive and maternal toxic dose exceeds 1 g/kgbw/day. Other *in vivo* assays such as the Chernoff-Kavlock protocol may be used in range finding for embryotoxicity studies (Wickramaratne, 1986) or may be used to supplement non-rodent embryotoxicity studies by providing reproductive toxicity data in an additional test species when this is desirable.

Only a few *in vitro* assays have been adequately validated for a broad spectrum of chemicals but many are sufficiently validated to be used in certain circumstances to select materials for further testing. Procedures using intact embryos (especially of mammalian species) can, with limitations, be expected to be sensitive to embryotoxic, including teratogenic, mechanisms which act on the mammalian embryo *in utero*. Primary cell cultures derived from embryos appear to be sensitive to most teratogenic mechanisms. A number of the above procedures have been extensively tested and shown to predict teratogenic potential.

A drawback to all *in vitro* procedures is that maternal factors, excretion and the specific stages at which reproductive toxins acts are not taken into account. This does not preclude the use of these tests for screening specific

classes of chemicals where the above factors play no part in the biological activity.

Assays using established cell lines are not as successful for screening reproductive toxins since data on their validation suggest that they are not highly predictive. This is not unexpected since each assay evaluates only one aspect of development. Since teratogenesis, for example, has many causes, no single endpoint test can be expected to be predictive. It was assumed that these tests may be more useful when used as part of a series of tests but present investigation do not confirm this (NTP, 1986). The more comprehensive in vitro procedures using primary cell cultures or intact embryos could in the future be more useful for the detection of teratogenicity.

Although most in vitro procedures have been designed to detect teratogenicity rather than other embryotoxic effects, those procedures which use intact embryos (rodents or non-mammalian) also detect lethality and growth retardation. They thus provide the most comprehensive assessment of embryotoxicity and teratogenicity. This does not mean that other in vitro tests will not be predictive of embryotoxic effects other than teratogenicity but tests may need to be modified and further testing of chemicals may be required for their validation.

It would be of value if, whenever possible, alternative tests could be performed in parallel with the classical in vivo tests to permit their further validation.

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APPENDICES

APPENDIX 1

DEFINITIONS

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

Blastocyst - in the case of mammals the early embryo at the stage at which it is implanted into the wall of the uterus.

Embryo - the offspring in the early stages of intrauterine development (in humans until the end of the seventh or eight week of pregnancy). This is the stage of development in which there is rapid cell division and where the body structure and organs begin to be formed.

Embryo-and foetotoxicity - toxic effects on the developing embryo and foetus. This includes teratoganicity.

Foetus - the offspring in the post-embryonic period of intrauterine development (in humans after the seventh or eight week of pregnancy). This is the stage in which tissues and organs continue to develop and grow.

Implantation - fixation of the blastocyst in the uterine wall.

in vitro - in an artificial environment.

in vivo - within a living organism.

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

Reproducibility - the closeness of agreement of results when a test is applied several times under prescribed conditions.

Reproductive toxic hazard - an exposure level at which a chemical causes a reproductive toxic effect or a reproductive effect produced by exposure.

Reproductive toxic potency - relates to amount (dose or concentration) of a chemical required to produce a reproductive toxic effect.

Reproductive toxicity - is the inherent property of a chemical which enables it to produce a reproductive toxic effect.

Reproductive toxic risk - is the probability that a certain population will develop an increased incidence of a reproductive toxic effect, or that an individual will suffer a reproductive toxic risk.

Sensitivity - is the ability of a system to identify successfully a reproductive toxic chemical.

Specificity - is the ability of a system to identify a chemical which has no reproductive toxic activity.

Teratogenicity - interference of exogenous agents with intra-uterine development and growth which results in irreversible structural and functional abnormalities in the foetus.

Toxicity - is the inherent property of a chemical to cause an adverse biological effect.

Zygote - the fertilised ovum.

APPENDIX 2

ALTERNATIVE IN VIVO ASSAYS

TEST NAME	TEST SYSTEM	TEST PROCEDURE	ENDPOINT PARAMETER(S)	METABOLIC CAPABILITY	APPLICABILITY
A. MAMMALIAN TESTS					
Range-finding studies	Pregnant lab. animals	Select appropriate dose levels for full scale studies with a small number of animals	Maternal toxicity embryo-/foetogenesis	Yes	Routinely used for testing chemicals; covers the whole period of intrauterine development
Limit-test	Pregnant lab. animals	Test at a high dose concentration to indicate absence of embryotoxic effects	Maternal toxicity embryo-/foetogenesis	Yes	Routinely used for testing chemicals of low toxicity; covers the whole period of intrauterine development
Chernoff-Kavlock assay	Pregnant lab. animals	Test is based on assumption that pre-natal insults would manifest as reduced viability and/or impaired growth of progeny	Maternal toxicity embryo-/foetogenesis and part of postnatal development	Yes	Suggested for screening; investigates maternal and embryo-/foetotoxicity by a limited number of parameters

APPENDIX 2 (continued)

ALTERNATIVE IN VIVO ASSAYS

TEST NAME	TEST SYSTEM	TEST PROCEDURE	ENDPOINT PARAMETER(S)	METABOLIC CAPABILITY	APPLICABILITY
B. NON-MAMMALIAN TESTS					
Chick embryo-toxicity test (CHEST)	Fertilised hen's egg	Incubation of treated eggs	Morphologic development of early (CHEST I) or more advanced stages (CHEST II) of chick embryos	No	Used for screening
Frog embryo assay (FETAX)	Frog embryos	Frog embryos are assessed for lethality, growth and morphological effects	Morphologic development of mid to late blastula frog embryos	No	Used for screening
Fish embryo test	Fish embryo	Assessment of early life stage of embryos	Hatchability, survival, embryonic development and growth	No	Used for screening
Drosophila embryo	Drosophila embryo	Assessment of development in an intact system	Viability and abnormal development	Yes	Used for screening and mechanistic studies
Hydra	Polyps (adults), dissociated polyps	Assessment of the ability of dissociated adult Hydra cells aggregated into a pellet to form an adult Hydra	Study of regeneration process, Adult toxicity/concentration affecting development (A/D ratio)	Possible	Used for screening

For References, see text

APPENDIX 3

IN VITRO ASSAYS

TEST NAME	TEST SYSTEM	TEST PROCEDURE	ENDPOINT PARAMETER(S)	METABOLIC CAPABILITY	APPLICABILITY
A. WHOLE EMBRYO CULTURES					
Preimplantation embryo culture	Preimplantation mammalian embryos	Development of embryos from two-cell stage to the blastocyst stage	Embryolethality, cell division, hatching of blastocysts, chromosomal abnormalities	Possible	Mechanistic studies and genetic analysis
Postimplantation embryo culture	Postimplantation mammalian embryos	Early somite stage embryo development	Malformation, lethality, growth retardation, differentiation	Yes	Used for screening and for mechanistic studies
B. WHOLE ORGAN CULTURE					
Limb bud cultures	Mouse embryo forelimb and hindlimb buds	Culture of embryonic/ foetal limb buds	Growth retardation, differentiations, malformations (?)	Possible	Used for mechanistic studies
C. PRIMARY CULTURES OF EMBRYONIC CELLS					
Drosophila neuroblast-cell test	Drosophila neuroblast-cells	Cellular differentiation of neuro- and myoblasts from *Drosophila* embryos	Specific cellular toxicity	No	Used for screening

For References - see text

APPENDIX 3 (continued 1)

IN VITRO ASSAYS

TEST NAME	TEST SYSTEM	TEST PROCEDURE	ENDPOINT PARAMETER(S)	METABOLIC CAPABILITY	APPLICABILITY
Chick embryo neural crest and limb bud cells	Chick embryo neural crest and limb bud cells	Histological differentiation of neural crest cells and synthesis of proteoglycans from limb bud cells	Growth retardation, differentiation	Possible	Used for screening of specific chemicals
Chick embryo neural retina cell test	Chick embryo neural retina cells	Formation of aggregates cells of neural retina cells	Growth retardation, differentiation, cell/cell recognition	No	Possibly for screening and for mechanistic studies
Rat and mouse limb bud cells test	Rat and mouse limb bud cells	Bud and midbrain cells micromass culture of embryo limb bud and midbrain cells	Growth retardation, differentiation	Possible	Used for screening
CNS cells in micromass test	CNS cells in micromass	Histological differentiation and DNA synthesis	Growth retardation, differentiation	Possible	Used for screening
Rat brain cell test	Rat brain cells	Agglomeration of foetal midbrain cells	Growth retardation, differentiation, specific nerve cell enzyme activities	No	Used for mechanistic studies

For References - see text

APPENDIX 3 (continued 2)

IN VITRO ASSAYS

TEST NAME	TEST SYSTEM	TEST PROCEDURE	ENDPOINT PARAMETER(S)	METABOLIC CAPABILITY	APPLICABILITY
D.ESTABLISHED CELL LINES					
MOT	Mouse ovarian tumour cells	Measurement of adhesiveness	Adhesiveness, growth retardation	No	Used for screening
HEPM	Human embryonic palatal mesenchyme cells	Growth of HEPM cells	Growth retardation	No	Used for screening
Poxvirus proliferation assay	Poxviruses	Assessment of the ability of a cell monolayer to support growth of poxviruses	Growth retardation	No	Not sufficiently validated for screening or mechanistic studies
Metabolic cooperation assay	V79 and mouse limb bud cells	Assessment of intercellular communication via gap junctions of V79 and mouse limb bud cells	Cell/cell interactions	Possible	Not sufficiently validated for screening or mechanistic studies
Neuroblastoma cell differentiation assay	Mouse neuroblastoma cells	Differentiation of mouse neuroblastoma cells into neurons	Growth retardation, differentiation	No	Used for screening

For References - see text

APPENDIX 4

MEMBERS OF THE TASK FORCE

C. BAEDER (Chairman)	HOECHST D - Frankfurt
G. DASTON	THE PROCTER AND GAMBLE COMPANY USA - Cincinnati
J. HELLWIG	BASF D - Ludwigshafen
B. SCHMID	CIBA GEIGY, Zyma CH - Nyon
H. SCHOEN	SANDOZ CH - Basel
W.J. BONTINCK (Secretary)	ECETOC B - Brussels

APPENDIX 5

MEMBERS OF ECETOC SCIENTIFC COMMITTEE

I.F. PURCHASE (Chairman), Director, Central Toxicology Laboratory	ICI UK - Alderley Park
M. SHARRATT*, (Vice-Chairman), Toxicology Advisor	BP UK - Guildford
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Christine YANNAKAS

From:	Christine YANNAKAS
Sent:	08 June 2005 16:28
To:	P. Calow (6091) (E-mail); W. de Wolf (5677) (E-mail); C. D'Hondt (5614) (E-mail); J. Doe (7919) (E-mail); P. Douben (6821) (E-mail); T. Feijtel (6253) (E-mail); A. Flückiger (5552) (E-mail); T. Hutchinson (5532) (E-mail); G. Randall (5523) (E-mail); B. van Ravenzwaay (5500) (E-mail)
Cc:	Michael GRIBBLE; Theres Ziegler (E-mail); Marina Herbst (E-mail); Judith Burton (E-mail); Nadine Vandenwyngaerd (E-mail); Clare Greenman (E-mail)
Subject:	ECETOC - Eve of SC meeting
Importance:	High

Dear All,

Mike Gribble will be hosting an informal dinner on Monday 13th June for those who are in Brussels before the SC meeting.
Would you please let me know by the end of week whether you can join him at
Restaurant Le Jardin de Catherine
⌐ 7, Place Sainte Catherine
,00 - Brussels.
Time: 7.30 p.m.

Thank you for your quick reply.
Best regards.
Christine

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