To

‘Fruity Newty’
ROLE OF OXIDATIVE METABOLISM

IN THE BIOACTIVATION OF CHEMICAL

TERATOGENS, AN IN VITRO STUDY

WITH RAT EMBRYO CELLS

THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

AT THE UNIVERSITY OF SURREY

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December 1986
ABSTRACT

The potential role of embryonic cells in the IN VITRO bioactivation of teratogens was investigated, with limb bud mesenchyme cells, (LB), and mid brain cells, (CNS), derived from 13 day old rat embryos. The cells were cultured for 5 days and during this period differentiated into foci of chondrocytes (LB) or neurones (CNS). The development of the cells in culture mirrored the IN VIVO development and therefore can be used as a teratogen screen.

The presence of constitutive forms of cytochrome P450 isoenzyme forms b, c and d (Levine et al. 1978 nomenclature) were detected in both cell types after the 5 day culture period by immunocytochemistry. Isoenzyme cytochrome P450 b was found to be non-inducible, whereas isoenzymes c and d were found to be inducible by both transplacental administration of 3-methylcholanthrene (3MC) and β-naphthoflavone (BNF) and by IN VITRO coincubation with 3MC and BNF. There was a difference in the developmental profile of the appearance of isoenzyme forms b and c over the 5 day culture period.

Having established the presence of embryonic cytochrome P450's in the culture system the role of metabolism in the bioactivation of diphenylhydantoin (DPH) was investigated. Five approaches were used:
1. Modulation of cytochrome P450 activity IN VITRO by coincubation with a variety of inhibitors caused an increase in DPH toxicity to the extent of 13-82% in LB and 3-52% in CNS cells.
2. Modulation of cytochrome P450 activity IN VITRO by coincubation with inducers, only LB cells coincubated with 3MC caused a 21% increase in DPH toxicity.
3. Modulation of cytochrome P450 activity IN VITRO by transplacentally administered inducers; only LB cells derived from 3MC or βNF pretreated dams increased DPH toxicity, by 20 and 30% respectively. In addition, only LB cells from βNF pretreated dams had the ability to activate the pro-teratogen cyclophosphamide (CPA), (CPA is only toxic to LB cells in the presence of an external metabolising source).

4. Modulation of the formation of the potentially reactive arene oxide intermediate of DPH showed that although the degree of covalent binding could be modulated this did not correlate with the modulation in toxicity and it was therefore concluded that the arene oxide intermediate did not play an important role in DPH teratogenesis.

5. Identification by HPLC analysis of DPH metabolites formed by the cells IN VITRO. Cells (especially LB) were capable of hydroxylation and hydantoin ring cleavage.
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>DPH</td>
<td>diphenylhydantoin</td>
</tr>
<tr>
<td>p. hydroxy. DPH</td>
<td>p. hydroxy 5,5', diphenylhydantoin</td>
</tr>
<tr>
<td>m. hydroxy. DPH</td>
<td>m. hydroxy 5, 5', diphenylhydantoin</td>
</tr>
<tr>
<td>DPH acid</td>
<td>diphenylhydantoic acid</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclophosphamide</td>
</tr>
<tr>
<td>$^{14}$C - DPH</td>
<td>5-5-[$^{14}$C]-diphenylhydantoin</td>
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ACKNOWLEDGEMENTS

I would like to thank the three wise men, my supervisors Oliver, Terry and G3 for their suggestions, help, advice and patience over 3 years. I would also like to thank Kath for all the helping out and practical advice, Steve for his amazing animal handling, John for his time and persistence with the immunocytochemistry, Janet for her enthusiastic typing, Rossi for being a pain, Paul for always being there to drown my sorrows, Cliff for being a brick, everyone at the Stanley Arms, Roly and Alex for Western blotting and antibody donations, Jackie and Abigail for living with me, David for giving me moral support and putting up with my tempers and moods, my brother and sister for financial support and encouragement but most of all John Harvey-Jones and Co at ICI for letting me have such a useful and productive 3 years.

I would like to thank the Safety of Medicines Department at ICI Pharmaceuticals, Alderley Park, Macclesfield for their logistic and financial support in this Case Award project, in addition to the S.E.R.C.

PERSONNEL ASSOCIATED WITH THIS STUDY

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Teratology. Mrs Angela Sheard and Mr Andrew Russell, Teratology Unit, Safety of Medicines Department, Imperial Chemical Industries PLC, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire.

**Immunocytochemistry.** Antibody donations; Dr G Gordon Gibson, Dr C Roly Wolf, Imperial Cancer Research Fund, Western General Hospital, Edinburgh, Scotland (also for Western blotting technique). Professor Donald Davies and Dr Alan Boobis, Royal Postgraduate Medical School, Ducane Road, Hammersmith, London. Technique: Dr John Foster, Biochemical Toxicology Section, Central Toxicology Laboratory, Imperial Chemical Industries PLC, Alderley Park, Macclesfield, Cheshire.
CHAPTER 1: INTRODUCTION

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1.6 THESIS OBJECTIVE
1.1 INTRODUCTION

Teratology is the study of structural and functional abnormalities which arise during embryonic development. The word is derived from the Greek "teratos" meaning pertaining to monsters, and quite clearly refers to the IN VIVO situation. In this thesis, reference is made to the term "IN VITRO teratogenesis" and this term has no clear morphological significance, but rather is used to identify an IN VITRO end-point with possible IN VIVO correlative significance.

Drugs or chemicals taken during pregnancy, either for therapeutic purposes or by accident, probably play a relatively minor role in the production of congenital malformations in man as compared to genetic or environmental factors (e.g. infection, metabolic imbalances and radiation). It has been estimated that between 4-6% of birth defects in humans are chemically related (Wilson, 1964, 1973; Bowman and Rand, 1980). 65-70% of congenital birth defects is unknown, so a far higher percentage of defects may indeed be drug-related (Table 1.1).

Table 1.1 Cause of Drug-Induced Malformation in Man

<table>
<thead>
<tr>
<th>Known genetic transmission:</th>
<th>Incidence</th>
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<tr>
<td></td>
<td>20%</td>
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<table>
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<th>Chromosomal aberration:</th>
<th>3-5%</th>
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<table>
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<th>Environmental causes:</th>
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<tr>
<td>radiations</td>
<td>1%</td>
</tr>
<tr>
<td>infections</td>
<td>2-3%</td>
</tr>
<tr>
<td>maternal metabolic imbalance</td>
<td>1-2%</td>
</tr>
<tr>
<td>drugs and environmental chemicals</td>
<td>4-6%</td>
</tr>
</tbody>
</table>

Unknown. 65-70%

(adapted from Schardein 1985)
Because 4-6% of birth defects are attributable to chemicals, we do not know whether these drugs would have been considered potentially teratogenic prior to consumption. The overwhelming size of the 'unknown' category in the etiology of birth defects is somewhat less than reassuring and may be a result of chemical teratogenesis by specific agents being so difficult to establish. Information on drug and chemical hazards to the human conceptus is derived mainly from isolated case reports by physicians; only a few are based on epidemiological studies.

A current estimate is that over 2,800 chemicals have been tested for teratogenic potential and about 38% have been proved to be teratogenic in at least two laboratory species (Schardein 1985) (Table 1.2).

<table>
<thead>
<tr>
<th>Tested</th>
<th>Clearly Positive</th>
<th>Probably Positive</th>
<th>Possibly Teratogenic</th>
<th>Not Teratogenic</th>
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<tr>
<td></td>
<td>2,820</td>
<td>180</td>
<td>602</td>
<td>291</td>
</tr>
</tbody>
</table>

a In two or more species.

b Based on limited testing or positive in majority of species response.

c Equivocal or variable reaction, and/or less than obvious response.

† Derived from the data of Frankos, 1985.
TESTING METHODS FOR TERATOGENICITY

Traditionally the embryo was thought to be protected from toxic insult by the placenta. In 1915 the first evidence (Weller, 1915) that lead crosses the placenta was obtained from a group of women suffering from lead-sickness who gave birth to mentally retarded children. Subsequently in 1923, a group of women exposed to arsenic gave birth to smaller, less mentally alert babies (Underhill and Amatruda, 1923). Three further significant events led to the rejection of the concept of placental protection:

(i) The 1940 rubella epidemic in Australia (Gregg, 1941)
(ii) The 'Minamata disease' caused by methyl mercury (Matsumoto et al. 1965)
(iii) The thalidomide tragedy of the early 1960's (Lenz, 1961; Taussig, 1962)

Prior to the thalidomide episode, determination of the reproductive toxicity of pharmaceutical products was generally unregulated with the principal objective of determining drug influences on fertility, both in the male and female. The teratogenic action of thalidomide on the human embryo caused a re-evaluation of the screening of drugs for reproductive toxicity.

IN VIVO

The aim of IN VIVO test procedures is to assess the effects of chemicals on reproduction using common laboratory species as models. The guidelines have a number of common requirements.
(i) Chemical to be tested for effect on general reproductive performance as well as embryo development.

(ii) Preferred species are the rat, mouse and rabbit. Other species may be used when differences in metabolism or toxicokinetics between the preferred species and man are known, or to clarify an ambiguous result.

(iii) Sufficient numbers of animals to permit statistical evaluation.

(iv) The route of administration should parallel the route by which man is to be exposed.

(v) Chemicals administered at several dose levels (usually three).

(vi) The lowest dose should produce no observable effects and the highest dose should be the maximum maternal tolerated dose (MTD).

(vii) Adequate concurrent control groups.

Detailed protocols from various countries and leading regulatory agencies can be found in the following references:- Schardein, 1976; Palmer, 1981; Kelsey, 1982; H.S.C.E. reports, 1982; E.C.M. Guidelines on reproductive toxicity testing, 1981.

Chernoff and Kavlock (1982) have proposed an IN VIVO screen (in the mouse) in which compounds are administered throughout organogenesis at, or near the MTD and the newborn are examined for number and weight both at birth and three days after parturition. The rationale is that prenatal insults will manifest themselves postnatally as reduced viability and/or impaired growth. There was a good correlation with known teratogens (100%, 15 out of 15), but also a high incidence of false positive results with non-teratogens (33%
3 out of 9). A recent report (Khera, 1984) suggests that maternal toxicity itself may result in embryotoxicity, and since the Chernoff and Kavlock screen uses doses at or near the MTD this could account for the high percentage of false positives reported. This latter test is more expensive and time consuming than the IN VITRO screens described below, and is not necessarily more predictive.

1.1.1.2 IN VITRO screens for teratogens

In the quest for new, more pharmacologically active drugs, the need for early hazard assessment in the screening programme is essential. The answer would be an ideal IN VITRO test system that would be simple, rapid, yield a large number of interpretable and reproducible results (giving few 'false negatives' and 'false positives') and retain some relevance to mechanisms of teratogenesis. IN VITRO teratogen tests have problems that are not found with other IN VITRO toxicity tests. Firstly, the toxicological target is the embryo, a mass of rapidly dividing cells, simultaneously differentiating along divergent lines. As far as possible the test should reproduce these aspects of embryogenesis. Secondly, both maternal metabolism of xenobiotics and fetal-placental-maternal interactions are usually not present IN VITRO. Since some teratogens may require bioactivation to the active form, the inclusion of a reliable metabolising system or source of metabolites in the screen would extend the utility of the test procedure. This has been achieved as follows.
(i) Use of serum from whole animals treated with the test compound (Chatot et al. 1980; Klein et al. 1982; Schmid et al. 1982; Clapper et al. 1983).

(ii) IN VIVO pretreatment of the mother with inducers of the drug metabolising enzyme systems. For example, limb bud organ cultures from mice pre-induced IN UTERO with the inducer Aroclor 1254 could bioactivate the proteratogen cyclophosphamide whereas non-treated controls were refractory (Neubert and Bluth, 1981).

(iii) The coincubation with isolated hepatocytes, containing the metabolising enzymes (Manson and Simons, 1979; Muller et al. 1980; Brown and Kram, 1982; Oglesby et al. 1984).

(iv) Coincubation with hepatic microsomes, or Sg liver fraction plus cofactors usually from Aroclor 1254-induced male rodents or rabbits (Greenberg, 1982; Wilk et al., 1980; Schmid et al., 1981; Kitchin et al., 1981a, 1981b). In addition the following methods are proposed that might be experimentally useful but which have not yet been reported in the literature.

(a) IN VITRO co-treatment with inducers given that the embryo cells themselves contain inducible forms of the bioactivation enzymes.

(b) Addition of Sg fractions from pregnant females, both control and preinduced.

(c) Incubation of the test compound with an Sg fraction then addition of the filtrate to the IN VITRO test system.
(d) Addition of purified enzyme components of the drug metabolising system.

The most satisfactory way of taking the fetal-maternal-placental unit into account is to dose the mother with the test compound, prior to removing the embryos for culture (Kochaar, 1975; Beaudoin and Lowell-Fisher, 1981, Flint et al., 1984). This would then constitute a combined IN VIVO/IN VITRO approach.

The different types of IN VITRO teratogenic screens that have been described are summarized in Table 1.3 and the IN VIVO to IN VITRO correlation of tests with reported teratogens and non-teratogens are summarised in Table 1.4. The most effective screens (from Table 3) measure several quantifiable end points and have some degree of complexity i.e. they model IN VIVO mammalian embryogenesis as closely as possible. From Table 1.4, the most effective screens have a high percentage of IN VIVO to IN VITRO correlation (at least 90-95%), exhibit low incidences for both false negatives and false positives and have been tested on sufficient number of teratogens and non-teratogens to be meaningful.

Several reviews of testing exist, covering the advantages, disadvantages and experimental techniques of all the major categories of IN VITRO screens (Clayton, 1980; Shepard et al. 1983; Kimmel et al. 1982; Pratt et al. 1980; Freese, 1982; Neubert, 1982; Flint, 1980; Kochaar, 1980; Barrach and Neubert, 1980). In general it appears that IN VITRO screens lack a reliable drug metabolising/activating system.

It has been suggested (Kimmel et al. 1981) that S9 hepatic fractions from Aroclor 1254 induced male Sprague-Dawley rats should be included
## IN VITRO TESTS FOR TERATOGENS

<table>
<thead>
<tr>
<th>Level of Test</th>
<th>Test Details</th>
<th>End Point</th>
<th>Measured Effects</th>
<th>Duration of test</th>
<th>Major Advantage</th>
<th>Major Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular or Chemical</td>
<td>Blocking of Bennetts mercury orange stains for SH groups in liver slices</td>
<td>Lack of coloured stain</td>
<td>Chemical</td>
<td>1 day</td>
<td>None</td>
<td>Non-specific for teratogens, No embryo component in the system</td>
</tr>
<tr>
<td></td>
<td>(Szydlowska et al. 1980)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibition of tumour cell attachment to lectin-coated surfaces</td>
<td>Prevention of tumour cell</td>
<td>Inhibition of</td>
<td>Approx. 1 hour</td>
<td>Speed and good in vivo correlation to known teratogens</td>
<td>Cells are not derived from the embryo and high % of false positives occur</td>
</tr>
<tr>
<td></td>
<td>(Braun et al. 1979, 1980)</td>
<td>attachment</td>
<td>cell-cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>communications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral</td>
<td>Vaccina infected clonally derived monkey kidney cells (Keller and Smith, 1982)</td>
<td>Plaque formation as a measure of</td>
<td>Inhibition of</td>
<td>2 days</td>
<td>None</td>
<td>Cells are not derived from an embryo source, therefore tend to be non-specific for teratogens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>functional virus number</td>
<td>cell proliferation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level of Test</td>
<td>Test Details</td>
<td>End Point</td>
<td>Measured Effects</td>
<td>Duration of test</td>
<td>Major Advantage</td>
<td>Major Disadvantage</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------------------------------------------------------------</td>
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<td>------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Invertebrates</td>
<td>Artificial hydra embryos and dissociated hydra cells (Johnson et al. 1982)</td>
<td>Developed embryo</td>
<td>Morphology and differentiation</td>
<td>4 days</td>
<td>Inexpensive with a large synchronous population</td>
<td>Uses an invertebrate model. Not a teratogen-specific test.</td>
</tr>
<tr>
<td></td>
<td>Brine shrimp development (Sleet and Brendel, 1982)</td>
<td>Developed embryo</td>
<td>Morphology, embryogenesis, DNA and protein</td>
<td>2 days</td>
<td>Inexpensive with a large synchronous population</td>
<td>Uses an invertebrate model. Not a teratogen-specific test.</td>
</tr>
<tr>
<td></td>
<td>Ability of planarians to regenerate normal heads (Best and Morita, 1982)</td>
<td>Cephalic regeneration</td>
<td>Morphology and behavioural effects</td>
<td>2 days</td>
<td>Allows assessment of behaviour</td>
<td>Uses an invertebrate adult model. Not a teratogen-specific test.</td>
</tr>
<tr>
<td></td>
<td>Disaggregated drosophila embryos (Vardiabasis and Salvater 1984; Vardiabasis and Teplitz, 1982)</td>
<td>Number of myotubules and ganglia in culture</td>
<td>Differentiation and biochemical changes</td>
<td>3 days</td>
<td>Good in vivo correlation with known teratogens</td>
<td>Uses an invertebrate model</td>
</tr>
<tr>
<td>Level of Test</td>
<td>Test Details</td>
<td>End Point</td>
<td>Measured Effects</td>
<td>Duration of test</td>
<td>Major Advantage</td>
<td>Major Disadvantage</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
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<td>---------------------------------------</td>
<td>------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Treat drosophila eggs through to pupa stage with dosed medium (Schuler et al. 1982)</td>
<td>Adult flies score for wing notches and humeral bristle defects</td>
<td>Differentiation, embryogenesis and morphology</td>
<td>3 days</td>
<td>Allows treatment during metamorphosis</td>
<td>Defects are not easily reproducible and uses invertebrate model</td>
</tr>
<tr>
<td>Differentiating cell lines</td>
<td>Differentiating mouse NIE-115 neuroblastoma cells (Mummery et al. 1984)</td>
<td>Ability of viable cells to exclude trypan blue</td>
<td>Differentiation</td>
<td>7 days</td>
<td>Clear cut quantifiable end point</td>
<td>Limited development processes involved in the system</td>
</tr>
<tr>
<td>Primary cultures of differentiating embryo cells.</td>
<td>Chick neural crest (Wilk et al. 1980, Greenberg, 1982), mouse limb (Hassel et al. 1978, Hassel and Horigan, 1982), Rat limb and mid brain cells (Flint et al. 1984, Girling and Flint, 1984, Flint and Orton, 1985) mouse secondary palate (Greene and Pratt 1984) and mouse lung cells (Merker et al. 1981)</td>
<td>Qualitative and quantitative assessment of numbers of differentiating cells</td>
<td>Differentiation, biochemical properties, some degree of morphogenesis</td>
<td>Approx. 5 days</td>
<td>Clear cut quantifiable end points. Possible to expose embryo cells by an IN VIVO route before culture or with IN VITRO metabolism</td>
<td>Differentiation does not lead to organ development as in the whole embryo</td>
</tr>
<tr>
<td>Level of Test</td>
<td>Test Details</td>
<td>End Point</td>
<td>Measured Effects</td>
<td>Duration of test</td>
<td>Major Advantage</td>
<td>Major Disadvantage</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------------------------------------------------------------</td>
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<td>----------------------------------------</td>
</tr>
<tr>
<td>Organ culture</td>
<td>Limb buds of rats or mice (Shepard and Bass 1970, Kochaar 1980, 1982; Barrach and Neubert 1980)</td>
<td>Protein content, hydroxyproline content and general appearance</td>
<td>Biochemical properties and gross morphology (quantitative and qualitative)</td>
<td>3-8 days</td>
<td>Intact organ can be observed during morphogenesis</td>
<td>Not easily quantifiable</td>
</tr>
<tr>
<td>Whole embryo culture</td>
<td>Culture of rodent embryos normally between days 10-11 of gestation (New, 1967; Sadler et al. 1982; Jelinek, 1982; Fantel, 1982)</td>
<td>Developed embryo</td>
<td>Increases in DNA and protein, morphology and organogenesis</td>
<td>1-2 days</td>
<td>Uses whole embryo, organogenesis similar to IN VIVO throughout possible period of culture</td>
<td>Technique is skilled and not easily quantifiable. Only a short (24-48 h) critical period of exposure possible during organogenesis</td>
</tr>
</tbody>
</table>
## TABLE 1.4

**CORRELATION BETWEEN IN VIVO TERATOGENS AND IN VITRO TEST RESULTS**

<table>
<thead>
<tr>
<th>Level of Test</th>
<th>Test</th>
<th>Number of teratogens tested</th>
<th>% False negatives</th>
<th>Number of non-teratogens tested</th>
<th>% False positives</th>
<th>% Overall accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular</td>
<td>Inhibition of histochemical reaction with SH groups (Szdyłowska et al. 1980)</td>
<td>16</td>
<td>31</td>
<td>18</td>
<td>0</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Inhibition of tumour cell attachment to lectin coated surfaces (Braun et al. 1979, 1982)</td>
<td>102</td>
<td>20</td>
<td>28</td>
<td>25</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>6</td>
<td>22</td>
<td>36</td>
<td>82</td>
</tr>
<tr>
<td>Viral</td>
<td>Poxvirus morphogenesis (Keller and Smith, 1982)</td>
<td>42</td>
<td>14</td>
<td>9</td>
<td>11</td>
<td>92</td>
</tr>
<tr>
<td>Invertebrates</td>
<td>Planarian cephalic regeneration (Best and Morita, 1982)</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Drosophila embryo culture (Vardiabasis and Teplitz, 1982, Vardiabasis and Salvater, 1984)</td>
<td>45</td>
<td>4</td>
<td>Not stated</td>
<td>Not stated</td>
<td>90</td>
</tr>
<tr>
<td>Differentiating cell lines</td>
<td>Mouse N1E - 115 neuroblastoma cells (Mummery et al. 1984)</td>
<td>39</td>
<td>13</td>
<td>18</td>
<td>45</td>
<td>78</td>
</tr>
<tr>
<td>Level of Test</td>
<td>Test</td>
<td>Number of teratogens tested</td>
<td>% False negatives</td>
<td>Number of non-teratogens tested</td>
<td>% False positives</td>
<td>% Overall accuracy</td>
</tr>
<tr>
<td>-------------------------------------</td>
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<td>---------------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Differentiating embryonic cells</td>
<td>Chick neural crest (Greenberg, 1980)</td>
<td>11</td>
<td>18</td>
<td>5</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Chick limb bud and neural crest cells (Wilk et al. 1980)</td>
<td>11</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Rat limb bud and midbrain cells (Flint et al. 1984)</td>
<td>18</td>
<td>6</td>
<td>13</td>
<td>8</td>
<td>95</td>
</tr>
</tbody>
</table>

There are no values for whole embryo culture or organ cultures presented because the method is labour-intensive, with usually 3 or less compounds tested per publication.

a The overall accuracy is calculated as %
(unsuccessful predictions)
(total number of teratogens and non-teratogens tested)
in IN VITRO tests to standardise the activation system. Recently, the consensus workshop on IN VITRO teratogenicity testing recommended that test validation would be facilitated by testing specified xenobiotics with defined teratogenicity (Smith et al. 1983) and listed 47 compounds intended to serve as a basis for IN VITRO screens in the hope of identifying systems which will contribute to a more effective test programme.

The micromass cell culture system uses 13 day old rat embryo limb bud (LB) and mid-brain cells (CNS) representative of 2 critical tissues in the developing embryo (Flint et al. 1985; Flint, 1981; Girling and Flint, 1984). This is a well validated screen with 95% predictability with known teratogens yielding few false positives or false negatives (Table 1.4). This system is neither too complex nor too simple to either perform or validate. I have used this culture system to investigate the potential metabolic capabilities of rat embryo cells, and the possibility of modulating the toxicity of diphenylhydantoin (DPH).

1.1.2 DEVELOPMENT IN VIVO VS IN VITRO

A comparison will be made of the differentiation in the micromass cultures with the differentiation in the embryo in vivo.

Limb bud: The development of the limb skeleton starts with the formation of the mesodermal structures (somite, somatopleure). From here cells migrate into the limb bud (migration) and increase in number (proliferation). Cells in the core of the limb mesenchyme condense to form pre-cartilaginous blastemata (day 14/15 gestation in
the rat), and later secrete cartilaginous matrix to form the typical
cartilaginous limb skeleton. Late in fetal life the cartilage
skeleton is replaced with bone. The sensitivity of these different
steps to teratogens varies greatly. Blastema formation and the onset
of cartilage differentiation especially can be disturbed by
teratogenic substances (Merker, 1977). The most appropriate IN VITRO
tests for teratogenic hazard towards the skeleton will thus be those
in which blastema formation and differentiation occur.

Micromass cell culture is a system in which isolated limb bud
cells IN VITRO yield blastema cells, other mesenchymal cells, myoblast
cells and myotubes (Flint, 1981; Merker et al. 1980). Dense packing
in the blastema and the occurrence of numerous gap junctions are a
prerequisite for differentiation. Alterations in the cell density,
membrane or contact behaviour lead to disturbances in skeletogenesis.
Perturbation of these processes which are known to occur in the
IN VITRO micromass culture is considered to be a sensitive indication
of teratogenic hazard.

In conclusion the IN VITRO micromass culture offers a reliable and
accurate reflection of IN VIVO skeletogenesis.

Midbrain/Mesencephalon:– At day 13 of gestation in the rat the
neural tube in the head has closed (both at the anterior and posterior
end), and three brain vesicles have formed: the prosencephalon, the
mesencephalon and the rhombencephalon. The fate of each of these is
as follows: the prosencephalon divides into the telencephalon
(cerebral hemispheres) and the diencephalon (thalami, posterior lobe
of pituatory gland, pineal body and optic lobes), the mesencephalon
midbrain); the rhombencephalon which divides into the mylencephalon (medulla oblongata) and the metencephalon (pons and cerebellum).

At the time of closure the neural tube consists of a single layer of neuroepithelial cells (the germinal cells) which give rise to neuroblasts or more correctly immature neurones. In the micromass cell culture undifferentiated neuroepithelial cells from the mesencephalon are disaggregated and cultured, in small discrete islands. Cells that are destined to differentiate as neurones actually segregate from the other cells in the island and aggregate together in small clumps (Flint, 1983). Other cells flatten and attach to the substrate and resemble typical fibroblasts throughout the culture period. The clumps of preneurone cells spread out forming large irregular foci. Cells in the foci differentiate as a pure population of neurones identifiable by specific incorporation of $^{3}$H-labelled γ-aminobutyric acid incorporation or by labelling with a monoclonal antibody to neuronal GQ-ganglioside. The ganglioside is not found on the cell surface at the start of the culture period but emerges during the 5 days of culture. Similarly the antigen is not present in the embryonic mesencephalon IN VIVO at day 13 post coitum, only emerging later in the differentiated midbrain. There appears to be a DE NOVO synthesis which is paralleled IN VIVO and IN VITRO.

The formation of neurones, binding of neural cell projections (neurites) into nerve-like structures (confirmed by electron microscopy) the synthesis of neural cell antigen and $^{3}$H-γ-aminobutyric acid uptake studies confirm that the micromass cell culture system is an excellent model for IN VIVO neuronal differentiation (Flint, 1983), and that while cells are differentiating they are sensitive to the teratogenic insult.
1.2. **KEY FACTORS INVOLVED IN TERATOGENESIS**

1.2.1 **TIMING OF THE TERATOGENIC INSULT**

An important feature of chemically-induced teratogenesis is the timing of treatment. During the pre-implantation period, the conceptus is generally resistant to the production of congenital malformations, although wastage due to embryolethality may occur. Following implantation and during organogenesis, the embryo is extremely susceptible to the teratogenic effects of chemicals. As each organ system is progressively differentiated (Table 1.5) it becomes less susceptible so that teratogenic insult by the same chemical at different stages of organogenesis can produce entirely different results. In the post-organogenesis fetal stage of development, toxicity can take the form of growth inhibition leading to fetal death or impaired postnatal development. Teratogenesis itself is thus inextricably linked with cell and tissue differentiation. The length of organogenesis relative to the period of gestation varies between species (Figure 1.1). The commonly used laboratory animals (mouse, rat and rabbit) spend 38-48% of their total gestational time in organogenesis, and the human only 13%. However, because of the long gestation time in the human (35 days compared to 8-13 days in the rodent), this species is at risk longer.

1.2.2 **ROLE OF PLACENTAL TRANSFER**

Disposition in the embryo of a number of drugs and chemicals may be almost completely blocked by the placental structures early in gestation (Denker, 1976). It is not completely understood whether the placenta acts as a total barrier at this stage or whether it allows xenobiotic transfer in trace amounts (Denker, 1982). In late
gestation several drugs appear to cross the placenta and accumulate in specific organs of the fetus; examples are diphenylhydantoin in fetal myocardium, liver and kidney (Waddell and Mirkin, 1972) diethylstilbestrol in the reproductive tract (Miller et al. 1982) and diazepam in the liver and small intestine (Igari et al. 1982). The accumulation specificity may be due to (i) the ability of the organ to metabolise the xenobiotic or (ii) the affinity of that tissue/organ for the xenobiotic.

Table 1.5 Comparison of Developmental Events in Man and Experimental Animals*

<table>
<thead>
<tr>
<th>DEVELOPMENTAL EVENT</th>
<th>SPECIES GESTATIONAL AGE (DAYS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAN</td>
</tr>
<tr>
<td>Implantation</td>
<td>6-7</td>
</tr>
<tr>
<td>First somite</td>
<td>18-21</td>
</tr>
<tr>
<td>Right and left heart primordia</td>
<td>21</td>
</tr>
<tr>
<td>Liver diverticulum</td>
<td>26</td>
</tr>
<tr>
<td>Anterior neuropore closed</td>
<td>24-25</td>
</tr>
<tr>
<td>Anterior limb bud</td>
<td>27-28</td>
</tr>
<tr>
<td>Posterior limb bud</td>
<td>29-30</td>
</tr>
<tr>
<td>TOTAL GESTATIONAL TIME</td>
<td>267</td>
</tr>
</tbody>
</table>

* data taken from Schardein, 1985.
The numbers refer to the percentage of the gestation period spent in the sensitive phase of organogenesis.
There appears to be three mechanisms whereby chemicals are transferred across the placenta (Waddell and Marlowe, 1981; Krauer et al. 1980) namely:

(i) Passive transfer e.g. drugs, infectious agents and fatty acids
(ii) Carrier mediated transfer e.g. glucose
(iii) Active transfer e.g. methylmercury and iron

The majority of chemicals seem to be passively transferred across the placenta with lipid soluble molecules crossing much more readily than hydrophilic molecules (Freese, 1982; Waddell and Marlowe, 1981; Krauer et al. 1980; Goodman et al. 1982; Dvorchik, 1982). The extent of passage of hydrophilic molecules is strongly dependent on the size and structure of the molecules as well as on the species and gestational stage (Mihaly and Morgan, 1984).

1.2.3 DIRECT AND INDIRECT ACTION OF TERATOGENS

Very little work has been directed towards determining the actual mechanisms of teratogenesis. However, for the compounds studied to date the biochemical mechanisms of teratogenesis may be divided into two categories (additional information may be found in Juchau, 1981 and Beckerman and Brent, 1984).

(i) Direct Acting: i.e. those compounds (parent compound, reactive intermediate or metabolite), that inhibit synthesis and/or accumulation of essential cellular components or bind directly
to cellular components thereby altering function or structure of embryonic tissue (Table 1.6).

(ii) Indirect Acting: i.e. those compounds whose toxic effect on the maternal/embryo unit as a whole (rather than the embryo alone) lead to teratogenesis (Table 1.7).

1.2.4 FACTORS INFLUENCING THE EXPRESSION OF TERATOGENIC INSULT

The expression of a teratogenic effect is influenced by several factors, including genetic, hormonal (sex) and interspecies differences in metabolic capability.

(i) Genetic: The Ah locus is a cluster of genes thought to be the centre of the genetic regulation of some of the cytochrome P450 mediated mono-oxygenases (Nebert and Jensen, 1979; Denker and Pratt, 1981) one of the enzyme systems responsible for xenobiotic metabolism. Benzo(a)pyrene is metabolised by cytochrome P450 mono-oxygenases under the control of the Ah locus and the degree of responsiveness/susceptibility to both teratogenesis and enzyme induction differs in two mice strains (C57/BL6 = responsive strain, AKR = non responsive strain) (Shum et al. 1979; Legreverand et al. 1984). Responsive mice were found to have 50 times more hepatic cytosolic receptor (a protein controlling induction) than the non-responsive strain, (Galloway, et al 1980). Teratogens that show a correlation between strain sensitivity and increased Ah receptors in mice include 2, 3, 7, 8-tetrachlorodibenzop-
<table>
<thead>
<tr>
<th>Effect</th>
<th>Teratogen</th>
<th>Mechanism of Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme inhibition</td>
<td>Acetazolamide</td>
<td>Carbonic anhydrase</td>
<td>Hirsch et al. 1983</td>
</tr>
<tr>
<td></td>
<td>Cortisol</td>
<td>Lysosomal enzymes</td>
<td>Ads et al. 1983</td>
</tr>
<tr>
<td></td>
<td>Diphenylhydantoin</td>
<td>Ornithine decarboxylase</td>
<td>Parker and Netzloff, 1982</td>
</tr>
<tr>
<td>Disruption of microfilaments</td>
<td>Cytochalasin D</td>
<td>Disrupts microfilament</td>
<td>Fantel et al. 1981</td>
</tr>
<tr>
<td>and microtubules</td>
<td>Colchicine</td>
<td>Binds to tubulin</td>
<td>Yamada and Wessells, 1973</td>
</tr>
<tr>
<td></td>
<td>Vinblastine</td>
<td>Crystallises microtubular protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diazepam</td>
<td>Myosin heavy chain disruption</td>
<td>Bandman et al. 1978; Lee et al. 1984;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Marquez-Orozco et al. 1983</td>
</tr>
<tr>
<td>Altered synthesis</td>
<td>Tunicamycin</td>
<td>Inhibits protein glycosylation</td>
<td>Eto et al. 1981</td>
</tr>
<tr>
<td>of glycosoamino</td>
<td>Diazepam</td>
<td>Synthesis interference</td>
<td>Wee and Zimmerman, 1983; McDevitt et al. 1981;</td>
</tr>
<tr>
<td>glycans and collagen</td>
<td></td>
<td></td>
<td>Barlow et al. 1980</td>
</tr>
<tr>
<td></td>
<td>Diazoxo-norleucine</td>
<td>Binds to enzyme of amine transfer reactions</td>
<td>Eto et al. 1981</td>
</tr>
<tr>
<td></td>
<td>Salicylates</td>
<td>Altered synthesis</td>
<td>Kimmel et al. 1971</td>
</tr>
<tr>
<td></td>
<td>Glucocorticoids</td>
<td>Effect on extracellular-matrix</td>
<td>Pratt, 1983; Kochaar and Larsson, 1977;</td>
</tr>
<tr>
<td></td>
<td>Diphenylhydantoin</td>
<td>Synthesis interference</td>
<td>Jelinek and Peterka, 1983</td>
</tr>
<tr>
<td></td>
<td>Hypervitaminosis A</td>
<td>Enhanced degradation of proteoglycans</td>
<td>Shepard, 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kochaar and Larsson, 1977)</td>
</tr>
<tr>
<td>Uncouplers of oxidative</td>
<td>Aldrin</td>
<td>Uncoupler</td>
<td>Shepard, 1980; Altman and Katz, 1976</td>
</tr>
<tr>
<td>phosphorylation</td>
<td>Diethylstilbestrol</td>
<td>Uncoupler</td>
<td>Shepard, 1980; Altman and Katz, 1976</td>
</tr>
<tr>
<td></td>
<td>Rotenone</td>
<td>Uncoupler</td>
<td>Shepard, 1980; Altman and Katz, 1986</td>
</tr>
<tr>
<td>Effect</td>
<td>Teratogen</td>
<td>Mechanism of Action</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------</td>
<td>----------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>Methotrexate</td>
<td>Folate antagonist</td>
<td>Smith et al. 1983; Shepard, 1980</td>
</tr>
<tr>
<td></td>
<td>Hydroxyurea</td>
<td>Thymidylate synthetase inhibition</td>
<td>Schardein, 1976; Shepard, 1980</td>
</tr>
<tr>
<td></td>
<td>Diphenylhydantoin</td>
<td>Folate antagonist</td>
<td>Hanson, 1975, 1976; Goodman and Gillman, 1975</td>
</tr>
<tr>
<td></td>
<td>5-Flourouracil</td>
<td>Thymidylate synthetase inhibition</td>
<td>Smith et al. 1983; Schardein, 1976; Shepard, 1980</td>
</tr>
<tr>
<td>Altered biosynthesis</td>
<td>Puromycin</td>
<td>Protein synthesis inhibition</td>
<td>Juchau, 1981; Goodman and Gillman, 1975</td>
</tr>
<tr>
<td></td>
<td>Diphenylhydantoin</td>
<td>Increase in fibronectin</td>
<td>Hicks et al. 1983</td>
</tr>
<tr>
<td></td>
<td>Rifampicin</td>
<td>RNA synthesis inhibition</td>
<td>Juchau, 1981; Goodman and Gillman, 1975</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D</td>
<td>RNA synthesis inhibition</td>
<td>Ritter, 1977</td>
</tr>
<tr>
<td></td>
<td>Tetracyclins</td>
<td>Protein synthesis inhibition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>Protein synthesis inhibition</td>
<td></td>
</tr>
<tr>
<td>Alkylating agents</td>
<td>2-acetylaminofluorene</td>
<td>DNA synthesis inhibition</td>
<td>Druckery, 1973; Platzer et al. 1982</td>
</tr>
<tr>
<td></td>
<td>Ethylmethanesulphonate</td>
<td>DNA synthesis inhibition</td>
<td>Druckery, 1973; Platzer et al. 1982</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>DNA synthesis inhibition</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.6 continued

<table>
<thead>
<tr>
<th>Effect</th>
<th>Teratogen</th>
<th>Mechanism of Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor complex</td>
<td>TCDD</td>
<td>Effect on palatal epithelial cells</td>
<td>Pratt, 1983; Nebert and Jensen, 1979; Denker and Pratt, 1981</td>
</tr>
<tr>
<td></td>
<td>Diethylstilbestrol</td>
<td>Binding within fetal reproductive tract</td>
<td>Miller et al, 1982</td>
</tr>
<tr>
<td>Effect</td>
<td>Teratogen</td>
<td>Mechanism of Action</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------</td>
<td>------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Altered placental blood flow</td>
<td>Serotonin</td>
<td>Constriction of umbilical</td>
<td>Shepard, 1980; Goodman et al. 1982; Muther et al. 1977</td>
</tr>
<tr>
<td></td>
<td>Ergotamine</td>
<td>vessels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergotovine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetazolamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Morphine</td>
<td>Constriction of placental</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Codeine</td>
<td>vessels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Meperidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histamine</td>
<td>Constriction of umbilical</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Digoxin</td>
<td>artery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caffeine</td>
<td>Dilation of placental vessels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aminophylline</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sodium Nitrite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impaired placental transport</td>
<td>Trypan blue</td>
<td>Inhibition of pinocytosis</td>
<td>Beck and Lloyd, 1966</td>
</tr>
<tr>
<td></td>
<td>Cadmium</td>
<td>Damage to placenta</td>
<td>Miller, 1983</td>
</tr>
<tr>
<td>Temperature</td>
<td>Hyperthermia</td>
<td>Placental thickening and</td>
<td>Smith et al. 1983; Arora et al. 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td>necrosis antimitotic effect</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(heat shock proteins)</td>
<td></td>
</tr>
<tr>
<td>Maternal toxicity</td>
<td>Any compound that</td>
<td>Impaired nutrition, blood</td>
<td>Khera, 1984</td>
</tr>
<tr>
<td></td>
<td>has a lower</td>
<td>flow or oxygen to the</td>
<td></td>
</tr>
<tr>
<td></td>
<td>maternally toxic</td>
<td>embryo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dose than minimum</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>teratogenic dose</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
dioxin (TCDD) (Denker and Pratt, 1981; Nebert, 1980; Pinsky and Digeorge, 1965; McDevitt et al. 1981) glucocorticoids (Kasanagi, 1983; Jelinek and Peterka, 1983; Wee and Zimmerman, 1983; Kochaar and Laison, 1977) diphenylhydantoin (Sulik et al. 1980; Pratt, 1983; Fazel and Sulik, 1980; Atlas et al. 1980; Hansen and Hodes, 1982, 1983) and acetazolamide (Hirsch et al. 1983; Hackman and Hurley, 1983; Biddle, 1975). The sex of the animal also has an influence on susceptibility to teratogenesis. Methylmercury is an established teratogen in the mouse and decreases hepatic mono-oxygenase activity in the male offspring only (Robbins et al. 1978). In contrast, acetazolamide-induced teratogenicity seems to affect a greater percentage of female mice offspring (Scott, et al. 1972). Similarly the synthetic oestrogen, diethylstilbestrol, therapeutically used between 1950-1970 in order to prevent threatened miscarriage during the first trimester, caused human female off-spring to develop vaginal adenocarcinomas (Herbst et al. 1971) during sexual maturation (14-22 year olds). In addition, male offspring similarly exposed pre-natally, have been reported to have reduced sperm counts and a high incidence of sterility (Gill et al. 1976).

(ii) Hormonal: In general, the actions of a variety of drugs are more pronounced and persist longer in female rats than in males (Kato, 1974; Fueur, 1979, Kato and Kamataki, 1982, Gustafsson et al. 1980). One consequence of this is that toxic effects of drugs may be more pronounced in pregnant females. One of the contributing factors to this phenomenon is thought to be the
activity of microsomal drug metabolising enzymes, controlled by steroid hormones acting directly or indirectly on the hepatic drug metabolising system.

In view of the apparent deficiency of such enzyme systems in the embryo, fetus, neonate and perinate (Juchau, 1981; Kato et al. 1962; Pelkonen, 1980; Neubert et al. 1978; Gillette and Strip, 1975; Waddell and Marlowe, 1976, Pelkonen, 1977; Snell, 1982; Kimmel and Buelke-Sam, 1981; Filler and Lew, 1981; Pacifici et al. 1983) the role of the drug metabolising enzymes in teratogenesis at the early stage of organogenesis is at present unclear.

(iii) Species differences in metabolism: Table 1.8 shows the inter-species variations between the minimum teratogenic doses of some selected compounds. There are clear differences for example, between the mouse and rat. The mouse appears more sensitive to the teratogenic effects of diazepam, 2,5 diaminotoluene, caffeine, diphenylhydantoin and aspirin. In contrast, the rat is more sensitive to the effects of L-dopa. The interspecies variations are probably not due to differences in placentation but may well be due to differences in either xenobiotic biotransformation or pharmacokinetics.
Table 1.8  

Interspecies Differences Between the Lowest Teratogenic Doses

The following compounds have been given for between 3 and 6 days of the most sensitive phase of organogenesis for each species. The minimum teratogenic dose is the lowest maternal dose at which terata are observed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Species</th>
<th>Route of administration</th>
<th>Minimum teratogenic dose (mg/kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>Hamster</td>
<td>Oral</td>
<td>300-400</td>
<td>Juskiewicz, 1980</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>up to 500</td>
<td>Ackermann et al. 1970</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>300</td>
<td>Mulvihill, 1973</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>50-75</td>
<td>Mulvihill, 1973</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Rabbit</td>
<td>Oral</td>
<td>450</td>
<td>McColl et al. 1967</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>10</td>
<td>Mackier et al. 1975</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>10</td>
<td>Sullivan et al. 1977</td>
</tr>
<tr>
<td>Dichlorophenoxyacetic acid</td>
<td>Hamster</td>
<td>Oral</td>
<td>100</td>
<td>Collins and Williams, 1971</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>25, 50-100</td>
<td>Schwetz et al. 1971; Khera and McKinley, 1972; Courtney, 1977</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>177-221</td>
<td></td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>Human</td>
<td>Oral</td>
<td>-</td>
<td>Hansen, 1976; Hansen and Smith, 1975</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>100</td>
<td>Elmazar and Sullivan, 1981</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>45</td>
<td>Miller and Becker, 1975</td>
</tr>
</tbody>
</table>
Table 1.8 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Species</th>
<th>Route of administration</th>
<th>Minimum teratogenic dose (mg/kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>Human</td>
<td>Oral</td>
<td>-</td>
<td>Rosenberg et al. 1983; Saxen and Saxen, 1975; Safra and Oakley, 1975</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>up to 200</td>
<td>Beall, 1972</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>100</td>
<td>Miller and Becker, 1975</td>
</tr>
<tr>
<td></td>
<td>Guinea pig</td>
<td>Oral</td>
<td>280</td>
<td>Gill et al. 1981</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Rat</td>
<td>Oral</td>
<td>400</td>
<td>Goldman and Yakovac, 1963</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>15</td>
<td>Trasler, 1965</td>
</tr>
<tr>
<td></td>
<td>Cat</td>
<td>Oral</td>
<td>25</td>
<td>Khera, 1976</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>Oral</td>
<td>400</td>
<td>Robertson et al. 1979</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Oral</td>
<td>250</td>
<td>Earley and Hyden, 1964</td>
</tr>
<tr>
<td>L-Dopa</td>
<td>Rat</td>
<td>Oral</td>
<td>10</td>
<td>Samojlik et al. 1969</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>500</td>
<td>Staples and Mattis, 1973</td>
</tr>
<tr>
<td>2,5 Diaminotoluene</td>
<td>Rat</td>
<td>Sub-cutaneous</td>
<td>-</td>
<td>Burnett et al. 1976</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Sub-cutaneous</td>
<td>50</td>
<td>Inouye and Murakami, 1971</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>Human</td>
<td>Oral</td>
<td>50-300 mg/day</td>
<td>Lenz, 1962; McBride, 1961</td>
</tr>
<tr>
<td></td>
<td>Rat(^a)</td>
<td>Oral</td>
<td>up to 150</td>
<td>Schumacher et al. 1968a, 1968</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>up to 200</td>
<td>Fratta, et al. 1965</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>Oral</td>
<td>up to 200</td>
<td>Robens, 1970</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Oral</td>
<td>25-200</td>
<td>Shepard, 1980; Schumacher, 1970</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>Oral</td>
<td>10</td>
<td>Delahunst and Lassen, 1964</td>
</tr>
</tbody>
</table>

\(^a\) exact values unknown

Thalidomide may produce a teratogenic effect if given at a high dose by intravenous administration (see References Schumacher et al. 1968b, Parkhie and Webb, 1983)
1.3. KINETIC PHENOMENA IN PREGNANT ANIMALS

1.3.1 ALTERED PHARMACOKINETICS AND DRUG PLASMA PROTEIN BINDING DURING PREGNANCY

Pregnancy increases the clearance of drugs whose excretion is predominantly renal and related to creatinine clearance, but fails to change the clearance of oral drugs that are extensively metabolised by the liver, i.e. have a high hepatic extraction ratio (Dvorchik, 1982). Processes which affect the kinetics of renal clearance (renal plasma flow and glomerular filtration rate) are usually increased during pregnancy (Dean et al. 1980). The apparent volume of distribution is also altered due to increased plasma volume. Drug distribution and elimination is changed by an increase in body fat and a decrease in the concentration of plasma proteins. The net result is a tendency towards prolonged drug half-life (Boobis and Lewis, 1982). Absorption of drugs is also affected. In addition, delayed gastric emptying has been noted during pregnancy (approximately twice as long as compared to non-pregnant women) and reduced gastric motility can lead to prolonged disintegration of some tableted drugs (Krauer et al. 1980).

Most drugs are partially bound to plasma proteins, predominantly albumin. The albumin concentration falls in the first 4 months of pregnancy which reduces the binding capacity of a given volume of plasma, but since the plasma volume increases during pregnancy the total mass of albumin effectively remains constant. For highly bound drugs, the hypoalbuminaemia of pregnancy will produce a lower total plasma concentration, associated with a decrease of the bound drug fraction and corresponding increase of the free drug fraction in the plasma (Stock et al. 1980; Allen and Greenblatt, 1981). In contrast
to albumin, most globulin fractions rise during pregnancy. The total effect of changing patterns of plasma proteins and of physiological competitors for their binding sites is complex and varies from compound to compound (Krauer et al. 1980). Accordingly, altered pharmacokinetics during pregnancy is a well documented area in both humans and laboratory animals (Dean et al. 1980; Ramsay, et al. 1980; Lovecchio et al. 1981; Levy, 1981; Combes et al. 1963; Perucca and Crema, 1982; Cummings, 1983).

In humans there is little evidence of changes in drug metabolite profiles during pregnancy, or liver size/weight changes (Combes et al. 1963). However, the liver of the pregnant rat has been reported to increase by up to 40% of the normal weight and it has been suggested that this increase is an adaptive response to accommodate hydroxylation of the rising tide of sex hormones (Symons et al. 1982). Interestingly, the same group also noted a decrease (53-73%) in cytochrome P450-mediated reactions accompanied by liver weight changes prior to parturition (Symons et al. 1982).

1.3.2 PHYSIOLOGICAL INTERPLAY BETWEEN MOTHER AND FETUS

There appears to be four general pharmacokinetic models which represent the plasma drug concentrations in mother and fetus following a single oral dose to the mother (Waddell and Marlowe, 1981). The first model (Figure 1.2, Type 1) is applied to compounds that freely cross the placenta and distribute rapidly in a single compartment in the fetus. The compound must obey certain criteria in order for the concentration profile in the maternal/placental/fetal unit to follow first order kinetics including:-
FIGURE 1.2. PHYSIOLOGICAL INTERPLAY BETWEEN MOTHER AND FETUS

TYPE 1

LOG DRUG CONCENTRATION

MATERNAL

FETAL

TIME

ELIMINATION

PARENTR DRUG (NON POLAR)

PARENT DRUG (NON POLAR)

FETUS

PARENT DRUG (NON POLAR)

POLAR METABOLITE
(1) No preferential binding to either fetal or maternal plasma proteins
(2) Distribution must be unaffected by pH gradients
(3) Fetal plasma concentration is slightly higher than maternal at equilibrium

A concentration gradient must exist if the only route of elimination is passage back across the placenta for appropriate metabolism and/or excretion. An example of a drug following this pattern is carbamazepine.

The second type of profile (Figure 1.2, Type 2) requires a detectable interval in which fetal/maternal plasma concentration are in equilibrium. After this point the fetal concentration is always higher because transfer of the non-polar metabolites across the placenta back to the mother is low. This type of kinetics is a result of a large fetal compartment which may be a result of either fetal plasma binding or a pH gradient trapping the non-polar entities. Compounds that follow this kinetic profile include local anaesthetics and aspirin (Kimmel and Young, 1980).

In the third type (Figure 1.2, Type 3) fetal metabolism occurs and polar metabolites accumulate on the fetal side, creating a concentration gradient. These may, however, undergo minor elimination into the amniotic fluid and pass back across the placenta by pinocytosis. Compounds of this type include diazepam and oxazepam (Allen and Greenblatt, 1981; Ridd et al. 1983, Bakke and Haram, 1982, Idanpaan-Heikkila et al. 1971).
FIGURE 1.2.  
PHYSIOLOGICAL INTERPLAY BETWEEN MOTHER AND FETUS

TYPE 2

LOG DRUG CONCENTRATION

MATERNAL

FETAL

TIME

PARENT DRUG (NON POLAR)

NON POLAR METABOLITES

PARENT DRUG (NON POLAR)

NON POLAR METABOLITES

MOTHER

PLACENT

FETUS
TYPE 3

FIGURE 1.2.

PHYSIOLOGICAL INTERPLAY BETWEEN MOTHER AND FETUS

ELIMINATION

PARENT DRUG (NON POLAR)

MOTHER

PLACENT

FETUS

POLAR METABOLITE

NON POLAR METABOLITES

PARENT DRUG (NON-POLAR)

POLAR METABOLITE

LOG DRUG CONCENTRATION

TIME

MATERNAL

FETAL
The fourth type of kinetics (Figure 1.2 Type 4) exists when a very polar parent drug is unable to cross the placenta, and is therefore excluded from the embryo, for example quaternary ammonium salts.

It should be noted that these four types of kinetics and drug transfer between the mother and fetus are idealised situations. In reality it is more probable that a hybrid type of pharmacokinetic profile exists and that there is some accumulation and/or metabolism in the fetal compartment of the parent drug and its metabolites.
1.4 ENZYMEOLOGY OF TERATOGEN ACTIVATION

Studies of the interaction of chemicals with biological systems have shown that in many cases, the parent compound is not the chemical species responsible for the observed toxic effects. Rather, the parent compound is biotransformed, i.e., converted to a toxic reactive intermediate or metabolite. These reactions (metabolism or biotransformation) have been studied in maternal hepatic tissue and in tissues of importance to teratogenesis, including the placenta and fetal liver.

1.4.1 CYTOCHROME P450

Enzymes catalyzing oxidation, reduction and hydrolytic reactions of chemicals are those most frequently implicated in the bioactivation of teratogens, and they are collectively referred to as "Phase 1" or "Functionalisation" reactions. The most important of these enzymes appears to be the microsomal mono-oxygenases which have been strongly implicated in bioactivation mechanisms. The haemoprotein cytochrome P450, functions as an oxygenase for both xenobiotics and endogenous substrates (Schenkman and Gibson, 1982). Literally hundreds of drugs and chemicals can bind to (Schenkman et al. 1981) and be metabolised by this enzyme system (Gibson and Skett, 1986) which therefore occupies a central position in pharmacology and toxicology.

Exposure of experimental animals or humans to a wide variety of chemicals can result in increased rates of biotransformation catalysed by microsomal cytochrome P450. Such increases in rates appear in part attributable to enzyme induction (particularly cytochrome P450) as manifested by increased DE NOVO synthesis of enzyme protein. It
appears that there are at least two main categories of inducers (Conney et al. 1960; Gelboin and Blackburn, 1964) and that these induce different groups of cytochrome P450 isozymes. The prototype of one category ('phenobarbitone-type inducers') is phenobarbitone, (Conney, 1967) and 3-methylcholanthrene (3MC) is representative of the second class. Chemicals in the latter group include other polycyclic aromatic hydrocarbons, halogenated dioxins, flavones (such as β-naphthoflavone) and polyhalogenated hydrocarbons. Recently a third category of inducer has been described, the prototype being hypolipidemic agents such as clofibrate (Gibson et al. 1982; Tamburini et al. 1984). Other classes of inducers are being increasingly recognised. It is also important to note that cytochrome P450 induction is generally associated with the synthesis of a particular multiple form(s) (or isoenzyme) of the haemoprotein.

There are a number of inhibitors which can interfere with mono-oxygenase function by a variety of mechanisms (Netter, 1979; Alston, 1981; Halpert and Neal, 1981). For example, metyrapone binds to the haem iron of cytochrome P450, or in its vicinity and so reduces the binding of oxygen (Netter, 1979). Other inhibitors commonly used as tools to investigate cytochrome P450-dependent metabolism include SKF525A (Netter, 1979), ellipticine (Delaforge et al. 1980, Lesca et al. 1980), cimetidine (Bell et al. 1983; Henry et al. 1980) α-naphthoflavone (Wiebel et al. 1971) and piperonyl butoxide (Alston, 1981; Halpert and Neal, 1981).

1.4.2 PHASE II METABOLISM

Detoxification of chemicals containing aromatic rings by oxidation proceeds via a highly reactive nucleophilic, arene oxide
intermediate (Jerina and Daly, 1974) and may result in the formation of phenols. These arene oxides can also be enzymatically hydrated to unreactive dihydrodiols by epoxide hydrolase or conjugated with glutathione, by glutathione-S-transferase, to essentially unreactive species (Oesch, 1982). The presence of these enzymes has also been established in the human fetal liver (Peng et al. 1984; Pacifici et al. 1983). Other 'phase II' enzymes include glucuronyl transferase, sulphotransferase and acyl transferase, however, these are either present at very low levels or not detectable in both human and rodent fetal tissue (Juchau, 1981).

1.4.3 DRUG METABOLISM BY THE PLACENTA

Most of the drug biotransformation reactions present in the liver have also been reported in the placenta (Juchau, 1980). Examples are cytochrome P450 dependent reactions (Pelkonen and Karki, 1975), glucuronidation, sulphation, glutathione conjugation, epoxide hydratase, catechol-0-methylation and monoamine oxidase (Juchau, 1980). Human placental cytochrome P450 concentration is approximately 20% of that found in maternal liver (Connelly and Bridges, 1980; Burke and Orrenius, 1979). The placental mitochondrial cytochrome P450 concentration is several times greater than in the microsomal fraction (Meigs and Ryan, 1968).

There is some evidence for the multiple forms of cytochrome P450 in the placenta (Meigs and Ryan, 1968; Zachariah and Juchau, 1977; Zachariah et al. 1976). Placental cytochrome P450 appears to be inducible only by 3MC-type inducers (Juchau, 1980). The aryl hydrocarbon hydroxylase system (i.e. the polycyclic hydrocarbon
metabolising system) in the placenta appears to have a naturally high basal activity compared to other oxidation reactions (Juchau and Zachariah, 1975) and is highly inducible, especially by benzo(a)pyrene or cigarette smoking (Zachariah and Juchau, 1977; Zachariah et al. 1976; Juchau et al. 1973; Juchau et al. 1980b). However, detoxication of the benzo(a)pyrene metabolites by Phase II glutathione conjugation does not appear to be similarly induced (Manchester and Jacoby, 1982). The main role of placental cytochrome P450 appears to be in cholesterol side chain cleavage and biosynthesis of oestrogens from C19 endogenous steroids (Juchau, 1980; Connelly and Bridges, 1980; Meigs and Ryan, 1968; Zachariah and Juchau, 1977).

1.4.4 METABOLISM BY THE FETUS

Although the fetal liver of primates possesses a well-developed complex of xenobiotic metabolising enzymes (albeit less active than the adult), the fetal livers of many laboratory animals (rat, mouse, guinea pig, rabbit and hamster) are apparently deficient in the microsomal cytochrome P450 system. There is a late development of the smooth endoplasmic reticulum (Remmer and Merker, 1963).

Non-human primates and humans differ from the common laboratory animals with respect to the early appearance of the hepatic smooth endoplasmic reticulum and earlier development of cytochrome P450 isoenzymes profiles (Neubert et al. 1978a; Pelkonen, 1980). Studies on the stumptail monkey (Dvorchik and Hartman, 1982) and marmoset (Neubert et al. 1978b) have shown xenobiotic metabolising capacity at mid-gestation comparable with the human fetal capacity at the same stage of gestation (Dvorchik and Hartman, 1982). Human fetal
microsomes as early as the 13th week of gestation have the capability of metabolising diazepam (Ackermann and Richter, 1977; Nau et al. 1979).

Laboratory animals, with few exceptions, have a low capacity to oxidise drugs and foreign chemicals until after birth, whereupon this capacity increases until adult levels have been achieved. Oxidative enzymes are first detectable in the liver close to birth, and adult levels are attained after a few days (as in the guinea pig) or a few weeks (as in the rat). There also appears to be a sex difference in time of attainment of adult levels in the rat; the female requires 30 days and the male 40 days (Gillette and Strip, 1975).

For a more comprehensive analysis of the above aspect of developmental pharmacology than is possible here, the numerous reviews and articles on the subject should be consulted (Juchau, 1981; Pelkonen, 1980; Kimmel and Buelke-Sam, 1981; Juchau et al. 1980; Jaffe, 1983; Parkhie et al. 1982; Neubert et al. 1978b).

IN VITRO studies with cultured fetal rat heptocytes have shown that their ability to metabolise certain xenobiotics only appeared after day 18 of gestation (Yeoh et al. 1983; Worrell, 1984). Other studies with cultured mouse embryos (during early development) have indicated that the onset of polycyclic aromatic hydrocarbon activation coincides with blastocyst formation (Filler and Lew, 1981) and that by day 12 of gestation, benzo(a)pyrene hydroxylation is easily detectable (Galloway et al. 1980). Following IN VIVO pretreatment of the mothers, or IN VITRO exposure to inducers, induction of the microsomal oxidases of rat fetal hepatocytes can be demonstrated (Worrell, 1984; Cresteil et al. 1984; Mizokami et al. 1982). Mouse
limb bud organ cultures for example can activate the proteratogen cyclophosphamide following 3 days of Aroclor induction IN UTERO (Neubert and Bluth, 1981) whereas control cultures require an activating source.

Mizokami et al. (1982) found that pretreatment of the mother with 3-MC induced a phenobarbitone type of cytochrome P450 in fetal liver and not the cytochrome P448 type. However other workers have reported that exposure of the mother to 3MC, Aroclor and β-naphthoflavone resulted in expression of the cytochrome P448 isoenzyme(s) in rat fetal liver (Sunouchi et al. 1983). Recent work on whole rat embryos in culture (Faustman-Watts et al. 1984) has shown that prior IN UTERO exposure to 3MC permitted bioactivation of 2-acetylaminofluorene but not cyclophosphamide. Phenobarbitone induction had no effect. Studies presented later in the thesis have shown that IN UTERO exposure to β-naphthoflavone and subsequent micromass culture of limb bud cells (Flint et al. 1984; Flint, 1981; Flint and Orton, 1985) resulted in the activation of cyclophosphamide but that 3MC, phenobarbitone and hypolipidemic agents were without effect. As human embryo/fetal tissue mono-oxygenase activity is comparatively higher than that observed in common laboratory species and does not appear to be coupled with a correspondingly high level of the Phase II, detoxication enzyme system (Juchau, 1981) this may be important to chemical teratogenesis and therefore a cause for concern.
1.5 BIOACTIVATION OF TERATOGENS

1.5.1 COMPOUNDS THAT REQUIRE BIOACTIVATION

1.5.1.1 Cyclophosphamide

The bioactivation of cyclophosphamide (CPA) has been most extensively studied. Foley et al. (1961) first demonstrated that CPA required hepatic activation before (growth) inhibition of tumour cell growth could be observed, and it is now widely accepted that CPA-induced teratogenesis involves metabolic activation. The maternal liver is the site of bioactivation (Greenaway et al. 1982; Hales, 1981, 1982) via the cytochrome P450 dependent mono-oxygenases (Manson and Simons, 1979; Kitchin et al. 1981; Fantel et al. 1979; Manson and Smith, 1977; Manson, 1981; Torkelson et al. 1974) (Figure 1.3). This has been further confirmed by IN VIVO and IN VITRO studies with inducers and inhibitors of cytochrome P450-mediated metabolism (Table 1.9).

IN VIVO studies: In the rat, phenobarbitone pretreatment of the pregnant female increased CPA teratogenicity; pretreatment with β-naphthoflavone however, had either no effect or decreased the teratogenicity (Hales, 1981). In the mouse, phenobarbitone again increased teratogenicity and SKF525A decreased the response; in these animals, phenobarbitone decreased and SKF525A increased the fraction of unchanged drug in maternal plasma and the embryo (Gibson and Becker, 1968, 1971) indicating that a CPA metabolite is the ultimate teratogen. In contrast, acute or chronic administration of Aroclor 1254 (a polychlorinated biphenyl mixture) attenuated CPA embryotoxicity and teratogenicity; the kinetics and placental transfer of total radioactivity was unaffected by Aroclor 1254 following 14C-CPA administration (Welsch 1985).
Table 1.9  
Effect of Modulating Metabolic Pathways Involved in Teratogenic Activation

<table>
<thead>
<tr>
<th>Teratogen</th>
<th>Test System</th>
<th>Effect of Modulators on the Teratogenic Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide (CPA)</td>
<td>IN VIVO, mouse</td>
<td>PB increased and SKF525A reduced teratogenicity</td>
<td>Gibson and Becker, 1968, 1971</td>
</tr>
<tr>
<td></td>
<td>IN VIVO, rat</td>
<td>PB caused an increase and βNF had no effect</td>
<td>Hales, 1981a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thiol compounds reduced and thiol-depleting compounds increased the teratogenic effects</td>
<td>Hales, 1981b</td>
</tr>
<tr>
<td></td>
<td>IN VITRO, whole rat embryo culture</td>
<td>PB and Aroclor 1254 induced microsomes enhanced the teratogenic effect. βNF and 3MC induced microsomes had no effect</td>
<td>Juchau et al. 1985; Faustman-Watts et al. 1984</td>
</tr>
<tr>
<td></td>
<td>IN VITRO, mouse limb bud cultures</td>
<td>After IN UTERO pretreatment of the mothers with Aroclor 1254 the limb buds could activate CPA</td>
<td>Neubert and Bluth, 1978</td>
</tr>
<tr>
<td></td>
<td>IN VITRO, rat embryo micromass</td>
<td>βNF IN UTERO exposure permitted limb bud cells to activate CPA. PB and 3MC had no effect.</td>
<td>Brown et al. 1986</td>
</tr>
<tr>
<td>Teratogen</td>
<td>Test System</td>
<td>Effect of Modulators on the Teratogenic Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------</td>
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<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>2-Acetylaminofluorene (AAF)</td>
<td>IN VITRO, whole rat embryos</td>
<td>3MC and Aroclor 1254 induced microsomes were required for the teratogenic effect. IN UTERO exposure to 3MC allowed AAF activation by cultures alone; PB had no effect.</td>
<td>Faustman-Watts et al. 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Juchau et al. 1985.</td>
</tr>
<tr>
<td>Chlorcyclazine</td>
<td>IN VIVO, rat</td>
<td>SKF 525 A reduced the incidence of malformations</td>
<td>Posner et al. 1967.</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>IN VIVO, mouse</td>
<td>PB decreased and SKF 525 A increased the frequency of malformations</td>
<td>Harbison and Becker 1970</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Co-administration with a thiol-depleting agent (diethylmaleate) increased the frequency of malformation</td>
<td>Wells and Harbison 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Co administration with 1,2-epoxy-3,3,3-trichloropropene (an epoxide hydratase inhibitor) increased the frequency of malformations.</td>
<td>Wells and Harbison 1980</td>
</tr>
<tr>
<td>Teratogen</td>
<td>Test System</td>
<td>Effect of Modulators on the Teratogenic Effect</td>
<td>Reference</td>
</tr>
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</tr>
<tr>
<td>Adriamycin</td>
<td>IN VITRO, whole rat embryo</td>
<td>3MC and Aroclor 1254 induced microsomes enhanced the malformations; PB induced or control microsomes had no effect.</td>
<td>Fantel et al. 1975</td>
</tr>
<tr>
<td>Ethylenethiourea</td>
<td>IN VIVO, rat</td>
<td>PB had no effect and SKF 525 A increased frequency of malformations.</td>
<td>Khera, 1984, Khera and Iverson</td>
</tr>
<tr>
<td>Ethanol</td>
<td>IN VIVO, mouse</td>
<td>Co-treatment with an alcohol dehydrogenase inhibitor (4-methylpyrazole) increased the effect.</td>
<td>Samojlik et al. 1969</td>
</tr>
<tr>
<td>Caffeine</td>
<td>IN VIVO, mouse</td>
<td>BNF co-treatment decreased the effect</td>
<td>York et al. 1985</td>
</tr>
</tbody>
</table>

Abbreviations used are:  
- **PB** : Phenobarbitone pretreatment  
- **SKF525 A** : SKF 525 A pretreatment  
- **BNF** : β-naphthoflavone pretreatment  
- **3MC** : 3-methylcholanthrene pretreatment
IN VITRO studies. Incubation of whole rat embryos IN VITRO with CPA had no effect on embryotoxicity or teratogenicity (Fantel et al. 1979; Mirkes, 1985) but a 5 hour exposure in the culture medium with a metabolising system (S9-mix) is sufficient to elicit the teratogenic effect (Mirkes et al. 1983). The embryotoxic and teratogenic effect of CPA was increased in rat embryo cultured with liver S9 fraction from phenobarbitone (Juchau et al. 1985) or Aroclor 1254 pretreated rats (Faustman-Watts et al. 1984). β-naphthoflavone or 3-methylcholanthrene pretreatment had no effect (Juchau et al. 1985; Faustman-Watts et al. 1984). Interestingly, it was not possible to induce malformations in embryos transplacentally pretreated with phenobarbitone (or 3 methylcholanthrene) and then exposed to CPA IN VITRO (Juchau et al. 1985, Brown et al. 1986). However β-naphthoflavone was active (Brown et al. 1986).

The major reactive metabolites of CPA are acrolein and phosphoramide mustard (Figure 1.3). Phosphoramide mustard is a potent alkylating agent (Hales, 1981) and is presumed to be the ultimate teratogen (Mirkes et al. 1981; Nau et al. 1982. IN VIVO, acrolein, has been reported to be embryolethal but not teratogenic (Hales, 1982; Schmid et al. 1981). However, abnormalities are observed in whole rat embryos exposed to acrolein in serum free medium (Mirkes et al. 1984) suggesting that its extensive binding to protein may play an important role in reducing the teratogenic effect IN VIVO. Co-administration of CPA with thiol compounds IN VIVO to rats causes a decrease in teratogenicity whereas co-administration with a thiol depleting agent causes an increase in teratogenic effects (Hales, 1981b). 4-hydroperoxy CPA, which decomposes to phosphoramide mustard and acrolein (Hales 1982; Mirkes et al. 1984) causes dysmorphogenesis
BIOACTIVATION OF CYCLOPHOSPHAMIDE

Cyclophosphamide $\xrightarrow{\text{Liver microsomal oxidation (P-450)}}$ 4-hydroxy cyclophosphamide $\xrightarrow{\text{oxidase}}$ keto cyclophosphamide

4-hydroxy cyclophosphamide $\xrightarrow{\text{aldehyde oxidase}}$ aldo phosphamide $\xrightarrow{\text{aldehyde oxidase}}$ phosphoramid mustard (PAM) + acrolein $\xrightarrow{\text{carboxyphosphamide}}$ nitrogen mustard
in mouse limb-bud cultures (Barrach et al. 1978) similar to that observed with CPA IN VIVO. Thiol agents were without effect in vitro (Brown et al. 1986).

1.5.1.2 Acetylaminofluorene

IN VITRO studies. 2-acetylaminofluorene (2AAF) was initially found to be teratogenic to whole rat embryos IN VITRO only in the presence of a S9 mix from Aroclor or 3-methylcholanthrene treated rats (Faustman-Watts et al. 1983). A metabolite of 2AAF (N-hydroxy AAF), was found to be directly teratogenic to whole embryo culture IN VITRO (Faustman-Watts et al. 1983) giving the prosencephalic defects observed with 2AAF following IN VITRO activation. Rat embryos transplacentally exposed to 3-methylcholanthrene and subsequently treated during culture with 2AAF (in the absence of S9 mix) showed increases in malformation incidence; transplacental exposure to phenobarbitone had no effect (Juchau et al. 1985). Oxidative metabolites of 2AAF were detected during incubation of 2AAF with day 10 rat embryos transplacentally exposed to 3-methylcholanthrene (Juchau et al. 1985).

1.5.1.3 Benzhydrylpiperazine Derivatives

The benzhydrylpiperazine antihistaminic drugs, chlorcyclizine, cyclizine, bucyclizine, meclizine, hydroxyzine and norcyclizine are teratogenic in rats and mice (King, 1963; King et al. 1965, 1966). Cytochrome P450 mediated N-dealkylation of chlorcyclizine and meclizine forms a common metabolite, norchlorcyclizine (Narrod et al. 1965). In the rat, the incidence of cleft palate induced by chlorcyclizine, but not norchlorcyclizine, was reduced by SKF525A administration. This response was associated with an increased fetal
concentration of chlorcycline (6-12 fold) and a decrease in norchlorcyclizine, indicating that norchlorcyclizine is the ultimate teratogen (Posner et al. 1967).

1.5.1.4 Benzo(a)Pyrene

When benzo(a)pyrene is administered in the diet to susceptible strains of mice, the frequency of malformations is greater in those embryos of the Ah^d/Ah^d than the Ah^b/Ah^d genotype (Legreverand et al. 1984, Nebert, 1980). Ah^d and Ah^b refer to the dominant or recessive allele respectively encoding a cytosolic receptor that regulates the expression of cytochrome P448. The greater susceptibility of Ah^d/Ah^d embryos is associated with a higher level of toxic metabolites of benzopyrene (1,6- and 3,6-quinones) suggesting that the parent compound is not the teratogenic species.

1.5.1.5 Rifampin

Rifampin, an antibiotic, causes embryo growth retardation IN VITRO but no increase in neural tube defects. The addition of a liver microsomal preparation and cofactors to rat embryos cultured in the presence of rifampin resulted in increased numbers of neural tube defects which could be related to the abnormalities observed in fetuses exposed IN UTERO (Greenaway and Fantel, 1983). These results indicate that an unidentified metabolite, probably produced by a cytochrome P450-mediated reaction, is the ultimate teratogen.
1.5.1.6 Procarbazine

Procarbazine is not teratogenic to whole cultured rat embryos IN VITRO unless the serum from pretreated animals or an S9 mix is added to the culture medium (Schmid et al. 1983).

1.5.2 COMPOUNDS THAT MAY OR MAY NOT REQUIRE BIOACTIVATION

1.5.2.1 Aldrin

IN VIVO studies: Literature reports indicate that both aldrin and its major metabolite dieldrin are teratogenic but induce different malformations (Joy, 1977; Brandt and Hogman, 1980). Studies on the teratogenic effects of aldrin and its major metabolites at equimolar doses did not elucidate whether or not the parent compound was the more teratogenic entity (Ottolenghi et al. 1973).

IN VITRO studies: Aldrin (Flint et al. 1984) and dieldrin (Flint and Orton, 1985) inhibit differentiation of rat embryo LB and CNS cells in the absence of a metabolising system.

1.5.2.2 Diethylstilbestrol

IN VIVO studies: Diethylstilbestrol (DES) is a transplacental carcinogen and teratogen. The effect of this compound appears to be dependent on the amount of unchanged drug reaching the fetus (Slikker et al. 1983; Miller et al. 1982) although it has been recently reported that a metabolite may be the active species. (Balling et al. 1985; Gottschilich and Metzler, 1984; Maydl and Metzler, 1984).
1.5.2.3 Thalidomide

The main transformation of thalidomide in the body is generally considered to be spontaneous non-enzymic hydrolysis (Schumacher et al. 1968, 1970). However in a variety of experimental animals, the turnover rates in plasma and muscle tissue and are surprisingly constant, whereas the rates in the liver vary considerably.

IN VITRO studies: Differentiation of rat limb bud cells, cultured at low density was inhibited by thalidomide in the absence of a metabolising system (Flint et al. 1985). Metabolism has been shown however to play a role in thalidomide cytotoxicity. Gordon et al. (1981) reported that only microsomes from a thalidomide sensitive species were capable of producing toxic metabolites (arene oxide) in an IN VITRO lymphocyte system. Braun et al. (1979, 1982) measured adhesion of mouse ovarian ascites tumour cells and showed that thalidomide toxicity requires activation by hepatic microsomes, an NADPH-generating system and molecular oxygen, but that this activation was not species specific.

1.5.2.4 Diphenylhydantoin

Diphenylhydantoin (DPH)

Diphenylhydantoin acid

Proposed arene oxide intermediate

Epoxide hydratase

\( R_1 = OH \), \( R_2 = H \)  
\( p \)-hydroxy-DPH

\( R_1 = H \), \( R_2 = OH \)  
\( m \)-hydroxy-DPH

Dihydrodiol

ДРР-3,4 catechol

ДРР-3-O-methyl catechol
The metabolic profile of DPH IN VIVO in the rat is represented in Figure 1.4.

a. Evidence that an arene oxide intermediate of DPH metabolism is the ultimate teratogen.

1. In common with other aromatic hydroxylations it has been proposed that the hydroxylation of DPH to p. hydroxy DPH observed in rat and man, proceeds via an arene oxide intermediate (Wells et al. 1980; Spielberg et al. 1981). IN VITRO studies have shown that an oxidative metabolite of DPH (i.e. NADPH/O2 dependent) irreversibly binds to rat liver microsomes; binding could be increased by co-incubation with trichloropropene oxide, an epoxide hydratase inhibitor, and reduced by glutathione (Martz et al. 1977; Pantarotto et al. 1982; Wells et al. 1980) suggestive of formation of a reactive arene oxide. Similar findings were reported using a human leucocyte system (Spielberg et al. 1981).

2. There is a correlation between the teratogenic effect and amount of covalently bound material in the gestational tissue of the mouse following administration of 14C-DPH. Coadministration of trichloropropene oxide and DPH doubled the incidence of cleft palate and the extent of covalent binding (Martz et al. 1977). This experiment cannot be regarded as conclusive as the dihydriodiol metabolite was detected in maternal fluid samples indicating ineffective inhibition of epoxide hydratase.
b. Evidence that DPH is teratogenic per se.

1. Studies on the teratogenic response induced by the major metabolites at equimolar doses in mouse show that the maximum response of any of the metabolites was only 10% of that caused by DPH itself (Harbison and Becker, 1974). However this evidence is not conclusive as the dihydriodiol metabolite (or the arene oxide intermediate) have not been tested.

2. DPH is directly inhibitory in the micromass cell culture system (Flint and Orton, 1985) and directly teratogenic to whole mouse embryos (Bruckner et al. 1983).

3. DPH toxicity IN VITRO was increased by between 13-80% above control levels by co-incubation with a variety of cytochrome P450 inhibitors (Brown et al. 1986).

4. DPH teratogenicity was increased over 3-fold in A/J mice fed a purified diet compared to standard rodent chow. This difference is associated with an increased concentration of DPH in maternal blood (McClain and Rohrs 1985).

5. SKF 525A pretreatment increased DPH teratogenicity in the mouse. In this experiment, inhibition of DPH metabolism was indicated by a raised concentration of DPH and a lowered concentration of p. hydroxy. DPH in maternal plasma, but parent and metabolite concentrations in (whole) fetuses were however unaffected. In contrast, phenobarbitone treatment decreased DPH teratogenicity and this was associated with lowered concentration of DPH and raised concentration of p. hydroxy. DPH in the fetus (Harbison and Becker, 1970).
Other theories as to the ultimate teratogenic species of DPH include a DPH-protein complex/modified serum protein (Clapper et al. 1985, 1983) or the catechol metabolite (Billings and Fischer, 1985; Billings et al. 1985).

1.5.2.5 Chlorambucil

Chlorambucil is a bifunctional alkylating agent (Murphy, 1959) and is teratogenic when added directly to cultures of whole rat embryos (Mirkes and Greenaway, 1982). The addition of an S9 mix enhances the effect, although cytochrome P450 was shown not to be involved by selective inhibitor studies (Mirkes and Greenaway, 1982). It has been postulated that chlorambucil and/or its phenylacetic acid metabolite are responsible for the teratogenic effects.

1.5.2.6 Adriamycin

Adriamycin is a glycosidic anthracycline antibiotic commonly used in the treatment of a variety of tumours. The compound is teratogenic in the rat (Thompson et al. 1978) but not in the rabbit. Embryotoxicity was observed when cultured rat embryos were directly exposed to adriamycin. The response was enhanced when cultures were incubated with liver S9 fractions prepared from rats pre-treated with 3 methylcholanthrene or Aroclor 1254; the addition of S9 from control or phenobarbitione treated rats had no effect (Fantel et al. 1985). The data suggests adriamycin is metabolically activated, although the failure of carbon monoxide to inhibit the response with an S9 fraction from 3-methylcholanthrene pretreated animals argues against the involvement of a cytochrome P450-dependent oxygenation.
1.5.3 **COMPOUNDS NOT REQUIRING BIOACTIVATION**

Direct acting teratogens include:

1. the metals which form protein complexes that directly interfere with cell proliferation and organogenesis (Mottet, 1981).

2. Androgens, oestrogens and other steroids which bind to cytosolic receptors and are translocated as complexes into the nucleus where the complex binds to chromatin and ultimately disrupts protein biosynthesis (Muther et al. 1982; Pratt, 1983; Kochaar and Larssan, 1977; Jelinek and Peterka, 1983; Nebert and Jensen, 1979; Denker and Pratt, 1981; Juchau and Fantel, 1981).

3. D-oxo-norleucine which is thought to act through a receptor (Pratt, 1983).

4. Vitamin A which is more teratogenic than any of its metabolites (Kochaar, 1976; Newall and Edwards, 1981). When vitamin A was given at both teratogenic and non-teratogenic doses, the amount of retinol-binding protein transferred to mice fetuses was found to be directly proportional to the maternal dose of vitamin A (Kochaar, 1976) strongly suggesting bioactivation is not involved.

5. Ethylenethiourea is teratogenic both orally and subcutaneously in both rats and hamsters (Khera and Iverson, 1981; Khera et al. 1983; Khera, 1984). In both species, metabolic modulation studies showed that phenobarbitone pretreatment IN VIVO had no effect on the teratogenicity whereas SKF525A increased the incidence of the malformations. The increased teratogenicity with SKF525A suggests the parent compound is the ultimate teratogen.
6. Salicylic acid but not its putative metabolites causes dysmorphogenesis in cultured rat embryos (Greenaway et al. 1982, 1984). In the same series of experiments, pre-incubation of salicylic acid with a variety of biotransforming systems failed to generate a more toxic metabolite.

7. Cytochalasin D is directly embryotoxic in the rat embryo culture system but the effect is greatly reduced by incubation with an Aroclor 1254 pretreated rat liver enzyme preparation (Fantel et al. 1981), presumably due to metabolism of the active parent compound.

8. Ethanol but not its principle metabolite, acetaldehyde, causes fetal malformations in an IN VIVO mouse model (Samojlik et al. 1969). Furthermore co-treatment with the alcohol dehydrogenase inhibitor, 4-methylpyrazole, causes an increase in malformations (Samojlik et al. 1969). Malformations in the cultured rat embryo are found on direct exposure to ethanol, confirming that the alcohol is the ultimate teratogen (Brown et al. 1979; Wynter et al. 1984).

9. Acetazolamide is probably a direct acting teratogen as both intra-amniotic or intra-uterine injection of the compound gives rise to the typical pattern of limb abnormalities observed in the mouse following oral administration (Scott, 1970; Storch and Layton, 1973).

10. Caffeine is a direct acting teratogen as co-administration with β-naphthoflavone to cytochrome P450 inducible and non-inducible strains of mice showed that increased drug metabolism was concomitant with a decrease in caffeine teratogenicity (York et al. 1985).
11. Other Compounds: There remain a group of compounds which are teratogenic because of their effects on the mother or the extra-embryonic tissues rather than the embryo itself. One example is mirex which causes a significant decrease in blood flow to the uterus and placenta and this is related to its embryotoxicity (Buelke-Sam et al. 1983). Trypan blue which does not cross into the embryo (Wilson et al. 1959) inhibits visceral yolk sac lysosomal enzymes, effectively starving the embryo of essential nutrients (Beck and Lloyd, 1966). The teratogenic activity of at least 15 compounds can be accounted for by maternal toxicity caused at doses where teratogenicity was observed (Khera, 1984; Khera, 1985; Kavlock et al. 1985). When these compounds (insulin, tolbutamide, 6-aminonicotinamide, morphine sulphate, dinoseb, zinc-1,10-phenanthroline complex, maytansine, vincristine, nickel chloride, indole-3-acetic acid, di-(2-ethylhexyl)-phthalate, phencyclidine, 2-nitro-p-phenylenediamine, ethylene glycol monomethyl ether and N-phthaloyl-L-glutamic acid) were administered at non maternally toxic doses, no teratogenicity was observed.
1.6. **THESIS OBJECTIVE**

A rapid and accurate (> 90% predictability with known teratogens) teratogen screen has been achieved using cultures of differentiating limb bud mesenchyme (LB) and mid-brain cells (CNS) from 13 day post-conception rat embryos (Flint and Orton, 1984; Flint et al. 1985). For this project I have used these cultures to examine the potential metabolic capability of rat embryo cells and the role of metabolism in the IN VITRO teratogenic activity of diphenylhydantoin (DPH), a well documented and well metabolised (IN VIVO) teratogen.

Five approaches were used:

(i) modulation of cytochrome P450 activity IN VITRO by co-incubation with a variety of inhibitors at concentrations that were non-cytotoxic.

(ii) induction of cytochrome P450 activity by prior transplacental exposure.

(iii) modulation of the level of the putative epoxide metabolite.

(iv) immuno-cytochemical staining of the cells with both monoclonal and polyclonal cytochrome P450 antibodies.

(v) identification of the metabolites formed by the cells themselves IN VITRO.

The objective of my thesis is to show that rat embryo cells IN VITRO have a metabolic capability and that either this inherent or drug induced capacity plays an important role in chemical teratogenesis.
CHAPTER 2:

MATERIALS AND METHODS

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2.1 MATERIALS

2.1.1 COMPOUNDS

Test compounds of the highest purity commercially available were used. Diphenylhydantoin, ellipticine, glutathione, saccharin, cimetidine, sodium salicylate, inosine, 3-methylcholanthrene, phenobarbitone and β-naphthoflavone were obtained from Sigma Chemical Company, Poole, Dorset, U.K. Benzimidazole, metyrapone and α-naphthoflavone were obtained from Fluka, Flurochem, Glossop, Derbyshire, U.K. m-hydroxy DPH, p. hydroxy DPH, trichloropropene oxide, 5-p-methyl phenyl-5 phenyl hydantoin, 5-p-hydroxyphenyl-5-p-tolylhydantoin and 5-phenyl-5-hydroxyhydantoin were obtained from Aldrich Chemical Company, Gillingham, Dorset, U.K. N-acetyl-L-cysteine was obtained from B.D.H. Chemicals Ltd., Speke, Liverpool, U.K. Cyclophosphamide was obtained from Koch-Light Laboratories Colnbrook, Bucks, U.K. 5-cyclobutyl, 5-phenylhydantoin was obtained from Lancaster Synthesis Ltd., White Lund, Morecambe, U.K. 5, cyclohexyl, 5-phenylhydantoin, 5-cyclopentyl-5-phenylhydantoin, 5,5-dicyclopiprylhydantoin and 5-cyclopropyl-5 phenyl hydantoin were obtained from I.C.N. Pharmaceuticals Inc Life Sciences Group, Plainview, New York, U.S.A. 5-[ring-2-14C]-phenobarbitone was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. 5,5-[4-14C]-diphenylhydantoin and 3-[6-14C]-methylcholanthrene were obtained from New England Nuclear, Du Pont (UK) Ltd, Southampton, U.K. SKF525A was a gift from Smith Kline and French Labs Ltd, Welwyn Garden City, Herts, U.K. Ranitidine hydrochloride was a gift from the Glaxo Research Group, Ware, Herts, UK. Diphenylhydantoic acid, hydantoin,
5-5 dimethylhydantoin, 5-phenylhydantoin, 5,5. p-dichlorodiphenyl-
hydantoin, 5-p-hydroxyphenylhydantoin, 5-methylhydantoin, 5-methyl-5-
p-chlorophenylhydantoin, 5-amino-5 phenylhydantoin, 5-ethyl-5
phenylhydantoin, 5-phenyl-p-aminohydantoin, 5-phenyl-p-
chlorohydantoin, 5-hydroxy-5-methylhydantoin, 5-p.m-
dichlorophenylhydantoin, 5-o-hydroxyphenylhydantoin, 5-ethanoic acid
hydantoin, 5-5 diphenyl-p-dihydroxyhydantoin and 5 phenyl-5-
hydroxyhydantoin were synthesised at ICI Pharmaceuticals Division.
Culture media and sera were obtained from Gibco Biocult, Paisley,
Scotland.

2.1.2 ANTIBODIES

Pre-immune goat sera and horseradish peroxidase labelled anti-
species IgG antibodies were obtained from Miles Scientific, Slough,
Bucks., England. The cytochrome P450 antibodies were gifts from
Dr G G Gibson, Biochemistry Department, University of Surrey, England,
Dr C R Wolf, Department of Molecular Pharmacology and Drug Metabolism,
I.C.R.F., Western General Hospital, Edinburgh, Scotland and
Professor D Davies and Dr A Boobis, Royal Postgraduate Medical School,
Hammersmith Hospital London/CTL, Macclesfield. The types of
antibodies are described in Table: 2.1.
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<thead>
<tr>
<th>NAME AND TYPE OF ANTIBODY (Ab)</th>
<th>TISSUE SOURCE OF ANTIGEN</th>
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<tr>
<td>Surrey P450 (formalised as Levin form 'b')(^x), polyclonal</td>
<td>Phenobarbitone induced Wistar rat liver</td>
<td>rabbit</td>
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<tr>
<td>Wolf's PB(_3) (formalised as Levin form 'b')(^x), polyclonal</td>
<td>Phenobarbitone induced Sprague-Dawley rat liver</td>
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\(^x\) - Ryan et. al. (1982) and Wolf et. al. (1984)

\(^{xx}\) - Gibson et. al. (1982)
2.2 METHODS

2.2.1 CULTURE TECHNIQUE

Animals
Sufficient virgin Alderley Park albino rats were mated to provide six successful pregnancies per experiment. Conception was determined by the vaginal smear technique. The pregnant animals were maintained until the 13th day after noting a positive vaginal smear. The animals weigh 250-350 g on this day.

Euthanasia
Animals were sacrificed by exposure to CO₂.

Equipment for aseptic removal of the uterus
Sterilised blunt and sharp ended scissors.
Pair of forceps.
4" sterile glass or plastic petri dishes.

Method
The dead animal was immersed in a bath of diluted Savlon* (approximately 10 ml concentrate in 500 ml water) prior to dissection. The animal was laid on a clean surface covered with absorbant paper and an incision made with blunt scissors through the body wall, but not entering the peritoneum. The hole was expanded by retraction. A

* Savlon is a trademark, the property of Imperial Chemical Industries PLC.
Further incision was made in the peritoneum using sharp scissors and forceps. The uterus was then located in the lower abdomen and removed using scissors and forceps with as little adherent fat as possible. It was then transferred rapidly to a dry sterile petri dish.

**Embryo dissection equipment**

Laminar flow cabinet.

Zoom stereo dissecting microscope with transmitted and incident illumination.

1 pair of M19 Pascheff Wolff micro-scissors. (Microdissection equipment: Moria-Dugast, Paris).

1 M17 drilled spatula.

2 pairs of sharpened No 5 Watchmakers forceps.

4" glass or petri dishes.

**Solutions**

Earl's Balanced Salt Solution (Eagles modification - EBBS).

50% V/V horse serum and EBSS (HS-EBSS).

All solutions were sterile and used within twelve months or otherwise discarded.
All procedures were undertaken in a laminar flow cabinet, the surfaces of which were sterilised by wiping down with tissues soaked in 70% alcohol. Uteri were washed through three changes of EBSS in petri dishes. The following procedures took place using the dissecting microscope at x6 magnification. Each conceptus consisting of the embryo plus extra-embryonic membrane was removed to EBSS by tearing a small hole in the wall of the uterus with Watchmakers forceps. After all the conceptuses were removed from a uterus, the remaining tissue was discarded. Conceptuses pooled from all six animals were transferred using the drilled spatula to fresh HS/EBSS in a petri dish. HS/EBSS was warmed in a water bath, to approximately 37°C. Extra-embryonic membranes were removed with watchmakers forceps and discarded. The embryos were collected to one side of the petri dish. Approximately 10 - 12 embryos are found per uterus. At this stage of
development, embryos have 34 - 36 somites. Small embryos having less than 34 somites and large embryos having more than 36 somites were rejected. The mesencephalon and fore-limb bud were removed from the acceptable embryos using microdissection scissors and watchmakers forceps. See Figure 2.1.

Processing of tissues prior to culture of disaggregated cells

equipment

Sterile glass Pasteur pipettes.
Sterile glass pipettes with 0.7 mm tip diameter, produced by drawing conventional pasteur pipettes in a bunsen flame.
Test tube rack.
Sterile capped plastic test tubes, conical bottom.
Sterile universal containers.
Sterile disposable 10 ml syringes with removable plungers.
Sterile stainless steel Swinney filter holders (Millipore) with 10 um mesh nylon filter (Simon) in place.
Modified Fuchs-Rosenthal Haemocytometer.
Benchtop centrifuge (capable of 1000 rpm).

Solutions
Calcium and magnesium-free Earl's Balanced Salt Solution (CMF-Gibco). 1% solution of trypsin (Difco, 1:250) prepared by stirring for 30 - 60 minutes in CMF and then filtering through a millex GS 0.2 um pore filter. Hams F12 culture medium with supplements of 10% fetal calf serum, L-glutamine (584.6 mg/l), penicillin (106 IU per litre)
and streptomycin (100 mg/l). All solutions were used within twelve months.

Method
Tissues were transferred by Pasteur pipette to separately labelled test tubes. The HS/EBSS was aspirated off with a Pasteur pipette and tissues washed three times with CMF. About 15 ml of CMF was kept in a sterile universal container for this purpose. The tubes were incubated for 20 minutes at 37°C. Trypsin solution was followed by a brief wash in CMF which was immediately replaced by 1.3 ml of the complete Ham's medium (CM). Tissue fragments were then disaggregated into their component cells by repeated aspirations through the 0.7 mm internal tip diameter Pasteur pipette (15 - 20 times). Care was taken not to make a froth. The resulting cell suspension was then poured into a 10 ml disposable syringe with a Swinney filter holder attached. Replacing the plunger forces the suspension though the 10 um mesh nylon filter and into a fresh test tube. A suspension of single cells was thus obtained. 1ml of this suspension was transferred to a fresh test tube. The small amount of remaining suspension was diluted 1:9 with CM and counted with the Fuchs-Rosenthal Haemocytometer.

The estimated cell concentration was used to calculate the amount of medium required to produce in the case of midbrain, $5 \times 10^6$ cells per ml, and in the case of limb, $2 \times 10^7$ cells per ml. If these final concentrations can only be acheived in volumes of less than 1 ml culture medium, the 1 ml of suspended cells was then centrifuged at 1000 rpm for five minutes, the supernatant removed and the cells re-
suspended in the calculated volume of fresh medium. Otherwise, excess fresh medium was added to the original 1 ml to give the appropriate final cell concentration.

Culture preparation equipment
Eppendorf "Multipette" 4780.
Eppendorf "Combitips" (sterilised) (500 ul size).
Falcon Primaria (3801) sterile plastic petri dishes (35 mm diameter).

Method
The cell suspension was drawn up into a 500 ul "Combitip" fitted to the "Multipette" and delivered in 10 ul aliquots to 35 mm sterile plastic disposable petri dishes.

Cells were allowed to settle for two hours at 37°C, forming circular cell islands (6-8 mm) the culture dishes filled to a final volume of 2 ml with CM and other supplements described below. Five (10 ul) aliquots of midbrain and at least three (10 ul) aliquots of limb cell suspension were delivered to each dish. (See Figure 2.1.)

Sufficient dishes were prepared for assessment of a concentration response curve for the tested compound, (usually 7 dishes for each cell type).

One midbrain and one limb bud dish receive vehicle control and each concentration of the compound in 2 ml culture medium. The medium was not changed for the following five days of culture. Culture conditions were 37°C, 5% CO2, 95% air and 100% relative humidity.
Formulation of stock solutions of test compounds

Compound or inhibitors were formulated initially as a concentrated stock solution in either dimethyl sulphoxide (DMSO), ethanol (EtOH) or Earle's Balanced Salt solution (EBSS) according to solubility. The final concentration of these vehicles in culture medium were: DMSO, 0.5% V/V and 1.0% V/V EtOH or EBSS to 35 mm petri dishes (Falcon) containing 2 ml of culture medium followed by agitation. If the compound was insoluble (i.e. precipitates out) the concentrated stock was diluted until solubility in culture medium was achieved. A series of dilutions of the stock solutions were then prepared to give the desired final concentration range in culture medium.

Culture medium was brought to 37°C and gassed with 5% CO₂, 95% air in sterile beakers in a flow CO₂ Incubator (Flow Laboratories, Irvine, Scotland). Sufficient medium and beakers were made ready to include the range of concentrations of compound plus a beaker in a sterile laminar flow cabinet and medium was stirred on a magnetic stirrer.

Fixation and staining of cultures after 5 days of incubation

1. Midbrain cultures

Medium was removed from culture dishes by aspiration and replaced with about 1 ml of 10% formaldehyde solution per dish. Fixative was removed with running tap water after a minimum of twenty minutes fixation. Cells were then stained for one to three minutes with haematoxylin followed by washing under with tap water. All the cultures were air dried.
2. Limb cultures

After aspiration of the medium, cultures were fixed with 10% formaldehyde containing 1% cetylpyridinium chloride for a minimum of twenty minutes. Cultures were then left for 1 hour in 3% acetic acid and stained with 1% alcian blue in 0.1 N hydrochloric acid (pH 1.0) for a minimum of two hours. After washing with tap water, the cultures were air dried.

Measurement of cell differentiation with an automated colony counter

Equipment


Method

Operation of the colony counter was described in the manual supplied by the manufacturer. Each cell island was positioned under the lens of the television camera so that its image was presented on the TV monitor. The colony counter was then set to count the number of foci of darkly staining cells which correspond in midbrain cultures to neurones and in limb cultures to chondrocytes. These differentiated cell types were not present in the initial cell suspension prepared from the embryo but have differentiated in culture (Flint, 1983; Flint and Orton, 1984), thus paralleling the IN VIVO development profile.

By counting the number of darkly staining foci, an estimate was made of the total population of cells which have differentiated
during the period of culture (Flint et al, 1984).

**Analysis of results**

The mean and standard deviation of the number of differentiated foci per cell island was calculated for controls and each concentration of compound. Results were plotted and the concentration (IC50) of compound inhibiting differentiation by 50% of control level was calculated by interpolation, or with the inhibitors, the maximum concentration at which no effect on differentiation was observed (N.C.C.). All data analyses comparing treatment groups with their concurrent controls were performed using Student's t-test.

**Coincubation with inhibitors**

Once the N.C.C. levels were determined, inhibitors and test compound were coincubated over the 5 day culture period in the same dish. The volumes of vehicle were appropriately altered to compensate for two compounds in the same dish.

2.2.2. **TERATOLOGY STUDY**

Alderley Park strain albino female rats (200-250g) were mated and the first day of the appearance of a vaginal plug taken as day 1 of pregnancy. Dams were randomised into three groups of ten and maternal weight gain throughout pregnancy monitored. The dosing regimen was as follows; Group I - Tween 80 (solvent vehicle), Group II - 150 mg/kg DPH in Tween 80, Group III - 300 mg/kg DPH in Tween 80 (dosing
solutions for Groups II and III were ball-milled for 24 hours before dosing commenced. Dosing was by oral gavage at a volume of 10 ml/kg days 10-15 (inclusive) of gestation. The maternal weight gain during this dosing period was calculated separately from the total maternal weight gain during gestation and clinical observations were made throughout pregnancy. Animals were sacrificed on day 20 of gestation (one day prior to term) and each uterus was scored for number of live fetuses, resorptions, implantation sites, corpora lutea and pre-implantation losses. Other parameters measured were the ratio of male to female off-spring, litter mean and fetal body and placenta weight, empty uterus weight and amniotic fluid per fetus. Clinical observations of the dams were made throughout gestation and necropsy observations made for each dam at the termination of the study.

2.2.3 PLACENTAL TRANSFER OF RADIOLABELLED COMPOUNDS

$^{14}$C - DPH

24 pregnant female Alderley Park strain rats (250-350g) were injected i.p. with 100 mg/kg DPH (in Tween 80) containing 5.26% W/V $^{14}$C-DPH according to its weight. The dosing solution was ball-milled for 24 hours prior to injection (neither pH nor composition was altered during this preparation).

The dams were injected at 3 pm on day 12 of gestation so that 18 hours after the injection the dams were 13.5 days pregnant (this protocol is in accordance with the IN VIVO/IN VITRO method of Flint et. al. 1984). Groups of three animals were sacrificed at $\frac{1}{2}, 1, 2, 3, 4, 5, 6$, and 18 hours post injection and blood samples from each
dam were taken and plasma samples prepared. The number, dry weight and protein content of embryos per uterus were recorded.

Pooled, dry embryos from each uterus were oxidised in a Packard tri-carb sample oxidiser model 3375 and $^{14}$CO$_2$ collected into 12 ml optisorb scintillation fluid. The radioactivity in these samples and 100 µl aliquots of maternal plasma was then determined on an Intertechnique S.L.30 liquid scintillation counter. The µg/ml bound DPH to maternal plasma and ng/mg protein bound DPH in embryos was calculated by comparison of the radioactivity in the samples to a reference sample of known weight of $^{14}$C-DPH.

$^{14}$C-Phenobarbitone

The inducing dosing regimen was followed as per section 2.2.4. 0.1% PB in drinking water was supplied ad libitum and supplemented with $^{14}$C-PB so that each dam received over a 10 day period (gestational days 4-13) 0.75 µCi/day. Maternal plasma and oxidised embryo samples were prepared as described earlier and the concentration of bound $^{14}$C-PB calculated against a reference sample (as for $^{14}$C-DPH.).

$^{14}$C-3-Methylcholanthrene

The dosing regimen in section 2.2.4. was followed, each dam received 3.3 µCi/day $^{14}$C-3MC in a 25 mg/kg, i.p. injection on gestational days 10, 11 and 12. Samples of maternal plasma and oxidised embryos were prepared as previously described and the concentration of bound 3MC calculated against a reference sample (as for DPH).
2.2.4. TRANSPLACENTAL INDUCTION OF FETAL CYTOCHROME P450

The maternal dosing regimens for transplacental induction of
embryo cells were as follows:-

1) β-naphthoflavone, 35 mg/kg in arachis oil, dosing volume 5
ml/kg given intra-peritoneally on days 10, 11, 12 gestation.
2) 3-methylcholanthrene, 25 mg/kg in arachis oil, dosing volume 5
ml/kg given intra-peritoneally on days 10, 11, 12 gestation.
3) Sodium phenobarbitone given at 0.1% (w/v) in the drinking water
ad libitum on days 4 -13 of gestation.

2.2.5. IMMUNOCYTOCHEMICAL STAINING TECHNIQUE FOR CYTOCHROME P450

The method used to identify the presence and isoenzymes of
cytochrome P450s was the peroxidase anti-peroxidase (PAP) method
adapted from Wolf et. al. (1984).

Method

Air dried cryosat sections of whole embryos at gestational day 18
or cell cultures of LB and CNS grown on sterile petri dishes were
washed three times for 5 minutes in excess 0.15 M saline containing
0.05M, phosphate buffer pH 7.2. Samples were then dehydrated by
passage through graded aqueous methanol (v/v) (50%, 75%, 95%) and the
endogenous peroxidases inhibited by treatment with 0.01% hydrogen
peroxide in 95% aqueous methanol for 20 minutes. Samples were then
rehydrated by passage through graded methanol (95%, 75%, 50%) and
washed again in PBS followed by PBS supplemented with 1% bovine serum
albumin (BSA) and then incubated in non-immune goat serum (diluted
1:30 with PB, 1% BSA) in order to saturate the antigen binding sites. Samples were then incubated with the P450 antibodies or pre-immune sera for 24 hrs at 4°C followed by treatment with horseradish peroxidase labelled anti-species IgG (at 1:20 dilution in PBS, 1% BSA) for 20 minutes at room temperature. Unreacted antibodies were washed off by three successive washings for 5 minutes each in PBS, 1% BSA., and a fourth wash in 0.05M Tris buffer pH 7.6. Peroxidase activity was revealed by incubation for 20 minutes with 3,3 diaminobenzidine 0.05M pH 7.6 and 0.01% H₂O₂. After washing in Tris buffer, samples were counterstained with haematoxylin, dehydrated and mounted under cover slips.

Control incubations were performed by substitution of the first anti-serum with pre-immune goat serum.

Figure 2.2 shows the peroxidase/antiperoxidase immunocytochemical staining technique schematically.

2.2.5. IMMUNOLOGICAL ASSAY FOR EMBRYO CYTOCHROME P450 BY WESTERN BLOT

Electrophoretic Blotting Procedure

This method is an adaptation of Towbin et. al. (1979) and is currently used at Dr C.R.Wolf's lab (Imperial Cancer Research Fund, Edinburgh), where this work was carried out.

Protein estimations (Lowry et. al. 1951) were carried out on rat LB and CNS cells after the 5 day culture period. The cultures were obtained from either untreated or β-naphthoflavone - induced dams. 1 mg protein samples diluted 1:1 with 'boiling mix' (10% stacking gel, 2% sodium dodecyl sulphate, 5% β-mercaptoethanol, 10% glycerol and
METHOD

Immunocytochemical Staining Procedure

FRESH CULTURE

Wash and dehydrate

Block endogenous peroxidase with H₂O₂ for 30 min.

Rehydrate and wash

Non-immune goat serum

Anti-P₄₅₀ Ab

24 hr. incubation at 4°C

Pre-immune serum

Wash

Horseradish peroxidase labelled IgG

Wash

Diaminobenzidine in 0.05 M, pH 7.6 TRIS buffer

Wash, counterstain with haemotoxylin, dehydrate to xylene and mount slide.
0.005% bromophenol blue (W/V)), were subjected to electrophoresis. The conditions for electrophoresis were as follows:- Separating gel = 9% polyacrylamide, separating gel = 4.5% polyacrylamide, electrode buffer = 25 mM TMS 0.192 mM glycine/20% (V/V) methanol at pH 8.3. Gels were run at 20 mA/gel when the dye front was in the stacking gel, and at 30 mA/gel when in the separating gel. The proteins were then transferred to nitrocellulose sheets as follows. A sheet of nitrocellulose (0.45 µm pore size in roll form, Millipore) was briefly wetted with water and laid on a scouring pad (Scotch Brite) which was supported by a stiff plastic grid. The gel to be blotted was put on the nitrocellulose sheet and care taken to remove all air bubbles. A second pad and plastic grid were added and the 'sandwich' slotted into the blotting apparatus with the nitrocellulose sheet nearest the anode. The electroblot apparatus was filled with buffer (20 mM disodium phosphate 20% (V/V) methanol) and attached to a power pack source of 0.25A overnight.

**Staining for Protein**

The blot was stained with amido black (0.1% in 45% methanol/10% acetic acid) and destained with 90% methanol/2% acetic acid to ensure complete transfer of protein to the nitrocellulose sheet.

**Immunological Detection of Proteins on Nitrocellulose**

The electrophoretic blots were soaked in 3% bovine serum albumin in saline (0.9% NaCl, 10mM TRIS-HCl, pH 7.9) for 1-2 hours at room temperature to saturate additional protein binding sites. The blots were then rinsed in saline before incubation with the cytochrome
P450 antibodies (see table 2.1 for legends), appropriately diluted with buffered saline. The sheets were then washed in saline (about 4 changes during 1 hour) and incubated for 1 hour with 125I-labeled protein A (0.1 µCi/ml buffered saline) and washed until the rinses showed no radioactivity. The sheets were then incubated with the second (indicator) antibody directed against the immunoglobulins from the first antiserum (anti-species IgG horseradish peroxidase labeled). Finally the substrate, (3% 4-chloro-1-naphthol in methanol, Tris buffered saline and H2O2 at a ratio of 1:6.2x10^-6) was added and the bands allowed to develop. The reaction was stopped by rinsing in distilled water. The sheets were then thoroughly air-dried before autoradiographs were made by exposure of the sheets on Kodak X-Omat R film for 6 days.

2.2.7 COVALENT BINDING OF 14C-DPH TO LB AND CNS CELL PROTEINS

The arene oxide is the putative teratogen of DPH, formed IN VIVO by a cytochrome P450 mediated reaction (see Chapter 1.5.2.4). This reactive intermediate binds to cellular proteins irreversibly. This experiment tests the hypothesis that the arene oxide is formed IN VITRO by embryo cells and by modulation of the pathway may elucidate the role the of arene oxide in the metabolism of DPH.

DPH was added at 60 µg/ml culture medium (with 0.4 µCi 14C-DPH/dish). The amount of covalently bound radioactive material to either LB or CNS cultures was determined in 1) untreated cells 2) cells derived from β-naphthoflavone induced mothers 3) cells co-
incubated over a five day period with a non-cytotoxic concentration of the epoxide hydratase inhibitor, trichloropropene oxide 4) cells co-incubated over a five day period with 20 µg/ml culture medium DPH and benzimidazole, a cytochrome P450 inhibitor, also at non-cytotoxic concentration. 5) untreated cells with different concentrations of DPH.

The amount of non-specific/background binding was tested by co-incubating untreated LB and CNS cells after the five days of culture for 30 minutes with 0.4 µCi ¹⁴C-DPH per dish.

Method

After the five day incubation period with radio-labelled compound, (0.4 µCi/dish) the culture medium was removed from LB and CNS cells. Cells were then washed three times in Earle's balanced salt solution without phenol red in calcium and magnesium free solution. The cells were then incubated at 37°C for 30 mins in Earle's solution containing 0.1% (w/v) ethylene diamine tetraacetic acid (EDTA), to loosen the cells from the bottom of the petri dishes. Cells were then scraped off the bottom of the petri dishes with a spatula and centrifuged at 2,000 r.p.m. for 5 minutes (MSE bench centrifuge). The supernatant was removed and 3 ml of 20% (w/v) trichloroacetic acid (TCA) was added and the cells mixed well. The precipitated DNA was collected by centrifugation at 2,000 r.p.m. for 15 minutes. The pellet was washed twice more with 20% TCA and then washed 6 times in 8 ml of a 4:1 methanol:water solution, the pellet being resuspended and recollected
by centrifugation inbetween each wash. The pellet was then dissolved in 1 ml of 1 N NaOH, and a 0.1 ml aliquot removed for protein determination (Lowry et. al. 1951). The remainder (0.9 ml) was counted in 10 ml Readisolve E.P. (Beckman) in a scintillation counter (Intertechnique) to determine protein bound radioactivity.

This procedure is a modification of the methods used by Blake and Martz (1980), Martz et. al. (1977) and Orton and Lowery (1981).

2.2.8 EXTRACTION AND IDENTIFICATION OF DIPHENYLHYDANTOIN METABOLITES

Embryo cells derived from untreated mothers were coincubated with 60 μg/ml culture medium DPH supplemented with $^{14}$C-DPH (0.4 μCi/dish) for the 5 day culture period. Cells and culture medium were then analysed to see if the cells themselves IN VITRO could metabolise DPH.

Extraction Method

The culture medium from each cell type was removed and pooled after the 5 day period so that total volume was approximately 12 ml. The cells were then washed twice in excess Earle's balanced salt solution without phenol red in calcium and magnesium free solution. Cells were then removed from the bottom of the petri dishes (as described in section 2.2.7) and LB cells or CNS cells pooled separately. The cell samples were then sonicated by a model W.375 sonicator for 5 seconds at 50% duty cycle, scale set 3. The collected pooled media from each cell type and the two different cell types were then passed through individual sep-pack C18-cartridges (Waters
Associates) pre-wetted with methanol. The metabolites were then eluted with 2 x 5 ml methanol, and the methanol was evaporated off under oxygen free nitrogen.

**Identification of metabolites by high pressure liquid chromatography**

Sample residues were reconstituted in a small volume of methanol and injected on to a spherisorb S nitrile reversed phase column, 25 cm x 4.6 mm (Technicol Ltd, Stockport, Cheshire); mobile phase - methanol : water (5:95); Flow-2 ml/min; chart speed - 5 mm/min; range-0.64; scan-10 mV; wavelength-210 nm; instrument-Pye-Unicam with an LC-UV detector. (Chow et. al. 1980, Chow and Fischer 1981). Fractions corresponding to the elution times of standard DPH (and its metabolites) were collected (at 1 min intervals) and radioactivity estimated by liquid scintillation counting (section 2.2.7).

**Analytical thin layer chromatography (TLC)**

A reference standard of $^{14}$C-DPH, the p. hydroxy, and m. hydroxy metabolites, ring opened acid metabolite and cell and culture medium samples were applied to a silica gel GF (250µ) plate (Analtech) and developed in chloroform : methanol : glacial acetic acid (85 : 10 : 5) (Chang et al 1970). Plates were examined under 254 and 366 nm U.V. light and then spotted with $^{14}$C- radioactive ink to aid location of the autoradiograph. Plates were maintained in complete absence of light under a photographic plate (18 x 24 cm Agfa-Gevaert photographic plates, NDT Systems) for 4-7 days, then the photographic plates were
removed and processed (4 min in fixer, 30 seconds in acetic acid; 4 minutes in developer solutions). The spot of radioactive ink was lined up with the spot on the T.L.C. plate and the relative Rf values for DPH and its metabolites calculated.

A quantification of the percentage metabolism was estimated using a Berthold LB 2722-2, TLC plate Dunnschicht-Scanner II (scan speed 60 mm/hr, slit 1x10 mm). The total radioactivity per channel on the TLC plate was recorded and the percentage radioactivity (of total) calculated per band.
CHAPTER 3.

TERATOGENIC POTENTIAL OF

DIPHENYLHYDANTOIN

Contents

3.1 TRANSPLACENTAL PASSAGE OF DPH

3.2 EFFECT OF DPH ON DIFFERENTIATING RAT EMBRYO CELLS IN VITRO

3.3 EFFECT OF DPH GIVEN IN VIVO ON DIFFERENTIATING RAT EMBRYO CELLS IN VITRO (ex vivo test)

3.4 QUALITATIVE STRUCTURE - ACTIVITY RELATIONSHIP OF DPH AND ITS ANALOGUES IN VITRO

3.5 IN VIVO TERATOLOGY OF DPH IN THE RAT

3.6 DISCUSSION
3. TERATOGENIC POTENTIAL OF DIPHENYLHYDANTOIN

These diverse experiments with DPH have been drawn together in an attempt to understand its teratogenic activity. First of all it was necessary to establish whether the compound actually crossed the placenta, and then to characterise the IN VITRO effect of the compound on differentiating rat embryo cells. Then the IN VITRO response of a group of structurally related compounds was examined so that the sensitivity of the cells could be scrutinised. Finally, the IN VIVO teratology of DPH was examined.

* The rationale behind the choice of analogues was to try to block the position(s) of metabolism on the phenyl ring, thus showing whether metabolism was essential for teratogenic activity.

Post Scriptum

Just to re-emphasise that whilst I realise that 'teratogenic' refers to the IN VIVO situation for the purpose of this thesis reference is made to 'IN VITRO teratogenesis' as a means to identify an IN VITRO end-point with possible IN VIVO correlative significance.
3.1. **TRANSPLACENTAL PASSAGE OF $^{14}$C-DPH IN THE RAT**

Figure 3.1. illustrates the time course profile of radioactivity across the placenta of a single i.p. dose of $^{14}$C-DPH, the protocol having been described in Chapter 2.2.4.

Peak maternal plasma values of 47±10 µg/ml DPH were reached by 2 hours post-dose and maintained at that level until 4 hours post-dose, after which the profile of elimination followed apparent first order kinetics. $^{14}$C-Labelled material was still detectable (17±11 µg/ml) at 18 hours post dose. The profile of the C$^{14}$-labelled material in the embryo followed the maternal plasma profile.

A peak concentration of approximately 250±50 ng/embryo batch was obtained 2 hours post dose and elimination appeared to follow first order kinetics. $^{14}$C-Labelled material (58±7 ng/embryo batch) was still detectable 18 hours post-dose. The apparent half lives in both maternal plasma and whole embryos was calculated and found to be 8½ and 6 hours respectively for both compartments.

Analysis of the radiolabelled material in both maternal plasma and whole embryos 2 hours post dose confirmed that it was authentic $^{14}$C-DPH, the Rf values being the same for dosing solution and reference material (0.42). No other spots signifying a metabolite were present. However at 8 hours post dose, both maternal plasma and whole embryos contained a small amount of the p. hydroxy. DPH metabolite (Rf=0.125); the majority of the radiolabelled material was unchanged $^{14}$C-DPH.

The percentage of p. hydroxy. DPH in maternal plasma at 18 hours was 4.33-5.26% and in whole embryos 4.01-4.87% (see chapter 2.2.8 for analytical method).
3.2. EFFECT OF DPH ON DIFFERENTIATING RAT EMBRYO CELLS IN VITRO

Mid-brain (CNS) and mesenchymal limb bud (LB) cells were removed from 13 day embryos and cultures prepared as described in Chapter 2. Various concentrations of DPH were added directly to the culture medium in ethanol (10 μl of ethanol per ml culture medium, a non-toxic concentration). DPH was left in contact with the cells over the 5 day culture period during which time the CNS cells differentiated into foci of neurones and LB cells into foci of chondrocytes. At the end of the culture period, cells were processed as described in Chapter 2.1, and the number of differentiated foci per cell island recorded and plotted against concentration of DPH in the culture medium (Figure 3.2.). The concentration at which differentiation was inhibited by 50% (the IC50 value) in each cell type was estimated from the graph. The IC50 value for CNS was 67±18 μg/ml (n=9) and for LB was 91±4 μg/ml (n=9).

The data shows that CNS cells were more sensitive to the effects of DPH IN VITRO than LB cells as based on the relative IC50 values, (p<0.1) but that for both cell types the DPH IC50 value was < 100 μg/ml thus qualifying the compound as having an IN VITRO teratogenic effect (Flint and Orton 1984).

3.3. EFFECT OF DPH GIVEN IN VIVO ON DIFFERENTIATING RAT EMBRYO CELLS IN VITRO (EX VIVO TEST)

DPH was administered as a single i.p. or oral dose at the maximum maternally tolerated dose levels, 200 and 300 mg/kg respectively, to 3 pregnant dams per group at 3 pm on day 12 of gestation. Embryos were
Figure 3.1

TIME COURSE OF TRANSPLACENTAL PASSAGE OF A SINGLE I.P. DOSE OF 14C DPH

DPH CONCENTRATION
µg/ml maternal plasma

DPH CONCENTRATION
ng/embryo

0 1 2 3 4 5 6

Time (hrs.)

0 100 200 300

0 1 2 3 4 5 6

'Tapparent'

1/2 maternal plasma 8 hrs

'Tapparent'

1/2 embryos 6 hrs

0 1 2 3 4 5 6

Time (hrs.)
Figure 3.2

THE INFLUENCE OF DPH ON FOCI FORMATION IN LB AND CNS CELLS IN VITRO

FOCI NUMBER

pg DPH/ml culture medium

µg DPH/ml culture medium

CNS
LB
67
91
100
explanted 18 hours later (at 9 am on day 13 of gestation) from each group of dams and pooled. This method is in accordance with the EX VIVO method of Flint and Orton (1984). Cultures were prepared and foci number at the end of the 5 day culture period were compared to cultures derived from untreated dams. Results showed that there were no significant differences between foci number in either LB or CNS from orally or i.p. DPH dosed or untreated dams. In conclusion, DPH was considered a negative teratogen by the EX VIVO method of teratogen prediction.

3.4. QUALITATIVE STRUCTURE-ACTIVITY RELATIONSHIP FOR DPH AND SOME ANALOGUES IN VITRO

DPH and 28 other structurally similar compounds (Tables 3.1 and 3.2) containing an intact hydantoin ring, (the exception being DPH acid containing a carboxylic acid and amino functional group at positions 1 and 2 respectively), were chosen for this study. The structural differences were all at the 5 position of the hydantoin ring, either a single replacement of one or both phenyl rings, or substitutions directly on to one or both phenyl rings. The analogues were chosen for their substitutions on the phenyl ring(s) so that metabolism to the p. and m. metabolites could be blocked.

Concentration-response curves were obtained for all compounds over the 5 day incubation period with both LB and CNS cells, and where possible IC50 values calculated. Log p values, were also calculated and shown in Table 3.2, where p = partition coefficient ratio in an octanol-water reference system (p=co/cw where co is the compound
### Table 3.1

**NOMENCLATURE USED IN DPH STRUCTURE**

**ACTIVITY RELATIONSHIP STUDY**

<table>
<thead>
<tr>
<th>COMPOUND NAME</th>
<th>COMPOUND NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-cyclohexyl-5 phenylhydantoin</td>
<td>1</td>
</tr>
<tr>
<td>5-cyclopentyl-5 phenylhydantoin</td>
<td>2</td>
</tr>
<tr>
<td>5-p-methyl diphenylhydantoin</td>
<td>3</td>
</tr>
<tr>
<td>5-p-hydroxy, 5 p-methyldiphenylhydantoin</td>
<td>4</td>
</tr>
<tr>
<td>5-amino, 5 phenylhydantoin</td>
<td>5</td>
</tr>
<tr>
<td>5, 5 p-p-dichlorodiphenylhydantoin</td>
<td>6</td>
</tr>
<tr>
<td>p-hydroxy. DPH</td>
<td>7</td>
</tr>
<tr>
<td>m-hydroxy. DPH</td>
<td>8</td>
</tr>
<tr>
<td>5, 5 p-p-dihydroxy DPH</td>
<td>9</td>
</tr>
<tr>
<td>5-p-hydroxypheny DPH</td>
<td>10</td>
</tr>
<tr>
<td>5-methyl, 5-p-chlorophenylhydantoin</td>
<td>11</td>
</tr>
<tr>
<td>5-p-m-dichlorophenylhydantoin</td>
<td>12</td>
</tr>
<tr>
<td>5-p-chlorophenylhydantoin</td>
<td>13</td>
</tr>
<tr>
<td>5-o-hydroxyphenylhydantoin</td>
<td>14</td>
</tr>
<tr>
<td>5-cyclopropyl, 5-phenylhydantoin</td>
<td>15</td>
</tr>
<tr>
<td>5-cyclobutyl, 5-phenylhydantoin</td>
<td>16</td>
</tr>
<tr>
<td>5-hydroxy, 5-phenylhydantoin</td>
<td>17</td>
</tr>
<tr>
<td>5-methyl, 5-phenylhydantoin</td>
<td>18</td>
</tr>
<tr>
<td>5-ethyl, 5-phenylhydantoin</td>
<td>19</td>
</tr>
<tr>
<td>Hydantoin</td>
<td>20</td>
</tr>
<tr>
<td>Diphenylhydantoic acid</td>
<td>21</td>
</tr>
<tr>
<td>5-phenylhydantoin</td>
<td>22</td>
</tr>
<tr>
<td>5-methylhydantoin</td>
<td>23</td>
</tr>
<tr>
<td>5-p-aminophenylhydantoin</td>
<td>24</td>
</tr>
<tr>
<td>5-hydroxy, 5-methylhydantoin</td>
<td>25</td>
</tr>
<tr>
<td>5-ethanoic acid hydantoin</td>
<td>26</td>
</tr>
<tr>
<td>5, 5-dimethylhydantoin</td>
<td>27</td>
</tr>
<tr>
<td>5, 5-dicyclopentylhydantoin</td>
<td>28</td>
</tr>
</tbody>
</table>
### TABLE 3.2

**Graded Response of Structure – Activity Relationship**

<table>
<thead>
<tr>
<th>More Toxic</th>
<th>Same Toxicity</th>
<th>Less Toxic</th>
<th>Hardy Toxic</th>
<th>Non-Toxic</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td><img src="image5.png" alt="Structure 5" /></td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure 6" /></td>
<td><img src="image7.png" alt="Structure 7" /></td>
<td><img src="image8.png" alt="Structure 8" /></td>
<td><img src="image9.png" alt="Structure 9" /></td>
<td><img src="image10.png" alt="Structure 10" /></td>
</tr>
<tr>
<td><img src="image11.png" alt="Structure 11" /></td>
<td><img src="image12.png" alt="Structure 12" /></td>
<td><img src="image13.png" alt="Structure 13" /></td>
<td><img src="image14.png" alt="Structure 14" /></td>
<td><img src="image15.png" alt="Structure 15" /></td>
</tr>
<tr>
<td><img src="image16.png" alt="Structure 16" /></td>
<td><img src="image17.png" alt="Structure 17" /></td>
<td><img src="image18.png" alt="Structure 18" /></td>
<td><img src="image19.png" alt="Structure 19" /></td>
<td><img src="image20.png" alt="Structure 20" /></td>
</tr>
<tr>
<td><img src="image21.png" alt="Structure 21" /></td>
<td><img src="image22.png" alt="Structure 22" /></td>
<td><img src="image23.png" alt="Structure 23" /></td>
<td><img src="image24.png" alt="Structure 24" /></td>
<td><img src="image25.png" alt="Structure 25" /></td>
</tr>
<tr>
<td><img src="image26.png" alt="Structure 26" /></td>
<td><img src="image27.png" alt="Structure 27" /></td>
<td><img src="image28.png" alt="Structure 28" /></td>
<td><img src="image29.png" alt="Structure 29" /></td>
<td><img src="image30.png" alt="Structure 30" /></td>
</tr>
</tbody>
</table>

- Log $p = 2.47$
- Corrected log (IC$_{50}$ CNS) = 2.42
- Corrected log (IC$_{50}$ LB) = 2.56

**Formulae:****

\[ \text{IC}_50 = \log \left( \frac{\text{IC}_50}{\text{mol wt}} \right) \times 1000 \text{ (ie \ mu mol)} \]

**Symbols:**

- **a** = Log $p$
- **b** = Corrected log (IC$_{50}$ CNS)
- **c** = Corrected log (IC$_{50}$ LB)
- **-** = IC$_{50}$ not found.
concentration in octanol and CW the corresponding concentration in the water phase). Table 3.2 shows the values obtained for each compound, IC50 CNS and LB denoted 'b' and 'c' on the right hand side of each structure. Compounds are also grouped according to the degree of toxicity as compared to DPH as a reference standard. Compounds with 'b' and 'c' corrected log IC50 LB < 2.56 or IC50 CNS < 2.42 were considered more toxic, these compounds are 1-6, (see Table 3.1 for numerically corresponding nomenclature). (Corrected log IC50 means IC50/mol weight x 1000 i.e. µmol log) Compounds that were less toxic than DPH ie: those compounds with 'b' and 'c' values greater than those of DPH were compounds 10-16. Compounds that were considered hardly toxic were 17, 18 and 19 with 'b' and 'c' values significantly greater than DPH. Compounds that were classed as non-toxic were 20-28 and generally IC50 values were either greater than the compounds maximum solubility or above the concentration response range investigated.

Given the biological significance of log p ie: that it is an indicator of lipid solubility and hence accessibility of compound to the cell, graphs were plotted for both LB (Figure 3.3) and CNS (Figure 3.4) cells of log IC50, the indication of toxicity against log p. Regression line analysis for each graph was made:-

\[
\text{LB Log } \frac{1}{\text{IC50}} = 3.94 \pm 0.2 - 0.52 \log p, \text{ where } 3.94 = \text{ intercept on x axis, -0.52 = slope. (n=12 observations, correlation coefficient } r^2 = 89\%, \text{ s.e}=0.26, F=41 \text{ (where } F=\text{goodness of fit) probability > } 99.9\%).
\]
CNS  \( \log \frac{1}{IC50} = 3.81 (\pm 0.12) - 0.53 \log p \), where 3.81 = intercept on x axis, -0.53 = slope. (n=13 observations, correlation coefficient \( r^2 = 94\% \), s.e = 0.18, F=100 (where F=goodness of fit) probability > 99.9%).

The above data shows that for both CNS and LB cells, there is a definite correlation between toxicity and lipophilicity, the more lipophilic the compound (ie: greater log p value) the more toxic that compound is ie: the smaller the log IC50 value).

3.5 IN VIVO TERATOLOGY OF DPH IN THE RAT

The IN VIVO teratology study of DPH was set up as described in Chapter 2.2.2. Group I animals received solvent vehicle only, Group II 150 mg/kg DPH and Group III 300 mg/kg DPH.

The necropsy details for each group are summarised in Table 3.3. A dose related change in maternal weight gain during gestation and over the dosing period (both significant at \( p < 0.001 \) level) was observed. Mean litter, fetal body and placental weights were significantly decreased (p <0.001) in the top dose group and both doses showed significant decrease (p <0.001) in empty uterus weight and mean amniotic fluid per fetus.

Clinical observations (Table 3.4) of the Group III dams shows that 70% of the animals suffered piloerrection and 50% suffered alopecia in the latter stages of gestation. Necropsy observations of Group III dams showed 30% of the animals with no abnormalities
CORRELATION BETWEEN LIPOPHILICITY AND TOXICITY FOR STRUCTURALLY RELATED HYDANTOINS IN LB CELLS

Footnote: Substitutions are all on the 5 position of the hydantoin ring
CORRELATION BETWEEN LIPOPHILICITY AND TOXICITY FOR STRUCTURALLY RELATED HYDANTOINS IN CNS CELLS

Log P v Log IC_{50} CNS (µ moles)

*indicates compounds which maybe partially ionized at pH7

Footnote: Substitutions are all in the 5 position of the hydantoin ring
### TABLE 3.3.
**NECROPSY DETAILS FROM THE DPH TERATOLOGY STUDY**

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Group I (n=10)</th>
<th>Group II (n=10)</th>
<th>Group III (n=9)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Weight Gain (Days 0-20 of gestation) (g)</td>
<td>150.74 (±11.40)</td>
<td>109.95* (±16.88)</td>
<td>33.81* (±46.34)</td>
</tr>
<tr>
<td>Maternal Weight Gain (Days 10-15 of gestation) (g)</td>
<td>33.85 (±4.18)</td>
<td>-5.85* (±21.44)</td>
<td>-25.93* (±15.76)</td>
</tr>
<tr>
<td>Number of Live Fetuses/Dam</td>
<td>12.8 (±2.3)</td>
<td>11.8 (±2.8)</td>
<td>12.1 (±2.7)</td>
</tr>
<tr>
<td>Number of Resorptions/Dam</td>
<td>0.5 (±0.6)</td>
<td>0.9 (±0.5)</td>
<td>1.2 (±1.0)</td>
</tr>
<tr>
<td>Number of Implantation sites/Dam</td>
<td>13.3 (±2.2)</td>
<td>12.7 (±2.7)</td>
<td>13.3 (±2.7)</td>
</tr>
<tr>
<td>Number of Corpora Lutea/Dam</td>
<td>14.8 (±1.5)</td>
<td>14.1 (±2.3)</td>
<td>14.7 (±0.7)</td>
</tr>
<tr>
<td>Number of Pre-implantation Losses/Dam</td>
<td>1.7 (±1.9)</td>
<td>1.5 (±2.0)</td>
<td>1.6 (±2.6)</td>
</tr>
<tr>
<td>Sex Ratio, Male : Female/Dam</td>
<td>0.485 (±0.135)</td>
<td>0.455 (±0.120)</td>
<td>0.463 (±0.063)</td>
</tr>
<tr>
<td>Litter Mean Fetal Body Weight (g)</td>
<td>3.383 (±0.183)</td>
<td>3.286 (±0.598)</td>
<td>2.419* (±0.440)</td>
</tr>
<tr>
<td>Litter Mean Placental Weight (g)</td>
<td>0.509 (±0.064)</td>
<td>0.490 (±0.040)</td>
<td>0.395* (±0.057)</td>
</tr>
<tr>
<td>Placental Index b</td>
<td>15.02 (±1.54)</td>
<td>15.24 (±2.17)</td>
<td>16.97 (±4.60)</td>
</tr>
<tr>
<td>Empty Uterus Weight (g)</td>
<td>5.10 (±0.67)</td>
<td>4.58* (±0.79)</td>
<td>4.13* (±0.52)</td>
</tr>
<tr>
<td>Mean Amniotic Fluid/Fetus Weight (g)</td>
<td>1.128 (±0.307)</td>
<td>0.867* (±0.586)</td>
<td>0.852* (±0.108)</td>
</tr>
</tbody>
</table>

* = Statistically different from controls at p<0.001 level

a = One dam died

b = (total placenta weight of litter / total fetal weight of litter) as a percentage.
detected, 30% with renal pelvic cavitation and 60% with patchy lungs (Table 3.5). This evidence supports the data in Table 3.1. that Group III animals showed signs of maternal toxicity. Group II and I dams showed no abnormalities either as clinical observations or at necropsy. Examination of the gross appearance, soft tissues and palates of each fetus did not show any malformations. However 40% of the litters from Group III dams exhibited domed-shaped heads probably attributable to reduced fetal size and fetal growth retardation.

This study has shown that there was a dose related effect on maternal and embryo toxicity but that orally administered DPH is not teratogenic in Alderley Park albino rats under the present experimental conditions.

3.5. DISCUSSION

The results presented in this chapter have shown that whilst DPH is not teratogenic IN VIVO in the Alderley Park strain rat by the oral route it does cross the placenta and reach the embryo. In contrast, DPH inhibits differentiation of A.P. strain rat embryo cells IN VITRO. Furthermore the rat embryo cells IN VITRO have the ability to distinguish between IN VIVO teratogenic and non-teratogenic hydantoin ring derivatives within a homologous series.

DPH is a well established mouse, rabbit and human teratogen but negative in the cat and dog (Schardein, 1986). Harbison and Becker (1972) however have reported reduced fetal body weight, increased incidences of cleft palate, hydrocephalus, hydronephritis and shortening of the long bones of the appendicular skeleton in fetal
### TABLE 3.4.

**CLINICAL OBSERVATIONS OF THE TOP DOSE GROUP IN THE**

**IN VIVO TERATOLOGY STUDY OF DPH IN THE RAT**

<table>
<thead>
<tr>
<th>FEMALE NUMBER</th>
<th>OBSERVATION</th>
<th>DAYS OF PREGNANCY ON WHICH OBSERVATION MADE</th>
</tr>
</thead>
<tbody>
<tr>
<td>003185</td>
<td>NAD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0-19</td>
</tr>
<tr>
<td></td>
<td>PILOERECTION</td>
<td>20</td>
</tr>
<tr>
<td>003191</td>
<td>NAD</td>
<td>0-12,18,19</td>
</tr>
<tr>
<td></td>
<td>PILOERECTION</td>
<td>13-17,20</td>
</tr>
<tr>
<td>003196</td>
<td>NAD</td>
<td>0-13</td>
</tr>
<tr>
<td></td>
<td>ALOPECIA</td>
<td>14-20</td>
</tr>
<tr>
<td></td>
<td>PILOERECTION</td>
<td>20</td>
</tr>
<tr>
<td>003199</td>
<td>NAD</td>
<td>0-18</td>
</tr>
<tr>
<td></td>
<td>ALOPECIA</td>
<td>19,20</td>
</tr>
<tr>
<td>003201</td>
<td>NAD</td>
<td>0-18</td>
</tr>
<tr>
<td></td>
<td>PILOERECTION</td>
<td>19,20</td>
</tr>
<tr>
<td>003203</td>
<td>NAD</td>
<td>0-12,17-20</td>
</tr>
<tr>
<td></td>
<td>BLOOD ON SNOUT AND/OR FOREPAWS</td>
<td>13-16</td>
</tr>
<tr>
<td>003207</td>
<td>BITE (SEE DESCRIPTION)</td>
<td>0,1</td>
</tr>
<tr>
<td></td>
<td>NAD</td>
<td>2-11</td>
</tr>
<tr>
<td></td>
<td>PATCHY HAIR GROWTH</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>ALOPECIA</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>PILOERECTION</td>
<td>13-20</td>
</tr>
<tr>
<td></td>
<td>STAINS AROUND MOUTH</td>
<td>20</td>
</tr>
<tr>
<td>003209</td>
<td>NAD</td>
<td>0-17</td>
</tr>
<tr>
<td></td>
<td>ALOPECIA</td>
<td>18-20</td>
</tr>
<tr>
<td></td>
<td>PILOERECTION</td>
<td>18-20</td>
</tr>
<tr>
<td>003214</td>
<td>NAD</td>
<td>0-17</td>
</tr>
<tr>
<td></td>
<td>ALOPECIA</td>
<td>18-20</td>
</tr>
<tr>
<td></td>
<td>PILOERECTION</td>
<td>19,20</td>
</tr>
</tbody>
</table>

<sup>a</sup> NAD = no abnormalities detected
TABLE 3.5.

NECROPSY OBSERVATIONS OF THE TOP DOSE GROUP IN THE
IN VIVO TERATOLOGY STUDY OF DPH IN THE RAT

Group III DPH  3000 MG/KG

<table>
<thead>
<tr>
<th>FEMALE NUMBER</th>
<th>DAY FEMALE KILLED</th>
<th>OBSERVATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>003185</td>
<td>20</td>
<td>Spots / patches on lungs</td>
</tr>
<tr>
<td>003191</td>
<td>20</td>
<td>Patchy lungs</td>
</tr>
<tr>
<td>003196</td>
<td>20</td>
<td>Patchy lungs</td>
</tr>
<tr>
<td>003199</td>
<td>20</td>
<td>NAD$^a$</td>
</tr>
<tr>
<td>003201</td>
<td>20</td>
<td>NAD</td>
</tr>
<tr>
<td>003203</td>
<td>20</td>
<td>NAD</td>
</tr>
<tr>
<td>003207</td>
<td>20</td>
<td>Patchy lungs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal pelvic cavitation</td>
</tr>
<tr>
<td>003209</td>
<td>20</td>
<td>Reduced stomach size</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mottled liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small intestine empty</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large intestine empty</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Patchy lungs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal pelvic cavitation</td>
</tr>
<tr>
<td>003214</td>
<td>20</td>
<td>Renal pelvic cavitation</td>
</tr>
</tbody>
</table>

$^a$ NAD = No abnormalities detected
rats of the Sprague-Dawley (Simonsen) strain when the mother is treated with 150 mg/kg DPH i.p. on days 10-15 gestation.

My study was conducted by the oral route (repeated i.p. injections to pregnant animals was not done to minimise stress and discomfort) so a direct comparison of results from both studies where, different strains and routes of administration were used, is not possible. The conclusion may be drawn that DPH is a possible strain specific rat teratogen but is definitely fetotoxic.

It would appear that the inter-species variation in DPH teratogenesis is not wholly related to the metabolism of the compound as the principle urinary metabolite of an oral dose of DPH to both rats and humans is the p-hydroxy DPH metabolite and its glucuronide conjugate (Wells and Harbison 1980). Both these species and the mouse have detectable levels of the dihydrodiol, catechol and o-methyl-catechol metabolites in the urine following administration of the drug (Glazko 1973). The dog, a resistant species, is the only one to exhibit a different metabolic profile, its major metabolite being m.hydroxy DPH. A further similarity between species, whether resistant or susceptible to DPH teratogenesis is that high levels of the unchanged drug and low levels its ring opened acid metabolite have been detected in human, rat and dog urine.

Studies with $^{14}$C-DPH dosed to pregnant mice (day 17.5 gestation) showed that 6 hours after the dose the majority of radioactivity in the embryo was unchanged DPH, and only 0.2-5% of the radioactivity was associated with the p.hydroxy. DPH. My studies by TLC/autoradiographic analysis of the radiolabelled material in
maternal plasma and whole rat embryos (day 13.5 gestation) confirm this; 2 hours post-dose radioactivity was 100% 14C-DPH, 8 hours post dose 95-96% radioactivity was 14C-DPH, and 4-5% radioactivity was 14C-p-hydroxy.DPH.

Given that there appear to be no appreciable differences between species in metabolic profiles nor amount of metabolite produced, that placentation differs little between species (Juchau 1981), that the clinical condition of epilepsy itself is not the cause of terata (Finnell and Chernoff 1984), it would appear that the interspecies variability to DPH teratogenesis remains largely unexplained.

DPH is considered teratogenic in a variety of IN VITRO systems including; whole mouse embryo culture (Sadler et.al. 1982, Brown et.al. 1979), rat embryo cell culture (Flint et.al. 1984, Flint and Orton 1985, Brown et.al. 1986 a,b.) and human fetal palatal mesenchyme cells (Pratt and Willis 1985). IN VITRO systems are ideal for elucidating the mechanisms of teratogenesis as the complication of the role of maternal metabolism and the physiological barrier of the placental unit are omitted. That DPH is considered a positive IN VITRO teratogen in a system utilizing Alderley Park strain rats (but not IN VIVO) implies that insufficient levels of DPH reach the embryo itself from the mother IN VIVO. The concentration at which differentiation of rat embryo cells IN VITRO is inhibited by 50% is 91±4 µg/ml for LB cells and 67±18 µg/ml for CNS cells, while the peak concentrations achieved after a single i.p. dose to the mother in whole embryos is 0.30 ± 0.05 µg/embryo. IN VITRO the compound remains in contact with the cells at a high, steady-state concentration for 5
days whilst IN VIVO embryos are exposed to a low concentration (apprent $t_{1/2}=8$ hours). The IN VIVO picture becomes more complex as DPH is known to induce its own metabolism (Glazko 1973) and therefore the passage of DPH itself is diminished as the concentration decreases due to metabolism on the maternal side resulting in less available DPH for transfer to the embryo. The question of the rat's resistance to DPH teratogenicity IN VIVO is probably not as simple as the concentrations reaching the embryo not being sufficient to elicit a response, but may be a combination of insufficient concentrations and the sensitivities of the embryo cells themselves. Table 1.8 in Chapter 1 highlights the minimum doses at which a teratogenic effect is observed for teratogens between different species and illustrates that certain species are more sensitive to a particular teratogen that others e.g. thalidomide, the most sensitive species being man and rabbit.

The human therapeutic plasma concentration range for DPH is 10-20 $\mu$g/ml ($t_{1/2}=8-12$ hours) (Hvidberg and Dam 1976). The peak plasma concentration in the rat following a single i.p. dose is approximately 50 $\mu$g/ml, the mean plasma concentration 20-40 $\mu$g/ml over a 12 hour period (apprent $t_{1/2}=8$ hours), so that the concentration in rat plasma is double that for human at any given time point. However the human is probably more susceptible than the rat to DPH teratogenesis, implying that for human embryo cells it is not the concentration of DPH but the inherent sensitivity of the cells themselves which governs the teratogenic outcome.

Structure-activity studies with five hydantoin derivatives in the mouse IN VIVO (Brown et.al. 1982) have shown that there is a common
mechanism for teratogenicity of the closed ring hydantoins, of lethality for the substituted hydantoins and that potencies were determined primarily by lipid solubility. In my study with 28 hydantoin derivatives with differentiating rat embryo cells IN VITRO the same conclusions can be drawn i.e. the intact hydantoin ring with at least one phenyl ring attached at the 5 position is required for a toxic effect and that this is accompanied by high lipophilicity (a high log p value). The two graphs (Figures 3.3 and 3.4) illustrate the correlation between the two parameters (toxicity vs log p).

Other points regarding the structure-activity relationship of this series of hydantoins compared to DPH can be made and include:-

1) Compounds that were non-toxic (numbers 20-28) had an aliphatic group at position 5 on the hydantoin ring and negative log p values. Exceptions to this were DPH acid (21) which had 2 phenyl rings at position 5 but a broken hydantoin ring and 5-p-aminophenylhydantoin (24) which had a (substituted) phenyl ring at position 5, however both compounds had negative log p values. Compound 28 had 2 cyclopropyl rings which may rupture in the culture medium and thus alter the log p value to a negative value corresponding to the other aliphatically substituted compounds. 5-phenylhydantoin (22) was totally non toxic IN VITRO and has been reported as a negative IN VIVO human teratogen (Finnell and Dilberti 1983), however it had a low but positive log p value. This observation implies that there may be a receptor-mediated effect which requires the presence of 2 phenyl rings since the
only difference between this compound and DPH is an extra phenyl ring at position 5 in the latter compound, which makes DPH 3 times more lipophilic than 5-phenylhydantoin.

2) Compounds that were weakly toxic to the cells (17-19) had single phenyl rings at the 5 position and an aliphatic group (in place of the second phenyl ring of DPH) and moderately low log p values.

3) Compounds that were less toxic than DPH (10-16) all had a single phenyl ring at the 5 position. Compounds 15 and 16 had cyclopropyl and cyclobutyl rings respectively at the other 5 position, which being sterically strained probably ruptured over the 5 day culture period rendering them as aliphatic groups. Compounds 11, 12 and 13 had a Cl group at the para position on the phenyl ring, 12 and 13 had only hydrogen at the other 5 position and compound 11 a methyl substitution, compound 12 was dichloro-substituted on the phenyl ring, para and meta. Compounds 12 and 13 can be directly compared to compound 22 and compound 11 compared to 18 as they are chlorinated substitutions on the phenyl ring. The result of a chlorine substitution appears to both increase log p values and toxicity.

Compounds 10 and 14 are mono-hydroxy substitutions of the phenyl ring at the para and ortho positions respectively and can be compared to compound 22 (5-phenylhydantoin). The result of a
hydroxy substitution appears to lower the log p values (from a positive one for 22 to a negative one for both 10 and 14) but toxicity is increased. This observation implies that the intrinsic activity of the hydroxy analogues is greater than that of the mono-phenyl-hydantoin.

All compounds that were classed as less toxic than DPH and had lower log p values indicating that they were less lipophilic than DPH.

4) Compounds that were equi-toxic with DPH were hydroxy derivatives (7-9). Compounds 7 and 8 were mono-hydroxy substituted at para and meta positions respectively, compound 9 was dihydroxy substituted, one on each phenyl ring at the para position. All compounds were substantially less lipophilic than DPH, but were equitoxic, implying that despite less accessibility to the cells the compounds were intrinsically more toxic than DPH itself.

5) Compounds that were more toxic to the cells than DPH were compounds 1-6. Compounds 1 and 2 contained a phenyl ring and saturated 6 or 5 membered rings respectively and were substantially more lipophilic than DPH. Compound 3 was methyl substituted at the p position on one of the phenyl rings; this substitution substantially increased the log p value and hence rendered the compound more toxic than DPH. Compound 4 was the p hydroxy derivative of compound 3, the hydroxy substitution decreased the log p value but did not alter the toxic potential
again implying that the intrinsic activity of the phenolic compound was greater than that of the non-hydroxy substituted compound. Compound 5 contained a single phenyl group and an amino function, both at the 5 position and a low log p value, however the compound was more toxic than DPH. It is probably that this compound is partially ionised at pH7 and therefore the log p value is not an accurate value for the IN VITRO/IN SITU situation. Compound 6 contained both phenyl rings and was the p-dichloro analogue of DPH, this compound was the most lipophilic and most toxic compound of the series.

In summary it would appear that the potency of a drug is controlled by the amount of free drug at the receptor, except for the phenolic derivatives, it would appear that accessibility of the compound to lipid bound receptors in embryo cells (which is governed by lipid solubility, log p) is the prime factor determining teratogenic outcome.

The teratogenic potential had 89% correlation coefficient with LB cells and 94% with CNS. This study has shown the ability and sensitivity of rat embryo cells IN VITRO for the prediction of teratogenic hazard, even structurally similar compounds within a homologous series, however it remains to be seen whether the prediction for all the compounds holds up IN VIVO.
The overall conclusion is that DPH teratogenicity in the rat IN VIVO/EX VIVO versus micromass IN VITRO is probably a function of embryo cell protection in the whole animal, however the micromass IN VITRO system is very useful for structure/activity analysis.
CHAPTER 4

IMMUNOCYTOCHEMICAL IDENTIFICATION
OF EMBRYONIC CYTOCHROME P450'S

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4.1.3. EFFECT OF CO-INCUBATING DPH AND BENZIMIDAZOLE

4.2 IN VIVO AND IN VITRO COMPARISON OF EMBRYOIC CYTOCHROME P450'S AT DAY 18 OF DEVELOPMENT.

4.3 DEVELOPMENTAL PROFILE OF EMBRYOIC CYTOCHROME P450'S IN VITRO.

4.4 IMMUNOLOGICAL ANALYSIS OF EMBRYOIC CYTOCHROME P450'S BY WESTERN BLOTTING.

4.5 DISCUSSION
4. IMMUNOCYTOCHEMICAL IDENTIFICATION OF EMBRYO CYTOCHROME P450'S

If embryo cells are to have the capacity to activate teratogens then it is essential to demonstrate the presence of the metabolising enzymes. The availability of specific antibodies provided a sensitive method of detecting the cytochrome P450 metabolising enzymes. The technique of immunocytochemical staining using various cytochrome P450 antibodies (see Chapter 2. for the technique and the antibody characteristics) was used to identify the constitutive levels of cytochrome P450's in 5 day old cultures of LB and CNS cells. The amounts of cytochrome P450's in the cells following induction was also investigated. This chapter provides the first evidence for the presence and inducibility of cytochrome P450's in differentiating rat embryo cells IN VITRO.

4.1 CONSTITUTIVE AND INDUCIBLE LEVELS OF EMBRYONIC CYTOCHROME P450

4.1.1. IN VITRO INDUCTION

The highest non-cytotoxic concentrations (N.C.C) of PB, 3MC and BNF IN VITRO were found by the method described in Chapter 2.2.1. (Table 4.1.). LB and CNS cells were coincubated over the 5 day culture period in the absence and presence of each inducer and the presence of cytochrome P450 isoenzymes detected by the degree of peroxidase staining (signified by brown coloured stain) with haematoxylin counterstain (blue) against the different antibodies was scored visually. The scoring system was as follows, 0 = absent, (+) = less than minimal, + = minimal, ++ = moderate, +++ = strong.
Table 4.1.

**DOSSING LEVELS FOR INDUCERS BOTH IN VITRO AND IN VIVO**

<table>
<thead>
<tr>
<th>INDUCER</th>
<th>CONCENTRATION ((\mu g/ml))</th>
<th>PROTOCOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN VITRO HIGHEST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NON CYTOTOXIC</td>
<td>IN VIVO DOSSING</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>250</td>
<td>0.1% (W/V) in drinking water during days 4-13 of gestation</td>
</tr>
<tr>
<td>3MC</td>
<td>0.5</td>
<td>25 mg/kg i.p. on day on days 10-12 of gestation</td>
</tr>
<tr>
<td>(\beta)NF</td>
<td>2.5</td>
<td>35 mg/kg i.p. day on days 10-12 of gestation</td>
</tr>
</tbody>
</table>
Table 4.2.a shows the results obtained from untreated cells and cells coincubated IN VITRO with either PB, 3MC or βNF. Results indicated that there was a minimal constitutive level of the cytochrome P448 isoenzymes in both LB and CNS cells. This level was induced to moderate level in CNS cells by both 3MC and βNF. In LB cells the level was moderately inducible by 3MC and strongly inducible by βNF, as gauged against all 3 P448 antibodies. Fig 4.1 illustrates the comparison between a. untreated cells, pre-immune serum  b. constitutive level  c. 3MC induced and  d. βNF induced for LB cells against the P450c antibody. Fig 4.2 shows the same comparison of treatments against the same antibody but forembryonic CNS cells. Fig 4.3 shows the same comparison of IN VITRO induction with the inducers in LB cells stained against the CTL/Hammersmith 3:4:2 monoclonal antibody and Fig 4.4 the same comparison with 3:4:2 antibody but for CNS cells.

CNS and LB cells both showed a moderate constitutive level of the PB-type P450 isoenzyme but this was not inducible. Fig 4.5 illustrates the constitutive level of P450 (picture b) and the level following coincubation with PB IN VITRO (4.5.c) in LB cells stained against the Surrey-derived PB P450b antibodies. Fig 4.6 shows the same but with CNS cells.

Both constitutive and inducible cytochrome P450's were localised around the foci of differentiated chondrocytes (LB) and neurones (CNS) in the cells that were differentiating; very little P450 was detected in the cells that had differentiated. This implies that teratogen bioactivation must therefore be undertaken only by susceptible differentiating cells.
### CONSTITUTIVE AND IN VITRO INDUCIBLE LEVELS OF EMBRYONIC CYTOCHROME P450's

<table>
<thead>
<tr>
<th>Cytochrome Type</th>
<th>12(N) Antibody to</th>
<th>( \beta_{NF} )</th>
<th>Unrelated</th>
<th>PB</th>
<th>3MC</th>
<th>PB</th>
<th>3MC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>LB</td>
<td>CNS</td>
<td>LB</td>
<td>CNS</td>
<td>LB</td>
</tr>
<tr>
<td>( P_{448} )</td>
<td>CTL/Hammersmith 3:4:2 ( ^b )</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( P_{448} )</td>
<td>R. Wolf, 3MC1 ( ^c )</td>
<td>+</td>
<td>(4)</td>
<td>/</td>
<td>+</td>
<td>(4)</td>
<td>+</td>
</tr>
<tr>
<td>( P_{448} )</td>
<td>R. Wolf, 3MC2 ( ^c )</td>
<td>+</td>
<td>+</td>
<td>/</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( P_{450} )</td>
<td>Surrey PB - P450 ( ^d )</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>( P_{450} )</td>
<td>R. Wolf, PB1 ( ^e ) ( ^f )</td>
<td>(4)</td>
<td>O</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>( P_{450} )</td>
<td>R. Wolf, PB2 ( ^e ) ( ^f )</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>( P_{450} )</td>
<td>R. Wolf, PB3 ( ^e ) ( ^f )</td>
<td>(4)</td>
<td>O</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

\( O = \) absent, (4) = barely present, + = minimal, ++ = moderate, +++ = strong

---

**a.** The antibodies to cytochrome P450 isoenzymes were derived from three separate laboratories. Dr G Gibson, University of Surrey, Dr C R Wolf, University of Edinburgh and Prof. D Davies, R.P.M.S., Hammersmith, London, as indicated in Table 2.1.

**b.** A monoclonal antibody derived from \( \beta_{NF} \) induced mouse.

**c.** Polyclonal antibody derived from 3MC induced Sprague Dawley rat liver microsomes.

**d.** Polyclonal antibody derived from PB induced Wistar rat liver microsomes.

**e.** Polyclonal antibody derived from PB induced Sprague Dawley rat liver microsomes.

**f.** It should be noted that no consistent nomenclature P450, and the mixed nomenclature is that of CTL/Hammersmith, Wolf et al. 1984, Ryan et al. 1982, and Gibson et al. 1982. Where possible, a consistent nomenclature is given in parenthesis according to Levin et al. 1978.
CONSTITUTIVE AND IN VITRO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448 IN LB CELLS (VS: P450 'c' ANTIBODY)

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, minimal ++)

c. cells co-incubated with 3MC (moderate +++)

d. cells co-incubated with BNF (strong ++++)
CONSTITUTIVE AND IN VITRO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448 IN CNS CELLS (VS P450 c' ANTIBODY)

a. untreated cells, pre-immune sera
b. untreated cells (constitutive level, minimal+)
c. cells co-incubated with 3MC (minimal+)
d. cells co-incubated with βNF (moderate, ++)
CONSTITUTIVE AND IN VITRO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448 IN LB CELLS (VS CTL/HAMMERSMITH 3:4:2 ANTIBODY)

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, minimal ±)

c. cells co-incubated with 3MC (minimal ±)

d. cells co-incubated with βNF (moderate, ++)
CONSTITUTIVE AND IN VITRO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448
IN CNS CELLS (VS CTL/HAMMERSMITH 3:4:2 ANTIBODY)

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, minimal+)

c. cells co-incubated with 3MC (minimal+)

d. cells co-incubated with βNF (moderate, ++)
LEVELS OF PB-TYPE CYTOCHROME P450 IN UNTREATED AND INDUCED LB CELLS (VS SURREY-DERIVED P450 ANTIBODY)

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, P450, moderate)

c. cells co-incubated with PB in vitro (moderate)

d. in utero exposure to PB (moderate)
LEVELS OF PB-TYPE CYTOCHROME P450 IN UNTREATED AND INDUCED CNS CELLS (VS SURREY- DERIVED P450 h ANTIBODY)

a. untreated cells, pre-immune sera

b. untreated cells
   (constitutive level, P450, moderate ++)

c. cells co-incubated with PB in vitro
   (moderate ++)

d. in utero exposure to PB (moderate, ++)
4.1.2. TRANSPLACENTAL INDUCTION

The maternal dosing regimen for transplacental induction of cytochrome P450's is described in Chapter 2.2.3. (values are given in Table 4.1) and was known to induce maternal cytochrome P450, as gauged by increased peroxidase staining of the maternal liver.

13 day embryos from either untreated or pre-induced mothers were explanted and LB and CNS cell cultures prepared; the embryos therefore effectively received an IN UTERO exposure to the various inducers. After a subsequent 5 day culture period cells were processed by the immunocytochemical staining technique (Chapter 2.2.5.) against the various cytochrome P450 antibodies and scored for intensity of peroxidase stain. The code for scoring the degree of stain intensity is described in section 4.1.1. and is given in Table 4.2.b along with the scoring results.

The results showed a minimal constitutive level of the cytochrome P448 isoenzyme in both cell types and that this level was moderately inducible in LB cells (but not CNS) by 3MC. However, the levels were strongly induced in both CNS and LB cells by bNF. Fig 4.7 illustrates the comparison between a. untreated cells, pre-immune sera, b. constitutive level of cytochrome P448, c. 3MC induced and d. bNF induced LB cells against CTL/Hammersmith 3:4:2 antibody. Fig 4.8 shows the same comparison but for CNS cells. Fig 4.9 shows the same comparison of pre-treatment with LB cells stained against P450C antibody and Fig 4.10 the same but for CNS cells.

There was a moderate constitutive level of the phenobarbitone-type cytochrome P450 present in cultures of both untreated LB and CNS cells, however this level was not increased by a prior IN UTERO
Table 4.2.b

CONSTITUTIVE AND IN UTERO INDUCIBLE LEVELS OF

EMBRYONIC CYTOCHROME P450'S

<table>
<thead>
<tr>
<th>Cytochrome Type</th>
<th>Antibody to P450 Antigen</th>
<th>Untreated</th>
<th>PB</th>
<th>3MC</th>
<th>BNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>CNS</td>
<td>LB</td>
<td>CNS</td>
</tr>
<tr>
<td>P445 b</td>
<td>CTL/Hammersmith 3:4:2 b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P448 b</td>
<td>R. Wolf, 3MC1 (Levin d) b</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>P448 b</td>
<td>R. Wolf, 3MC2 (Levin c) b</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>P450 b</td>
<td>Surrey PB - P450 (Levin b) c</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>P450 c</td>
<td>R. Wolf, PB1 (Wexman PB1) c</td>
<td>(+)</td>
<td>O</td>
<td>(+)</td>
<td>O</td>
</tr>
<tr>
<td>P450 c</td>
<td>R. Wolf, PB2 (PB2) c</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>P450 c</td>
<td>R. Wolf, PB3 (Levin b) c</td>
<td>(+)</td>
<td>O</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

*= absent, (+) = barely present, + = minimal, ++ = moderate, +++ = strong

a. Antibodies were the same as in Table 4.2.a. and footnotes a-f have the identical meaning again as given in Table 4.2.a.
CONSTITUTIVE AND IN UTERO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448 IN LB CELLS (VS CTL/HAMMERSMITH 3:4:2 ANTIBODY

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, P448, minimal+)

c. 3MC induced (minimal, +)

d. βNF induced (strong, +++)
CONSTITUTIVE AND IN UTERO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448

IN CNS CELLS (VS CTL/HAMMERSMITH 3:4:2 ANTIBODY

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, P448, minimal +)

c. 3MC induced (minimal, +)

d. βNF induced (strong, +++)
CONSTITUTIVE AND IN UTERO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448
IN LB CELLS (VS $^{P450 \text{c}'} \text{ antibody}$)

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, P448, minimal+)

c. 3MC induced (moderate, ++)

d. ßNF induced (moderate, ++)
CONSTITUTIVE AND IN UTERO INDUCIBLE LEVELS OF EMBRYO CYTOCHROME P448

IN CNS CELLS (VS P450 'c' ANTIBODY)

a. untreated cells, pre-immune sera
b. untreated cells
   (constitutive level, minimal, +)
c. 3MC induced
   (minimal, +)
d. BNF induced
   (moderate, ++)
exposure to PB. Fig 4.5.b. and d. shows the comparison between constitutive and PB IN UTERO exposed LB cells stained against the Surrey-derived P450b antibody, and Fig 4.6.b. and d. the same but with CNS cells.

Both constitutive and inducible cytochrome P450 was localised around the foci of differentiated chondrocytes (LB) and neurones (CNS) in the differentiating cells.

4.1.3 EFFECT OF DPH AND BENZIMIDAZOLE ON EMBRYO CYTOCHROME P450's

Chronic administration of DPH and benzimidazole have both been shown to cause induction of cytochrome P450 in rat liver. IN VITRO co-incubation of LB/CNS cells over a 5 day period with either DPH or benzimidazole did not increase the intensity of staining over the constitutive levels when stained against either Surrey-derived 450b or CTL/Hammersmith 3:4:2 antibodies. Fig 4.11 illustrates staining for a) untreated cells, pre-immune sera, b) constitutive, c) DPH co-incubation d) Benzimidazole co-incubation of LB cells vs. CTL/Hammersmith 3:4:2 antibody. Fig 4.12 illustrates the same treatments but with CNS cells.

4.2. IN VIVO AND IN VITRO COMPARISON OF EMBRYONIC CYTOCHROME P450's AT DAY 18 OF DEVELOPMENT

Rat embryos from untreated mothers were explanted on day 18 of gestation, as this corresponds to the stage of development at the end of the culture period (for the IN VITRO system embryos were explanted on day 13 of gestation and cultured for 5 days, giving a total of 18 days of development). Embryo mid-brain and fore-limbs were removed
LEVELS OF CYTOCHROME P448 IN UNTREATED AND VARIOUSLY CO-TREATED LB CELLS (VS. CTL/HAMMERSMITH 3:4:2 ANTIBODY)

- a. untreated cells, pre-immune sera
- b. untreated cells (constitutive level, P448, minimal, 4)
- c. Co-incubated with DPH (minimal, 4)
- d. Co-incubated with benzimidazole (minimal 4)
LEVELS OF CYTOCHROME P448 IN UNTREATED AND VARIOUSLY CO-TREATED
CNS CELLS (VS. CTL/HAMMERSMITH 3:4:2 ANTIBODY)

a. untreated cells, pre-immune sera

b. untreated cells (constitutive level, P448, minimal, 4)

c. Co-incubated with DPH (minimal, 4)

d. Co-incubated with benzimidazole (minimal 4)
and placed immediately in liquid nitrogen, cryostat sections were then cut and the sections stained against either Surrey-derived-P450c or CTL/Hammersmith 3,4,2 antibodies. Fig 4.13 shows the presence of both major forms of cytochrome P450 in day 18 embryo, at constitutive levels similar to those from IN VITRO CNS/LB cells. (compare to Figs 4.3 b/4.4 b and Fig 4.5 b/4.6 b) The localisation of cytochrome P450's was in the perichondrial tissue in both day 18 embryos and cultured LB cells (indicated by arrows). There also appeared to be some cytochrome P450 present in the myogenic tissue from day 18 embryos (Fig 4.13 a and c). Mid-brain sections revealed the presence of cytochrome P450's in the connective tissue of the brain and in neurofibrils which correlated to the localisation in CNS cells cultured IN VITRO (Fig 4.13 b and d). The phenobarbitone-type isoenzyme of cytochrome P450 was present in both tissues at a higher level than the P448 isoenzyme (as gauged by intensity of staining) which correlates with the IN VITRO observation (Table 4.2, moderate ++ for PB-type P450 and minimal + for P448). The untreated sections coincubated with pre-immune sera were completely devoid of brown staining (data not shown).

4.3 DEVELOPMENTAL PROFILE OF EMBRYO CYTOCHROME P450'S IN VITRO

Embryo cells that had been cultured for 0, 1, 2, 3, 4 and 5 days were stained against Surrey-derived-P450c and CTL/Hammersmith antibodies and the day on which staining was first detected (and the corresponding intensity) was recorded (Table 4.3 a).

Results showed that the phenobarbitone-type cytochrome P450 first appeared on day 1 of culture and plateaued on day 5 of culture for
LOCALISATION OF CYTOCHROME P450's IN LB AND CNS SECTIONS FROM DAY 18 EMBRYOS

a. Fore-limb section (VS Surrey P450b) (VS CTL/Hammersmith 3:4:2)

b. Fore-limb section

(c. Mid-brain section (VS Surrey PB-P450)

d. Mid-brain section (VS CTL/Hammersmith 3:4:2)
### Developmental Onset of Cytochromes P450 In Vitro

**TABLE 4.3 a**

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Days in culture for LB/CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Surrey-derived P450 b'</td>
<td>−</td>
</tr>
<tr>
<td>CTL / Hammersmith 3 : 4 : 2</td>
<td>−</td>
</tr>
</tbody>
</table>

**Effect of βNF in Utero Induction on Developmental Pattern of P448 Appearance in Vitro**

**TABLE 4.3 b**

<table>
<thead>
<tr>
<th>Treatment vs: 3 : 4 : 2 Ab.</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>βNF In Utero 35mg/kg, i.p., D. 10 – 12 gestation</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Code: − = absent, (+) = present, + = minimal, ++ = moderate, +++ = massive
both LB and CNS cells. The cytochrome P448 isoenzyme was first detected on day 3 of culture and reached a minimal (+) level on day 5 of culture (for both LB and CNS cells).

IN UTERO exposure to pNF did not cause a precocious development of cytochrome P448 (Table 4.3 b) but did increase the intensity of staining (minimal to moderate) over days 3-5 of culture for both cell types.

4.4 IMMUNOLOGICAL IDENTIFICATION OF EMBRYO CYTOCHROME P450'S BY WESTERN BLOTTING

The method of Western blot is described in Chapter 2.2.6 5 day old cultures of LB and CNS cells derived from either untreated or pNF induced mothers were pooled, homogenised and an aliquot electrophoresed, transferred to nitrocellulose paper and then stained against either a) Surrey derived-P450c antibody b) P450d antibody or c) Wolf 3MC2 antibody. Samples were then post-labelled with 125 I-labelled protein A and autoradiographs produced. The results are shown in Fig 4.14. The first track, LB cells from untreated mothers, the second track LB cells from pNF induced mothers, the third CNS cells from untreated mothers, the fourth CNS cells from pNF induced mothers and the fifth track (far right) corresponds to appropriately induced rat liver microsomes against which each antibody was directed. Results indicate that there was a high level of non-specific binding (probably due to high protein loading) but it was possible to demonstrate the presence of bands of embryonic proteins (cytochrome P450) corresponding to the major induced rat liver microsomal P450's (indicated by arrow). The untreated and pNF induced
WESTERN BLOT IDENTIFICATION OF EMBRYO CYTOCHROME P450's IN UNTREATED AND βNF TRANSPLACENTALLY INDUCED LB AND CNS CELLS

a-(vs Surrey PB-P450) b-(vs Wolf 3MC₁) c-(vs Wolf 3MC₂)

Code: LC = untreated LB cells  LB = βNF induced LB cells
      CC = untreated CNS cells  CB = βNF induced CNS cells
LB and CNS cells showed a uniform level of PB-P450 (vs Surrey PB-P450 antibody) that was unaffected by either cell type or induction (Fig 4.14 a). However both βNF induced LB and CNS cells showed a greater degree of staining intensity than cells derived from untreated mothers when stained against either P450c or P450d antibodies (Fig 4.14 b and c).

4.5 DISCUSSION

Results from this Chapter have shown that rat embryo cells have a) constitutive levels of cytochrome P448, and b) it was inducible by 3MC and βNF (by both immunocytochemical staining and increased levels of P448 protein detected by Western blotting) c) trans-placental βNF induction was more potent than IN VITRO co-incubation with βNF. d) DPH and benzimidazole do not act as inducers e) the IN VIVO distribution pattern and levels of cytochrome P450's are paralleled IN VITRO f) there was developmental regulation of the appearance of cytochrome P450's IN VITRO g) βNF does not cause a precocious development of cytochrome P448 but increases the levels.

Immunocytochemical evidence for the constitutive and non-inducible levels of PB-type cytochrome P450 show an anomaly (Table 4.2), in that Surrey PB-P450 which is formalised as Levin 'b' form parallels the observed staining intensity as Wolf PB2, not Wolf PB3, which is also formalised as Levin 'b' form. An unsuccessful attempt was made to distinguish the cross reactivity of all 3 antibodies by Ouchterlony double diffusion technique, so that it remains in unknown which antibody is the true Levin 'b' or which is a mixture or alternatively they are products of strain differences to PB induction.
Despite this anomaly all 4 PB-P450 antibodies showed the same result; LB and CNS cells have a constitutive level of PB-type P450 that was not inducible by PB co-incubation either IN UTERO or IN VITRO. However embryo cytochrome P448, which at a constitutive level was not as abundant as cytochrome P450, was found to be inducible by both 3MC and βNF. This isoenzyme was inducible by both transplacental exposure to inducers or by coincubation IN VITRO with inducers at non-cytotoxic concentrations. All three P448 antibodies showed that whilst 3MC induction IN VITRO was as potent as an IN UTERO exposure, for βNF this was not so. IN UTERO exposure caused a greater staining intensity (vs. CTL/Hammersmith 3:4:2 antibody). This difference in inducibility by BNF after IN UTERO exposure indicates either a) that maternally mediated metabolism of βNF possibly enhanced its induction potential in embryo cells or b) a higher dose of βNF was delivered to the embryos after IN UTERO exposure. For 3MC, maternal metabolism apparently did not appear to be an important factor for its induction potential. The observation that embryo cytochrome P450 was non-inducible whilst cytochrome P448 was, is reflected in the literature; fetal rat hepatocytes were found to increase substrate turnover only when exposed to cytochrome P448 inducers (Worell 1984, Cresteil et.al. 1984, Mizokami et.al. 1982). In addition, mouse limb bud organ cultures could activate cyclophosphamide after IN UTERO exposure to Aroclor 1254 but were refractive with PB pretreatment (Neubert and Bluth 1981). Furthermore, whole rat embryos could activate 2-acetyl aminofluorene after 3MC pretreatment but not after PB treatment (Juchau et.al. 1985).
The antibodies that were directed against PB induced rat liver microsomal cytochrome P450 showed differences in the constitutive level of the haemoprotein in both cell types and this difference may have been due to each antibody recognising different epitopes on the same protein. The antibodies that were directed against βNF-inducible P448 (CTL/Hammersmith 3;4,2) and 3MC-inducible P448 (P450c and P450d) all showed a similar low constitutive level of the cytochrome P448 isoenzyme in LB and CNS cells. However induction by either IN UTERO or IN VITRO exposures showed that LB cells consistently contained more of these cytochrome P448 isoenzyme than CNS cells i.e. by all 3 antibodies. There also appeared to be little difference in either constitutive or induced levels scores and this indicates that the antibodies may be recognising the same or a very similar epitope on the cytochrome P448 proteins.

The observation that both IN VITRO and IN VIVO localisation of embryo cytochrome P450's appeared to be mainly in the differentiating cells around either the differentiated foci of chondrocytes (LB) or neurones (CNS) IN VITRO and perichondrial tissue (LB) or connective tissue (CNS) IN VIVO would tend to indicate that cytochrome P450b has an endogenous role in differentiation because of its localisation and lack of inducibility by PB. However the developmental regulation of each isoenzyme showed that cytochrome P450B appeared first (day 1 of culture) reaching a maximum on day 4 of culture and cytochrome P450c appeared later (day 3 of culture) reaching its maximum on day 5. However, cytochrome P450b attained a higher level as gauged by staining intensity. If the role of
cytochrome P450's was solely in differentiation of embryo cells it would be expected that the enzyme would be moderately active at a relatively early stage in culture. It is possible that the non-inducible PB-type cytochrome P450's have their major role in differentiation of cells. criteria. However that the cytochrome P448 isoenzyme (cytochrome P450c) appeared later in culture and was shown to be inducible, though no precocious development was observed by βNF induction, may indicate that the role of this isoenzyme is different from that of the PB-type cytochrome P450. It is postulated that this isoenzyme form (cytochrome P450c) is the active form of embryo cytochrome P450's capable of xenobiotic metabolism and that will be presented in subsequent Chapters to substantiate this conclusion.
CHAPTER 5.

MODULATION OF CYTOCHROME

P450 ACTIVITY IN EMBRYO CELLS

Contents

5.1 COMPARATIVE EFFECTS OF DPH AND ITS METABOLITES ON IN VITRO DIFFERENTIATION OF EMBRYO CELLS

5.2 EFFECT ON IN VITRO DPH RESPONSE BY COINCUBATION WITH INHIBITORS OF DRUG METABOLISM

5.3 MODULATION OF THE IN VITRO RESPONSE OF DPH AND CYCLOPHOSPHAMIDE BY INDUCERS.
   5.3.1 COINCUBATION WITH INDUCERS.
   5.3.2 MODULATION OF THE IN VITRO RESPONSE OF DPH AND CPA FOLLOWING TRANSPLACENTAL INDUCTION.

5.4 DISCUSSION
5. MODULATION OF CYTOCHROME P450 ACTIVITY IN EMBRYO CELLS

These sets of experiments were designed to show that the embryo cytochrome P450's identified immunocytochemically in Chapter 4. were functional in metabolising xenobiotics. It was also hoped that by modulation of the levels of embryo cytochrome P450's by either inhibition or induction, that ultimately the effect of DPH and CPA on rat embryo cell differentiation IN VITRO could also be modulated thus giving evidence for the role of embryo cytochrome P450's in chemically induced teratogenesis.

5.1 COMPARATIVE EFFECTS OF DPH AND ITS METABOLITES ON IN VITRO DIFFERENTIATION OF EMBRYO CELLS

DPH, its major hydroxy metabolite p.hydroxy.DPH, its minor hydroxy metabolite m.hydroxy.DPH and the ring opened acid metabolite diphenylhydantoic acid were added directly to the culture medium at the beginning of the culture period and left for five days with either LB or CNS cells. Concentration-response curves were prepared and the relative IC50s calculated (concentration at which compound inhibits differentiation by 50%). These results are summarised in Table 5.1, and show that DPH and its two hydroxy metabolites were equitoxic to LB and CNS cells IN VITRO. However the ring opened acid metabolite, formed by a non-P450 mediated reaction IN VIVO, was totally non-inhibitory to cells up to its maximum solubility. Furthermore the results showed that both DPH and the p.hydroxy metabolite were significantly (p<0.001 and p<0.01 respectively) more toxic to the CNS cells than LB cells.
Table 5.1

### COMPARATIVE EFFECTS OF DPH AND ITS MAJOR METABOLITES ON CNS AND LB CULTURESa IN VITRO

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>IC50 CNS (µg/ml)</th>
<th>IC50 LB (µg/ml)</th>
<th>NUMBER OF EXPERIMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>67 ± 18</td>
<td>91 ± 4b</td>
<td>9</td>
</tr>
<tr>
<td>p.hydroxy DPH</td>
<td>54 ± 11a</td>
<td>90 ± 9ab</td>
<td>6</td>
</tr>
<tr>
<td>m.hydroxy DPH</td>
<td>79 ± 8a</td>
<td>84 ± 9a</td>
<td>6</td>
</tr>
<tr>
<td>DPH acid</td>
<td>&gt; 250c</td>
<td>&gt;250c</td>
<td>6</td>
</tr>
</tbody>
</table>

a There were no significant differences between the IC50 of DPH and its major hydroxy metabolites in CNS or LB.

b Significant differences (p < 0.001 and < 0.01 respectively) were found between the IC50's of DPH and p.hydroxy DPH in LB cultures compared to CNS.

c Maximum solubility in culture medium.
5.2 EFFECT ON DPH RESPONSE IN VITRO BY COINCUBATION WITH INHIBITORS OF DRUG METABOLISM

The highest non-cytotoxic concentrations (NCC) found for the cytochrome P450 inhibitors are listed in Table 5.2. Inhibitors were coincubated at their N.C.C. with different concentrations of DPH over the five day culture period and IC50's calculated in CNS and LB cultures as before. Figure 5.1 shows, for a representative experiment, the different concentration response curves obtained from co-incubating DPH in the presence and absence of cimetidine with LB cells. In the presence of cimetidine there was a shift to the left indicating a more toxic response to DPH. Table 5.2 summarises the values obtained for LB and CNS IC50's ± standard error of mean. The percentage changes in toxicity of DPH with the inhibitors are represented graphically in Figure 5.2. The results showed that benzimidazole, ellipticine, aspirin and metyrapone were the most potent inhibitors to both LB and CNS cells, significantly increasing the toxicity of DPH by 67-82% in LB cells and 41-52% in CNS cells. a-naphthoflavone was a strong inhibitor to CNS cells increasing toxicity by 42% and only acted as a weak inhibitor in LB cells increasing toxicity by 22%. Cimetidine and SKF 525A whilst increasing toxicity by 15 and 3% respectively in CNS cells were found not to be statistically significant, however both compounds significantly increased DPH toxicity in LB cells by 48 and 13% respectively.

No significant differences were found in the DPH IC50 values with LB or CNS when coincubated with either ranitidine (Figure 5.3), sucrose or inosine (Table 5.2). No modulation of the IC50 values for the p-hydroxy. DPH metabolite was observed following coincubation with
Table 5.2
MODULATION OF DIPHENYLHYDANTOIN TOXICITY IN VITRO

| COMPOUND COINCUBATED WITH DPH | CNS CULTURES | | | LB CULTURES | | | |
|-------------------------------|---------------|------------------|------------------|------------------|
| | NON-CYTOTOXIC LEVEL (µg/ml) | IC50 DPH + COMPOUND COINCUBATED (µg/ml) | % INCREASE IN TOXICITYb | NON-CYTOTOXIC LEVEL (µg/ml) | IC50 DPH + COMPOUND COINCUBATED (µg/ml) | % INCREASE IN TOXICITYb |
| DPH alone | - | 67 ± 18 | - | - | 91 ± 4 | - |
| SKF 525A (SKF) | 3 | 64 ± 2 | 3 ± 1 | 3 | 79 ± 1x | 13 ± 2 |
| α-NAPHTHOFLAVONE (αNF) | 4 | 42 ± 5x | 37 ± 5 | 4 | 71 ± 9x | 22 ± 10 |
| CIMETIDINE (CIM) | 400 | 57 ± 9 | 15 ± 10 | 400 | 47 ± 3x | 48 ± 3 |
| METYRAPONE (MET) | 250 | 39 ± 8x | 41 ± 8 | 250 | 30 ± 4x | 67 ± 5 |
| ASPIRIN (ASP) | 50 | 36 ± 6x | 47 ± 8 | 50 | 28 ± 6x | 69 ± 6 |
| ELLIPTICINE (ELL) | 0.001 | 32 ± 2x | 52 ± 2 | 0.001 | 27 ± 2x | 70 ± 2 |
| BENZIMIDAZOLE (BEN) | 200 | 39 ± 6x | 42 ± 6 | 200 | 16 ± 6x | 82 ± 6 |
| SUCROSE | 600 | 64 ± 5 | 3 ± 4 | 600 | 90 ± 3 | 1 ± 3 |
| INOSINE | 500 | 66 ± 6 | 1 ± 6 | 500 | 89 ± 5 | 2 ± 5 |
| RANITIDINE | 750 | 65 ± 6 | 3 ± 6 | 750 | 93 ± 2 | 2 ± 2 |

* Mean and standard deviation from three experiments
* = significantly different from DPH IC50 (p<0.001, students t.test)
REPRESENTATIVE EXPERIMENT SHOWING THE
EFFECT OF CIMETIDINE ON THE CONCENTRATION-RESPONSE
OF DPH ON LB CELLS IN VITRO

Number of differentiated foci vs. µg diphenylhydantoin / ml culture medium

- IC50: 39 µg/ml
- IC50: 87 µg/ml

○ + cimetidine (400 µg/ml)
○ - cimetidine

C50: 39 µg/ml C50: 87 µg/ml
FIGURE 5.2

EFFECT ON THE TOXICITY OF DIPHENYLHYDANTOIN BY COINCUBATION WITH CYTOCHROME P450 INHIBITORS

\[
\% \text{ INCREASE IN TOXICITY} = \left( \frac{\text{IC50 DPH + inhibitor}}{\text{IC50 DPH alone}} \right) \times 100 - 100
\]

\( a \) = Inhibitors are abbreviated as in Table 5.2

\( x \) = \( p < 0.001 \)

DPH alone is considered 100%. The percentage increase in toxicity was therefore calculated by:

- Inhibitors are abbreviated as in Table 5.2
- \( a \) = Inhibitors are abbreviated as in Table 5.2
- \( x \) = \( p < 0.001 \)

\[
\% \text{ INCREASE IN TOXICITY} = \left( \frac{\text{IC50 DPH + inhibitor}}{\text{IC50 DPH alone}} \right) \times 100 - 100
\]
either α-naphthoflavone or cimetidine (Figure 5.4). The CNS cells were more sensitive than LB to p-hydroxy DPH even in the presence of the inhibitors (p<0.01) data not shown.

5.3 MODULATION OF THE IN VITRO RESPONSE OF DPH AND CYCLOPHOSPHAMIDE BY INDUCERS

5.3.1 COINCUBATION WITH INDUCERS

The highest non-cytotoxic concentrations for the inducers phenobarbitone (PB), 3-methylcholanthrene (3MC) and β-naphthoflavone were obtained in the same way as for inhibitors (see chapter 2.2.1.)

These values are listed in Table 5.3. Various concentrations of DPH were coincubated with N.C.C. levels of each inducer for 5 days with either LB or CNS cells. Results show that none of the inducers modulated the effect of DPH on CNS cells and only co-incubation with 3MC (not PB nor β.NF) could modulate the effect of DPH on LB cells. 3MC slightly increased the toxic effect of DPH IN VITRO (21% ± 8).

Cyclophosphamide (CPA) was totally non-inhibitory to either LB or CNS cells up to its maximum solubility (µg/ml) in the presence of any of the inducers, (data not shown).

5.3.2. MODULATION OF THE IN VITRO RESPONSE OF DPH AND CPA FOLLOWING TRANSPLENTAL INDUCTION

Results from the transplacental passage of radio-labelled 14C-PB and 14C-3MC show that radioactivity was present in the embryos. (Experimental procedure described in Chapter 2.2.3). Table 5.4 summarises the data and shows the percentage of the maternal dose in the embryos, and clearly demonstrates that both of these inducers of
Figure 5.3

EFFECT OF RANITIDINE ON THE IN VITRO RESPONSE
OF DPH WITH LB CELLS

\[
\begin{align*}
\text{IC50 : 95 } \mu g/ml \\
\text{IC50 : 92 } \mu g/ml
\end{align*}
\]

\( \mu g \) diphenylhydantoin/ ml culture medium

\text{a Representative experiment i.e. 1 of 3}
Figure 5.4

EFFECT OF CIMETIDINE ON THE IN VITRO RESPONSE OF
P. HYDROXY DPH WITH LB CELLS

- + cimetidine (400µg/ml)
- cimetidine

IC50: 79 µg/ml
IC50: 77 µg/ml

µg p. hydroxy DPH / ml culture medium

a Representative experiment i.e. 1 of 3
Table 5.3

MODULATION OF THE IN VITRO RESPONSE OF DPH BY COINCUBATION WITH INDUCERS IN VITRO

<table>
<thead>
<tr>
<th>INDUCER</th>
<th>CONCENTRATION (μg/ml)</th>
<th>DPH. IC50a (pg/ml)</th>
<th>DPH. IC50a (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NON-CYTOTOXIC</td>
<td>DPH. IC50a</td>
<td>DPH. IC50a</td>
<td></td>
</tr>
<tr>
<td>INDUCER</td>
<td>CONCENTRATION (μg/ml)</td>
<td>LB</td>
<td>CNS</td>
</tr>
<tr>
<td>DPH ALONE</td>
<td>-</td>
<td>91±4</td>
<td>67±18</td>
</tr>
<tr>
<td>PHENOBARBITONE</td>
<td>250</td>
<td>81±7</td>
<td>62±5</td>
</tr>
<tr>
<td>3-METHYLCHOLANTHRENE</td>
<td>0.5</td>
<td>70±12b</td>
<td>59±2</td>
</tr>
<tr>
<td>β-NAPHTHOFLAVONE</td>
<td>2.5</td>
<td>82±10</td>
<td>65±5</td>
</tr>
</tbody>
</table>

a = mean and standard error where n = 3 experiments.

b = Significantly different from DPH alone (p<0.02), 21% increase in toxicity.
drug metabolising enzymes can cross the placenta and reach the embryo. However the 14C radioactivity represents both parent compound and metabolite(s), given more time the proportion and identification would have been desirable. Neither 14C-PB nor 14C-3MC equilibrate with the embryo (ratio of maternal plasma to embryo < 1) but levels indicate exposure.

The dosing protocols for PB, 3MC and βNF are fully described in chapter 2.2.4. and doses were found not to impair either reproductive ability or cause any discernable embryo abnormalities. Results of the modulation of the IN VITRO response of DPH in the presence of LB or CNS cells derived from pre-induced mothers are summarised in Table 5.5. DPH IC50 was not modulated by CNS cells derived from any transplacentally induced embryos, however LB cells from either β-NF or 3MC pretreated dams showed an increased toxic response to DPH (30%±10 and 20%±5 respectively). Phenobarbitone was without effect.

Coincubation of CPA with either LB or CNS cells derived from the variously pretreated dams showed that only LB cells derived from β-NF pretreated mothers had the ability to activate CPA and hence show a toxic response. LB cells from 3MC or PB pretreated dams showed no response as did CNS cells from all 3 pretreatments, (data not shown). Figure 5.5 shows the dose response of LB cells from β-NF pretreated dams with CPA. The recorded IC50 was 44 µg/ml. This experiment was repeated twice more and confirmed that only LB cells derived from β-NF pretreated dams could activate CPA, however there was a large variability in IC50 values (12, 44 and 99 µg/ml), Table 5.6.
Table 5.4

TRANSPLACENTAL PASSAGE OF RADIOLABELLED INDUCERS

A.  

<table>
<thead>
<tr>
<th>PREGNANT ANIMAL</th>
<th>MATERNAL PLASMA CONCENTRATIONS (ng$^{14}$C-PB/ml)</th>
<th>EMBRYOS (ng$^{14}$C-PB/mg)</th>
<th>DISTRIBUTION RATIO EMBRYO/MOTHER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.32</td>
<td>0.96</td>
<td>0.1030</td>
</tr>
<tr>
<td>2</td>
<td>11.14</td>
<td>1.08</td>
<td>0.0969</td>
</tr>
<tr>
<td>3</td>
<td>9.23</td>
<td>0.81</td>
<td>0.0877</td>
</tr>
<tr>
<td>4</td>
<td>8.09</td>
<td>0.44</td>
<td>0.0543</td>
</tr>
</tbody>
</table>

Dams recieved 0.1% $^{14}$C-PB ($\approx 0.75 \mu$Ci/day) in the drinking water for 10 days (gestational days 4-13) and were killed at 8.00 am on day 13 of gestation.

B.  

<table>
<thead>
<tr>
<th>PREGNANT ANIMAL</th>
<th>MATERNAL PLASMA CONCENTRATIONS (ng$^{14}$C-3MC/ml)</th>
<th>EMBRYOS (ng$^{14}$C-3MC/mg)</th>
<th>DISTRIBUTION RATIO EMBRYO/MOTHER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.72</td>
<td>15.45</td>
<td>0.00041</td>
</tr>
<tr>
<td>2</td>
<td>1.67</td>
<td>6.15</td>
<td>0.00037</td>
</tr>
<tr>
<td>3</td>
<td>1.64</td>
<td>28.90</td>
<td>0.00176</td>
</tr>
</tbody>
</table>

Dams received 25 mg/kg/ day ($\approx 3.3 \mu$Ci/animal/day) i.p. dose for 3 days (gestational days 10-12) and were killed at 8.00 am on day 13 gestation. i.e. 24 hours after the last dose.
Table 5.5

MODULATION OF THE IN VITRO RESPONSE OF DPH
BY TRANSPLACENTALLY ADMINISTERED INDUCERS

<table>
<thead>
<tr>
<th>INDUCER</th>
<th>DOSING REGIMEN</th>
<th>DPH.IC50 (µg/ml)</th>
<th>DPH.IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>91±4</td>
<td>67±18</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>0.1% DRINKING</td>
<td>90±14</td>
<td>65±6</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>WATER D4-13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>25 mg/kg i.p.</td>
<td>73±5</td>
<td>57±3</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>D 10-12</td>
<td>(20*)</td>
<td></td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>35 mg/kg i.p.</td>
<td>64±12</td>
<td>56±5</td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>D 10-12</td>
<td>(30*)</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean and standard error where n = 3 expts'.

Figures in parenthesis are % increase in toxicity

* significantly different from DPH alone (p < 0.001); students t.test.
### Table 5.6

#### EFFECT OF INDUCERS ON CPA ACTIVATION IN VITRO

<table>
<thead>
<tr>
<th>Inducer</th>
<th>CPA IC50</th>
<th>CPA IC50</th>
<th>Inducer</th>
<th>CPA IC50</th>
<th>CPA IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN VITRO</td>
<td>CNS</td>
<td>LB</td>
<td>IN VIVO</td>
<td>CNS</td>
<td>LB</td>
</tr>
<tr>
<td>µg/ml</td>
<td>µg/ml</td>
<td>µg/ml</td>
<td>µg/ml</td>
<td>µg/ml</td>
<td></td>
</tr>
<tr>
<td>CPA alone</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>CPA alone</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>PB</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>PB</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>3MC</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>3MC</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>βNF</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>βNF</td>
<td>&gt; 1000</td>
<td>99,44,12</td>
</tr>
</tbody>
</table>
Figure 5.5

REPRESENTATIVE EXPERIMENT OF CYCLOPHOSPHAMIDE

ACTIVATION BY β-NAPHTHOFLAVONE PRETREATED LB CELLS

![Graph showing the effect of Cyclophosphamide on the number of differentiated foci. The graph shows a decrease in the number of foci as the concentration of Cyclophosphamide increases. The IC₅₀ is marked as 44 μg/ml, and (3MC + PB no effect) is noted.]
5.4 DISCUSSION

In summary results from this chapter have shown that modulation of cytochrome P450 activity in embryo cells by use of inhibitors IN VITRO and inducers IN VITRO and IN UTERO can subsequently modulate the IN VITRO response of DPH in both LB and CNS cells and CPA in LB cells.

Firstly, I have shown that, of the 3 metabolites tested IN VITRO only DPH acid was totally non-inhibitory to differentiating rat embryo cells. Both hydroxy-metabolites and DPH itself were approximately equitoxic, and it would therefore appear that the only completely detoxifying pathway of the ones studied, is cleavage of the hydantoin ring and formation of the acid product.

It is interesting to note that after both m and p hydroxylation of the phenyl ring of DPH, a chiral centre is generated thus yielding sterochemically distinct hydroxylated metabolites. While this has been recognised as a general facet of metabolism of this class of compounds (Kaminsky et al. 1984) it must be emphasised that this aspect was not addressed in my studies and is certainly an area that should be addressed in future work related to the IN VITRO teratogenic potential of hydroxylated DPH metabolites.

Recent reports (Clapper et al. 1985, Clapper and Klein 1986) postulate that it is either a maternal serum drug-protein complex that is the ultimate teratogen or the catechol metabolite (Billings and Milton 1985) or a free radical intermediate derived from a prostaglandin synthetase mediated bioactivation (Wells et al. 1986) or the arene oxide intermediate derived from a cytochrome P450 mediated reaction (see Chapter 1.5.2.4 for references and experimental
evidence). Since these reactive intermediates are unstable it was not possible to look at their effect directly IN VITRO. By modulating the metabolism to the reactive species it was possible to identify the most potent compound. The study with DPH co-incubated with a wide variety of fairly specific and broad cytochrome P450 inhibitors showed that (without exception) DPH toxicity was increased in the presence of metabolic inhibitors. This increase in toxicity was not due to physico-chemical factors such as pH change, osmolality or excess solvent vehicle (as neither inosine nor sucrose, 2 ethanol soluble non-teratogens, altered the inhibitory effect of DPH). Ranitidine which is pharmacologically and structurally similar to cimetidine but without the P450 inhibitory properties (Bell et.al 1983, Watts et.al. 1982), was without effect on DPH IC50 IN VITRO (Figure 5.3) whereas cimetidine caused an increase in toxicity (15% CNS, 48% LB). This observation strongly suggests that the increased effects of DPH when coincubated with cytochrome P450 inhibitors is due entirely to the inhibition of metabolism by the cytochrome P450 system.

This study implies that differentiating rat embryo cells, especially the LB cells have the ability to metabolise DPH.

The fact that p-hydroxy.DPH still represents a substantial teratogenic hazard IN VITRO implies that hydroxylation is not a completely detoxifying reaction unlike hydantoin ring cleavage to DPH.acid.

Direct coincubation IN VITRO of DPH with the various cytochrome P450 inducers showed that only LB cells co-incubated with 3MC increased DPH toxicity, β-NF and PB were ineffective. CNS cells were refractory with all inducers. It would appear that the only compound
to act IN VITRO was 3MC and the only responsive tissue LB. The N.C.C. level of 3MC was 0.5 µg/ml compared to PB (N.C.C. 250 µg/ml) and β-NF (N.C.C. 2.5 µg/ml). That 3MC was able to modulate DPH toxicity at such a low N.C.C. level implies that it is a very powerful inducer.

IN VIVO studies of the transplacental passage of radiolabelled PB and 3MC have shown that both these inducers cross the placenta at levels comparable with other compounds e.g. 14C-DPH distribution ratio embryo/mother on day 11 gestation after a single i.p. dose in the rat was 0.1 (Stevens and Harbison 1975). 14C-PB reached a higher distribution ratio embryo/mother (0.103 - 0.0543) than 14C-3MC (0.00176 -0.00041). This observation may be a result of 14C-PB being administered ad libitum in the drinking water and therefore samples were from constant exposure as opposed to 14C-3MC samples which were taken 24 hours after the last i.p. dose. Therefore the actual distribution ratios may have been more alike had sampling and dosing regimens been identical. Embryo cells from PB-induced dams could neither modulate DPH toxicity IN VITRO nor activate CPA. That PB was present in the embryos after maternal administration but was still unable to modulate teratogenic effects implies that PB is not an inducer of embryo cytochrome P450, although it was active in the maternal liver. Alternatively PB may induce embryonic cytochrome P450, but the specific isoenzyme(s) induced do not alter the teratogenic potential of DPH.

Transplacental exposure to both cytochrome P448 inducers (βNF and 3MC) caused increased toxicity of DPH in LB cells IN VITRO, however, only β-NF pretreated LB cells could activate CPA IN VITRO. Although a strict comparison of IN VIVO versus IN VITRO induction is not possible
(concentrations of inducers IN VITRO is limited by their direct toxicity), the effects of exposing embryos transplacentally to P448 inducers caused a greater response than by directly coincubating embryo cells with either 3MC or β-NF (no effect). It would appear that a maternally mediated reaction (or metabolism of the inducer) enhances the inductive effects on embryo cytochrome P448, especially with β-NF as transplacentally exposed LB cells had the ability (sufficient cytochrome P448) to activate CPA.

This study shows that embryo cytochrome P448 is inducible, especially by β-NF, but that this phenomenon is tissue specific as CNS cells were refractory to all transplacentally administered and IN VITRO inducers. The observation that cytochrome P450 was not inducible whereas cytochrome P448 was in embryo cells is in accordance with the literature (Pelkonen 1980, Juchau et.al. 1985, Mizokami et.al. 1982 and Worrell 1984).

The effects of DPH IN VITRO can be increased by modulation of the levels/activity of the cytochrome P450 system by both inhibitors and inducers, which makes it difficult to determine whether DPH itself or some metabolite(s) or both, is the ultimate teratogen. Inhibition of the cytochrome P450's increased the effects of DPH IN VITRO to a greater extent than inducers. The maximum reduction in IC50 (increased toxicity) with an inhibitor was benzimidazole and LB cells 82±6 and with an inducer, β-NF transplacentally induced LB 30±10. That inhibitors increased toxicity of DPH in both LB and CNS cells implies that it is DPH itself and not a metabolite which is the ultimate teratogen, however how effective or precise (for a particular isoenzyme, an inhibitor may block one pathway and consequently
increase another) the inhibition of cytochrome P450's was, remains a matter for speculation.

Only LB cells from cytochrome P448 induced dams had the ability to modulate DPH toxicity IN VITRO so implying for LB cells a metabolite of DPH is the ultimate teratogen. However CNS were refractory to the inductive effects of both 3MC and β-NF. It would seem that these inducers may be increasing the levels of p-hydroxy.DPH (which was found to be equitoxic with DPH IN VITRO) or producing another metabolite not generally formed by induced embryo cells eg, dihydrodiol or O-methyl-catechol. However these results illustrate the complexity of whether DPH itself or some metabolic product which is the ultimate teratogen; data from this chapter presents evidence for both cases. That p-hydroxy.DPH is equitoxic with DPH suggests that it could be both DPH and the p-hydroxy. metabolite; this will be considered in conjunction with the evidence from chapters 6 and 7.

The main conclusion from this chapter is that there are changes in compound toxicity following modulation of cytochrome P450 activity in embryo cells, by appropriate IN VITRO and IN VIVO manipulations.
CHAPTER 6:

ROLE OF THE ARENE OXIDE INTERMEDIATE OF DPH IN IN VITRO TERATOGENESIS

Contents

6.1 IRREVERSIBLE BINDING OF $^{14}$C-DPH TO EMBRYO CELL PROTEINS

6.2 MODULATION OF THE DPH EPOXIDATION PATHWAY IN VITRO

6.3 DISCUSSION
6. ROLE OF THE ARENE OXIDE INTERMEDIATE OF DPH IN IN VITRO TERATOGENESIS

The major metabolites of DPH are the p. and m. hydroxy derivatives of the phenyl group at position 5 of the hydantoin ring. These metabolites could have been derived by isomerisation of an epoxide intermediate. Epoxides are reactive species and may be the causative agent in DPH toxicity and covalent binding to embryo proteins has been used as a measure of reactive DPH arene oxide metabolite formation. The assignment of an epoxide as the reactive species of DPH has been examined by increasing its formation (via P450 inducers), decreasing its detoxification (via epoxide hydrolase inhibition) and increasing its detoxification (via thiol supplementation).

6.1 IRREVERSIBLE BINDING OF $^{14}$C-DPH TO EMBRYO CELL PROTEINS

Since the yield of protein from cultured embryo cells was so small (6 uteri = 72 embryos = 350 µg protein in LB or 100 µg protein in CNS) 12 uteri embryos were pooled for each experiment. Each of the different coincubations or pretreated cells had its own control, cells derived from untreated mothers coincubated over the same period of time with the appropriate concentration of DPH and the same fixed concentration of $^{14}$C-DPH. It was found that a 30 minute incubation of 5 day old cultured cells with $^{14}$C-DPH did not give rise to any covalent binding (data not shown).

A concentration-response of covalent binding for DPH at 20, 40 and 60 µg/ml (and a fixed concentration of 0.4 µCi $^{14}$C-DPH/dish) with LB and CNS cells showed that increasing concentrations of DPH did not significantly alter the degree of covalently bound metabolites over
Table 6.1

IRREVERSIBLE BINDING OF $^{14}$C-DPH TO EMBRYO CELL PROTEIN

<table>
<thead>
<tr>
<th>Concentration of DPH (µg/ml)</th>
<th>Cell type</th>
<th>DPH equivalents$^a$</th>
<th>DPH equivalents$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mol x 10^{-15}/mg</td>
<td>protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>LB$^b$</td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CNS$^c$</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>LB</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CNS</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>LB</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CNS</td>
<td>2.15</td>
</tr>
</tbody>
</table>

$^a$ calculated by reference $^{14}$C-DPH, 1 D.P.M. = 6 x 10^{-15} moles DPH.

$^b$ Pooled cells from 12 uteri, 48 islands yielded 673 µg protein and 245 D.P.M.

$^c$ Pooled cells from 12 uteri, 48 islands yielded 403 µg protein and 157 D.P.M.
Table 6.2

THE EFFECTS OF COINCUBATION AND TRANSPLACENTAL INDUCTION OF
ON IRREVERSIBLE BINDING OF 14C DPH TO EMBRYO CELLS

<table>
<thead>
<tr>
<th>CO-TREATMENT</th>
<th>CELL TYPE</th>
<th>DPH equivalents(^a) Mols x 10(^{-15})/mg protein</th>
<th>CONTROL(^b) Mols x 10(^{-15})/mg protein</th>
<th>DPH binding(^c) (% CONTROL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LB</td>
<td>7.92</td>
<td>20.15</td>
<td>32.6 ± 8.2</td>
</tr>
<tr>
<td>BENZIMIDAZOLE</td>
<td>LB</td>
<td>9.33</td>
<td>10.56</td>
<td>15.82</td>
</tr>
<tr>
<td>(200 (\mu)g/ml)</td>
<td>LB</td>
<td>5.73</td>
<td>16.47</td>
<td></td>
</tr>
<tr>
<td>with DPH at 20 (\mu)g/ml</td>
<td>LB</td>
<td>3.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>33.38</td>
<td>97.03</td>
<td>67.7 ± 27.2</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>0.48</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>1.37</td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>1.32</td>
<td>3.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>46.76</td>
<td>20.12</td>
<td>176.75</td>
</tr>
<tr>
<td>TCPO (1 (\mu)g/ml)</td>
<td>LB</td>
<td>31.62</td>
<td>26.85</td>
<td>± 55.4</td>
</tr>
<tr>
<td>with DPH at 60 (\mu)g/ml</td>
<td>LB</td>
<td>23.51</td>
<td>16.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>90.98</td>
<td>37.73</td>
<td>298.25</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>3.76</td>
<td>1.00</td>
<td>± 114.45</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>5.38</td>
<td>3.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>6.00</td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>43.32</td>
<td>20.11</td>
<td>175.5</td>
</tr>
<tr>
<td>β-NF IN UTERO</td>
<td>LB</td>
<td>29.35</td>
<td>27.02</td>
<td>± 61.3</td>
</tr>
<tr>
<td>exposure with</td>
<td>LB</td>
<td>23.24</td>
<td>16.44</td>
<td></td>
</tr>
<tr>
<td>DPH at 60 (\mu)g/ml</td>
<td>LB</td>
<td>37.71</td>
<td>15.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>48.81</td>
<td>37.72</td>
<td>255.75</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>4.23</td>
<td>1.00</td>
<td>± 158.3</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>3.62</td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>5.36</td>
<td>1.47</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a}\) calculated by reference 14C-DPH, 1 DPM = 6 \times 10\(^{-15}\) mol s.

\(\text{b}\) The appropriate binding data of DPH in the absence of coincubation or transplacental induction.

\(\text{c}\) Calculated for each separate experiment \((n=4)\), mean and standard error.
this limited substrate range (Table 6.1) and resulted in 2.02 - 2.50.

pmol of DPH bound/mg protein.

The effect of two different coincubations and βNF pretreatment on

the amount of metabolite irreversibly bound to embryo LB and CNS cells

was examined. - Benzimidazole at its highest non cytotoxic

concentration (200 μg/ml) with DPH at 20 μg/ml, trichloropropene oxide

(TCPO) at its highest non-cytotoxic concentration (1 μg/ml) and DPH at

60 μg/ml, and β-NF pre-induced cells with DPH at 60 μg/ml. The

results are summarised in Table 6.2. The results show that

coincubation with benzimidazole causes a decrease in the amount of

covalently bound 14C-DPH metabolites to 33% of the control value in LB

and to 67% in CNS cells. Coincubation with TCPO increases the amount

of covalent binding by 1.76 fold in LB and 2.98 fold in CNS cells over

cells cultured with DPH in the absence of the inhibitor.

Transplacentally β-NF induced embryonic cells increased covalent

binding of DPH by approximately the same amount as TCPO, i.e. 1.76

fold in LB and 2.55 fold in CNS cells relative to the control,

covalent binding in embryonic cells derived from untreated mothers.

6.2 MODULATION OF THE EPOXIDE PATHWAY IN VITRO

Coincubation of LB or CNS cells with either TCPO or thiol

supplementing agents (N-acetyl-cysteine or glutathione) at their

highest non-cytotoxic concentrations with different concentrations of

DPH did not result in alteration of DPH toxicity expression as

assessed by the IC50 value (Table 6.3).
Table 6.3

**EFFECT ON DPH TOXICITY BY MODULATING THE DETOXIFICATION OF THE ARENE OXIDE INTERMEDIATE**

<table>
<thead>
<tr>
<th>COMPOUND CO-</th>
<th>COMPOUND ADDED</th>
<th>IC50 OF DPH</th>
<th>IC50 DPH +</th>
</tr>
</thead>
<tbody>
<tr>
<td>INCUBATED AT NON-CYTOTOXIC LEVEL (µg/ml)</td>
<td>(µg/ml)(^a/b)</td>
<td>(µg/ml)(^a/b)</td>
<td></td>
</tr>
</tbody>
</table>

| DPH ALONE | - | 67 ± 18 | 91 ± 4 |
| TRICHLORO- PROPENE OXIDE | 1 | 63 ± 7 | 90 ± 4 |
| N-ACETYL CYSTEINE | 1000 | 62 ± 5 | 86 ± 5 |
| GLUTATHIONE | 100 | 63 ± 4 | 90 ± 3 |

\(^a\) Mean and standard error from 3 experiments

\(^b\) No significant differences found between all test and control values, in both CNS and LB cells.
6.3 DISCUSSION

Results from this Chapter have shown that the degree of irreversible/covalent binding of $^{14}$C-DPH to rat embryo cells IN VITRO can be modulated by both inducers and inhibitors of cytochrome P450 and an expoxide hydrolase inhibitor (TCPO). However, TCPO and thiol supplementing agents did not modify the toxicity of DPH IN VITRO.

It is to be expected that an inducer of the cytochrome P450 system such as β-NF would increase the oxidative metabolism (ring hydroxylation) of DPH and since in the case of DPH, hydroxylation is thought to proceed via the reactive arene oxide intermediate (see Chapter 1.5.2.4 and Figure 1.4) the amount of reactive intermediate available for covalent binding to essential proteins would also be expected to increase. In my study this was observed; β-NF transplacentally induced LB cells had 1.75 fold greater covalent binding than the controls and CNS cells 2.55 fold greater.

Similarly an inhibitor of the cytochrome P450 system such as benzimidazole would be expected to have the reverse effect and this was observed. Benzimidazole coincubated at a non-cytotoxic concentration with LB reduced the covalent binding of $^{14}$C-DPH by 32% relative to the control and CNS by 67%. It should be noted that whereas βNF caused an increase in covalent binding and benzimidazole a decrease, both compounds caused an increase in DPH toxicity as gauged by a decrease in IC50 (Results from Chapter 5).

The variability in covalent binding of controls and cells coincubated with TCPO or benzimidazole or transplacentally induced cells was large but this was to be expected since the binding to rat liver microsomes (a more consistantly reproducible system) of $^{14}$C-
practical metabolites also produced great variation and standard error (120% control values) (Orton and Lowery 1981).

One of the detoxification pathways for reactive arene oxide intermediates is hydration to the dihydrodiol metabolite, mediated by the enzyme epoxide hydrolase (Oesch 1982). TCPO, which is an inhibitor of this enzyme, would be expected to cause an increase in the amount of reactive arene oxide available for covalent binding and in this study this was observed; LB cells had $176.75 \pm 55.45\%$ the amount of $^{14}$C-DPH bound relative to controls and CNS $298.25 \pm 114.45\%$. This study of the irreversible binding of $^{14}$C-DPH to rat embryo cells has shown that the enzymes regulating metabolism to, and detoxification of, the arene oxide are not only present but functional at an early stage of organogenesis IN VITRO.

My findings are in accordance with the literature, that both human (Pelkonen 1980) and rat (Pelkonen and Karki 1975) embryos were capable of epoxidation at early stages of gestation. However despite this biochemical evidence preliminary immunocytochemical work on the identification of the epoxide hydrolase antibody (using an antibody to rat liver epoxide hydrolase raised in the goat) proved negative, but this was probably due to incorrect/insufficient concentrations of either the 1st or 2nd antibody in the technique (Chapter 2.2.5), and therefore these results must be considered tentative.

Coincubation of DPH with either TCPO or thiol supplementing agents (which detoxify the arene oxide by conjugation with thiol groups to an essentially non-reactive intermediate) (Oesch 1982), were found to be ineffective at modulating the response of either LB or CNS cells to DPH IN VITRO, as gauged by altering the IC50 values and
therefore not able to modify DPH toxicity. However TCPO was found to increase the degree of covalent binding of 14C-DPH in both LB and CNS cells. This finding implies that despite the effect of TCPO on the degree of covalent binding, the arene oxide does not play an important role in the toxicity of DPH IN VITRO. This concept will be further discussed in Chapter 8.
CHAPTER 7:

IDENTIFICATION OF $^{14}$C-DPH METABOLITES
FORMED IN VITRO BY EMBRYO CELLS

Contents

7.1 Identification of DPH metabolites in rat embryo cells.

7.2 Identification of DPH metabolites formed in the culture media by rat embryo cells.

7.3 Discussion
7. IDENTIFICATION OF $^{14}$C-DPH METABOLITES FORMED IN VITRO BY EMBRYO CELLS

$^{14}$C-DPH was incubated with rat embryo cells (pooled from 12 untreated mothers) at its maximum non-cytotoxic concentration, 50 μg, 0.2 μCi/ml $^{14}$C-DPH culture media. After the five day incubation period potential metabolites were extracted from LB and CNS cells and LB and CNS culture media by the methods described in chapter 2.2.8. The extracts were then analysed by an H.P.L.C. method with U.V. detection and the radioactivity in the fractions counted by a liquid scintillation technique, see chapter 2.2.7 for method details. The control experiment was 50μg; 0.2 μCi/ml $^{14}$C-DPH incubated for 5 days in culture medium in the absence of rat embryo cells.

The retention times and minimal levels of detection for each of the metabolites (p.hydroxy. DPH, m.hydroxy. DPH, DPH acid) and DPH itself were recorded and are presented in Figure 7.1. The most polar metabolite, DPH acid, eluted first, retention time 2 mins, minimum level of detection (m.l.d) at 0.0.8 sensitivity was 1 ng/ml. The subsequent order of elution was p.hydroxy.DPH, (6 mins, m.l.d. 10 ng/ml) m.hydroxy.DPH (7.5 mins, m.l.d. 10 ng/ml) and DPH (14 mins, m.l.d. 2 μg/ml). Figure 7.2. illustrates the HPLC trace and relative radioactivity of DPH incubated with culture medium in the absence of cells and shows that no radioactive metabolites were produced.

7.1 IDENTIFICATION OF DPH METABOLITES IN RAT EMBRYO CELLS

Analysis of the extracts from LB and CNS cells are shown in Figure 7.3. LB cells showed that the majority of the radio-activity was present as $^{14}$C-DPH (71.5%) but that by area under curve
**HPLC Analysis of DPH and its Metabolites**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (mins.)</th>
<th>Minimum Level of Detection at 0.08 Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A - DPH acid</td>
<td>2</td>
<td>1 ng/ml</td>
</tr>
<tr>
<td>B - p-hydroxy DPH</td>
<td>6</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>C - m-hydroxy DPH</td>
<td>7.5</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>D - DPH</td>
<td>14</td>
<td>2 ng/ml</td>
</tr>
</tbody>
</table>
Analysis of Extract From Incubating 14C—DPH in Culture Medium in the Absence of Embryo Cells

HPLC deflection (cm)
--- (Peak height) ---

Time (mins.)

(DPM sample - DPM blanks)
Corrected DPM's
Analysis of $^{14}$C-DPH Metabolites in Embryo Cells

**LB Cells**

**CNS Cells**
comparison, 3.5% of radioactivity was DPH-acid and 5.3% p.hydroxy.DPH. An unidentified peak at 20 mins represented 19.6% of the radioactivity. CNS cells showed that the majority of radioactivity was 14C-DPH (92.5%), of the total. The only other two metabolites present were identified as DPH acid (3.2%) and p.hydroxy.DPH (4.3%).

7.2 IDENTIFICATION OF DPH METABOLITES FORMED IN THE CULTURE MEDIA

BY RAT EMBRYO CELLS

Analysis of the extracts from the culture media from LB and CNS cell incubates are shown in Figure 7.4. Both sets of media extracts showed, by HPLC and radioactivity in the corresponding eluants, the presence of DPH acid and p.hydroxy.DPH metabolites. LB cells showed that 79.5% of the total radioactivity was 14C-DPH, 17% p. hydroxy. DPH and 3.4% DPH acid. CNS cells showed that 93.7% of total radioactivity was 14C-DPH, 5.4% p.hydroxy. DPH and 0.8% DPH acid.

7.3 DISCUSSION

Results from this chapter indicate that rat embryo cells IN VITRO have the ability to metabolise DPH by both ring opening to the acid metabolite and hydroxylation to the p.hydroxy.DPH metabolite and that LB cells have a greater metabolic capacity than CNS. This conclusion is based on relative retention times on the HPLC and comparison to authentic standards, and it is recognised that, time permitting a more positive identification of the metabolites by physical methods (e.g. mass spectrometry) would have been desirable. DPH alone with media did not show any metabolites present (Figure 7.2) This confirmed that the observed peaks from HPLC analysis/radioactivity in eluants were genuine metabolites formed by the cells themselves.
Fig. 7.4.

Analysis of $^{14}$C - DPH Metabolites in Culture Media

**LB Media**

![Graph showing analysis of $^{14}$C - DPH metabolites in LB Media.]

**CNS Media**

![Graph showing analysis of $^{14}$C - DPH metabolites in CNS Media.]

- Solvent
- DPH
- p.OH DPH
- DPH acid
- Time (mins.)
- Corrected DPM's
- HPLC deflection (cm)

**Note:** The graphs illustrate the analysis of $^{14}$C - DPH metabolites in LB and CNS media over time, showing peaks for Solvent, DPH, p.OH DPH, and DPH acid.
The method of HPLC analysis employed a reverse phase C18 carbon-nitrile column which meant highly polar compounds eluted first with high resolution and sensitivity of detection. Extracts from the samples of 14C-DPH shows that peaks were more retarded (average peak of DPH from samples was at 18 minutes compared to 14 minutes with standard pure DPH) and the peak shapes were broader and more flattened. However the retention time of DPH that had been extracted from a 5 day incubation period with culture medium in the absence of cells showed that its retention time lengthened to 16.5 minutes. It is not understood why culture medium should cause this effect, since, serum proteins were previously removed by Sep-pak filtration (see method in chapter 2.2.8).

LB cells had 3.5%, and CNS 3.2% radioactivity present as the DPH acid which implies equal abilities of both types of cells to rupture the hydantoin ring to form the acid product. However LB cells had 5.3% compared to 4.3% radioactivity in CNS cells as the p.hydroxy.DPH metabolite which tends to indicate that LB cells were slightly more effective at hydroxylation than CNS.

LB cells also had an unidentified metabolite that was less polar than DPH and represented 19.6% of the total radioactivity. This metabolite could not have been the dihydrodiol or catechol since these polar metabolites should have eluted around the same time as the p.hydroxy.DPH metabolite (Chow et al 1980, Chow and Fischer 1981). It could possibly be a protein-bound DPH fraction that was not completely removed by the Sep-pak extraction procedure, a novel metabolite or artifact. Interestingly this peak was only found in LB cells and not the medium.
Analysis of the media from LB cell incubates showed that 17% of the total radioactivity was the p-hydroxy.DPH metabolite compared to 5.4% with the CNS and LB cells had 3.4% compared to 0.8% in CNS of DPH acid. This indicates that LB cells are more active at producing each metabolite than the CNS cells since the metabolites would pass across the membranes to a more dilute medium.

Based on biliary excretion data, pregnant rats (days 19-21 gestation) convert at least 50% of an oral dose of DPH to p-hydroxy DPH within 5 hours (Vore et al. 1978). In the 2nd trimester of pregnancy humans also convert 48% \pm 21 of an oral dose of DPH to p-hydroxy DPH within 6 hours of an oral dose (Kochenour et al. 1980).

In conclusion these results have shown that whilst intra-cellular levels of metabolites were approximately equal in LB and CNS cells the extra-cellular concentrations indicated that LB cells were more effective at hydroxylation and hydantoin ring opening than CNS cells. Also LB cells had over the 5 day incubation period approximately \( \frac{1}{3} \) the metabolic capacity for p-hydroxy.DPH formation compared to maternal liver over a 5 hour period; (for a comparable time period LB cells had 0.014% metabolic capability of the maternal liver).
SUMMARY AND CONCLUSIONS

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8.1 EVIDENCE FOR PRESENCE AND FUNCTIONALITY OF RAT EMBRYO CYTOCHROME P450's

Results from this thesis have shown the first qualitative evidence, by means of immunocytochemistry (Chapter 4), of the presence of cytochrome P450's in differentiating rat embryo cells IN VITRO. The implications of detecting these enzymes at such an early stage of organogenesis in the rat are far reaching. Historically, rodent embryos are known to be late developers of smooth endoplasmic reticulum and hence relatively deficient in microsomal cytochrome P450's compared to human or primate embryos (Remmer and Merker 1963). Accordingly, detection of constitutive, and in some cases inducible, levels of cytochrome P450's in rat embryo LB and CNS cells would almost certainly suggest their presence, and probably at higher levels in human embryo cells at the same stage of organogenesis.

Isoenzymes of cytochrome P450 (PB-inducible form 'b' and 3MC-inducible type forms 'c' and 'd') were constitutively present in untreated embryo cells, however, only the cytochrome P450 'c' and 'd' isoenzymes were found to be inducible. This observation was in accordance with related published literature (Mizokami et al 1982, Sunouchi et al 1983, Faustman-Watts et al 1984, Juchau et al 1985), and maybe associated with the fact that cytochrome P450 'c' and 'd' isoenzymes are the only isoenzymes inducible in cultured cells (Guengerich 1986). The placenta, (part of which is contributed by the embryo), is also reported to only have inducible cytochrome P450 'c' and 'd' forms despite having a constitutive level of cytochrome P450 'b' (Juchau 1980), and so indirectly consolidate my findings.
The method of Western Blotting was used to confirm that the increased levels of staining in cells observed by immunocytochemistry with exposure to cytochrome P450 'c' and 'd' form inducers was cytochrome P450 'c' protein. Results showed that in fact βNF pre-exposure of both LB and CNS cells resulted in more protein present in the bands corresponding to purified rat liver microsomal P450 'c'. The levels of PB inducible P450 'b', whilst present at a moderately high constitutive level were refractory to induction. Given these findings in conjunction with the staining results, the conclusion can be made that rat embryo cytochrome P450 'c' is inducible but PB-inducible P450 'b' is not.

The ontogeny of the two major forms of cytochrome P450 in culture over the 5 day incubation period showed that the (non-inducible) PB-type P450 'b' appeared first and reached a higher steady-state level than the (inducible) P450 'c' form. It would be tempting to speculate that PB-type P450 'b' has an endogenous role and hence, despite having the correct epitope to be identified by immunocytochemical staining, would not respond to PB induction.

The P450 'c' isoenzyme appeared later in the culture period (midway, on day 3), and this developmental time pattern was unaltered by prior IN UTERO exposure to βNF. Only increased levels were observed at the end of the culture period. This result indicates that induction of embryo cell cytochrome P450 'c' is a process involving 'pre-programming' and that embryo cells 'memorise' the βNF exposure, perhaps because the gene coding for P450 'c' has not yet been expressed and so induced levels are seen 4 days after the last exposure, i.e. day 12 IN UTERO = last exposure, day 3 IN VITRO = increased level and first appearance.
That DPH toxicity IN VITRO was modulated by coincubation with cytochrome P450 inhibitors, inducers and transplacentally administered inducers would indicate that the embryo cytochrome P450's observed immunocytochemically and detected by Western Blotting were also functional (i.e. capable of xenobiotic metabolism). All of the cytochrome P450 inhibitors tested that were coincubated with LB cells caused an increase in DPH toxicity (results from Chapter 5), but only 5 out of the 7 caused an increase in toxicity with CNS cells. Modulation of DPH toxicity with cytochrome P450 inducers was only observed with LB cells (3MC but not βNF IN VITRO and transplacentally administered 3MC and βNF). Phenobarbitone was without effect. Similarly only LB cells derived from βNF pretreated dams could activate CPA, all other inducers were without effect and CNS cells were refractory to all forms of co-treatment with inducers. Modulation of compound toxicity provided indirect assessment of metabolism in the embryo cells. Direct evidence was provided by detection of DPH metabolites. A greater percentage of metabolites of DPH were formed by LB cells than CNS cells (Chapter 7) not only indicates that embryo cells IN VITRO have a sufficient constitutive level of xenobiotic metabolising enzymes to hydroxylate DPH, but that some tissues have a greater metabolic potential and capability than others, and that the cultured cells parallel the IN VIVO metabolism of DPH.

In conclusion my results have shown 1) a constitutive level of embryo cytochrome P450's 2) only embryo cytochrome P450 'c' and 'd' are inducible and 3) LB cells are more biochemically active and more susceptible to cytochrome P450 'c' inducers than CNS cells.
8.2 ROLE OF METABOLISM IN THE BIOACTIVATION OF DPH

DPH has well documented teratogenic effects in man (Hanson and Smith 1975, Hanson 1976), rabbit (Schardein 1983) and mouse (Harbison and Becker 1970, Wells and Harbison 1980, Finnell and Chernoff 1984), however, the data for rat is equivocal (Harbison and Becker 1972 and results from Chapter 3.1). The issue of species sensitivity (viz rat versus others) with regard to selective metabolite formation was discussed in chapter 3.5 and showed that there were no obvious metabolic differences in the metabolism of DPH that could account for the observed differences in species sensitivity (Glazko 1973). My IN VIVO DPH teratology study conducted in the Alderley Park rat strain, DPH proved to be negative despite showing signs of an embryotoxic effect, but direct coincubation of DPH with differentiating rat embryo cells IN VITRO showed that the compound inhibited differentiation of embryo cells. It was concluded that an insufficient concentration of DPH (or its metabolite(s)) reached the embryo for a teratogenic effect to be observed IN VIVO.

A quantitative structure-activity study with 28 hydantoin ring analogues illustrated not only that embryo cells IN VITRO were capable of distinguishing between structurally related compounds but that the degree of toxicity to embryo cells was closely correlated to lipophilicity which tends to argue against metabolism playing a role in toxicity.

Given the information that DPH was a positive IN VITRO rat teratogen and that the embryo cells IN VITRO could distinguish between structurally similar compounds then the study of coincubating the major metabolites of DPH direct with the cells should be potentially
able to distinguish the ultimate teratogenic entity. However, both
the hydroxy (m and p) metabolites were equitoxic with DPH and only the
ring opened acid product was totally non-inhibitory (up to its maximum
solubility in culture medium). It was, therefore, concluded (on the
basis of the metabolites studied) that the only completely detoxifying
pathway IN VIVO is hydantoin ring cleavage to the acid product and
that hydroxylation of DPH results in the formation of an equitoxic
compound. However, it must be emphasised that in light of the
previously discussed sterochemical aspects of DPH hydroxylation,
different isomer production and inherent toxicities may have a role to
play in the IN VITRO system described in this thesis.

It is important at this point to re-emphasize the dichotomy in
the literature as to whether DPH itself or the metabolically derived
arene oxide intermediate, or the hydroxy (m and p) metabolites being
the ultimate teratogen. Evidence that shows DPH itself is the
ultimate teratogen includes:-

a) DPH is directly toxic to rat embryo cells (Flint and Orton
1985) and whole mouse embryos IN VITRO (Bruckner et al 1983), however,
evidence from Chapter 7 would indicate that embryos are capable of
limited xenobiotic metabolism IN VITRO and therefore in reality
exposure may have been to DPH and metabolites derived by the embryo
cells themselves.

b) A/J mice fed a purified diet had decreased hepatic cytochrome
P450 levels, increased maternal blood DPH concentrations and showed a
3 fold increase in the teratogenic effects (McClain and Rohrs 1985).
However, it cannot be excluded that the purified diet may also have
cau sed disturbances in endogenous metabolism leading to hormone
imbalance and hence an increased susceptibility to teratogens.
c) SKF 525A pretreatment increased both maternal plasma concentrations of DPH and teratogenicity whilst PB pretreatment decreased maternal plasma DPH concentrations and teratogenicity (Harbison and Becker 1970). However, embryo levels of either DPH or p.hydroxy.DPH were unaffected. Dihydrodiol levels were not reported.

d) Studies with the major metabolites at equimolar doses on the teratogenic response of the mouse showed that the maximum response of any of the metabolites tested was only 10% of that of DPH itself (Harbison and Becker 1974). The dihydrodiol metabolite, however, remains to be examined.

Evidence that the arene oxide intermediate is formed during DPH metabolism is irrefutable (Claesen et al 1984, Milton et al 1985, Pantarotto et al 1982, Wells et al 1980, 1983, Spielberg et al 1981) but the evidence showing this metabolite as the ultimate teratogen is sparse and obviously circumstantial. Martz et al 1977 showed a correlation between the degree of covalently bound $^{14}$C-DPH to gestational tissue and teratogenicity in the mouse by use of modulation of epoxide hydrolase pathway. However, in their studies neither inhibition of epoxide hydrolase (by TCPO) nor sequestering of the arene oxide (by glutathione) was complete as HPLC analysis revealed traces of free dihydrodiol metabolite present in gestational tissue and maternal plasma.

Given that the literature stands divided as to the role of metabolism in the bioactivation of DPH, I shall now summarise the evidence obtained from Chapters 5, 6 and 7.
Coincubation of a variety of inhibitors of cytochrome P450 with DPH IN VITRO caused an increase in the toxic effect as gauged by lowering of the IC50's of DPH in both LB and CNS compared to IC50's of DPH alone. The effects were greater in LB cells compared to CNS. There did not seem to be any correlation between the potency of each inhibitor and its effect on DPH toxicity modulation (Table 5.2). The most potent inhibitors were benzimidazole, (a broad non-specific cytochrome P450 inhibitor), ellipticine (a specific IN VITRO P450 'c' inhibitor) and aspirin (which is not a classical P450 inhibitor but acts via its major metabolite gentisice acid in inhibiting the P450-driven prostaglandin synthesis pathway) it is also a mild cyclo-oxygenase inhibitor. Given that one of the current theories of DPH teratogenesis is that a free radical intermediate generated by a prostaglandin synthetase mediated bioactivation, is the ultimate teratogen (Wells et al 1986), aspirin, which would inhibit this metabolite formation, was found to be peculiarly effective at modulating DPH toxicity, despite its weak cytochrome P450 inhibition properties. This result may indicate that indeed such a PG synthetase derived metabolite may be involved in DPH teratogenesis. However, despite extensive efforts the literature does not appear to document the prostaglandin synthetase/cylo-oxygenase inhibition properties of the other inhibitors tested.

The weakest modulator of DPH toxicity was SKF.525A. The mode of inhibition of this compound is thought to be via a metabolite (Netter 1979) and hence if the embryo cells were unable to metabolise SKF.525A then the inhibition would be ineffective. Cimetidine was a moderately strong modulator of DPH toxicity IN VITRO whereas its
pharmacologically similar analogue ranitidine (which does not have P450 inhibition properties) was not. This indicates that indeed it is the enzyme inhibition properties of the inhibitors which was responsible for the observed increases in DPH toxicity following co-incubation. This set of experiments would tend to indicate that DPH itself is the ultimate teratogen. Coincubation of DPH with a variety of cytochrome P450 inducers IN VITRO showed that only 3MC and LB cells could produce an effect, resulting in increased toxicity. Following transplacental administration of the inducers, again only LB cells, derived from BNF or 3MC pretreated dams showed any response (a 30 and 20% increase in toxicity respectively). These modulations in DPH toxicity following induction would indicate that indeed a metabolite is the ultimate teratogen and conflicts with the results from inhibitor studies. However, it should be noted that only cytochrome P450 'c' inducers in LB cells could cause an effect and that this effect was a 20 - 30% increase in toxicity (depending on the inducer) compared to 13 - 82% increase in toxicity with various inhibitors in LB cells and 3 - 52% increase with various inhibitors in CNS cells. CNS cells were refractory to modulation of DPH toxicity by inducers and yet immunocytochemically were shown to be induced (Chapter 4) but were capable of DPH toxicity modulation when coincubated with inducers. That inhibitors increased the toxicity of DPH in both LB and CNS cells would indicate that DPH itself is the ultimate teratogen yet how effective or precise (one pathway may be blocked and consequently another increased) the inhibition of cytochrome P450's was remains a matter for speculation. It would seem that inducers may be increasing the levels of p.hydroxy.DPH formed IN VITRO (which was
found to be equitoxic with DPH) and may be producing another metabolite(s) not generally formed by uninduced cells, e.g. free radical intermediate, dihydrodiol or o-methyl catechol, which may have been more toxic than DPH itself. However, this theory was not confirmed by H.P.L.C. analysis. It was found that uninduced cells, especially LB, were capable of limited p-hydroxy DPH and DPH acid production. This result coupled with the fact that LB cells were more resistant to the effects of DPH IN VITRO than CNS would indicate that hydroxylation to the p-hydroxy metabolite is a detoxification pathway, but not a completely detoxifying process since p.OH DPH is equitoxic with DPH.

The role of the arene oxide intermediate in DPH teratogenesis appears to be insignificant since modulation of the epoxide hydrolase pathway did not result in altered DPH toxicity IN VITRO. However, covalent binding (an indication of the level of arene oxide metabolite produced) was found to alter with various co-treatments and this did not correlate with the modulation of toxicity. Benzimidazole which increased DPH toxicity IN VITRO by 82 ± 6% in LB cells caused a 32% decrease in covalent binding. Similarly, benzimidazole caused a 42± 6% increase in DPH toxicity with CNS cells but a 67% decrease in covalent binding. Transplacental exposure to βNF caused a 30% increase in DPH toxicity in LB cells and a 175% increase in covalent binding, no modulation of DPH toxicity in CNS cells but a 255% increase in covalent binding. TCPO (an inhibitor of epoxide hydrolase, the enzyme responsible for enzymatic detoxification of the arene oxide intermediate to the dihydrodiol) was totally ineffective at modulating DPH toxicity IN VITRO in either LB or CNS cells but
caused a 176% and 298% increase in covalent binding in each cell type respectively. In summary, the degree of covalent binding was found not to correlate with the degree of DPH toxicity IN VITRO, and so it is concluded that, despite evidence for the formation of the arene oxide intermediate IN VITRO, it does not play an important role in DPH teratogenesis.

In conclusion, the role of metabolism in the bioactivation of DPH remains partially unresolved. It has been established that the p-hydroxy metabolite is equitoxic with DPH and that hydroxylation is not a completely detoxifying reaction, only hydantoin ring cleavage to the acid product is totally detoxifying. Inhibition studies would tend to indicate that DPH itself is the ultimate teratogen while inducer studies would tend to indicate that a metabolite, but probably not the arene oxide intermediate, is the ultimate teratogen. To explain DPH teratogenesis is not simply a question of parent compound vs metabolite as the ultimate teratogen but may be the result of more than one metabolite and a combination of DPH itself.

8.3 FUTURE WORK

The results of this thesis have established the presence of cytochrome P450 and potential xenobiotic metabolising capabilities of embryo cells, especially LB cells, the logical extension of the project should be firstly to identify how much embryonic metabolism contributes to chemically induced teratogenesis, by expanding the number of examples eg. diazepam, CPA and aldrin. As a phenomenon it is important to remember that human embryos have greater levels of cytochrome P450 than rodents, and that exposure to environmental
chemicals and cytochrome P450 'c' and 'd' inducers is more likely in the human (i.e. smoking or dietary contaminants).

Besides examining embryo metabolism of other compounds the specific areas in which the importance of the role of embryonic metabolism could be further investigated are:-

1) The relative levels of metabolites produced by transplacentally induced embryo cells and cells coincubated with cytochrome P450 inhibitors.

2) The function of the non-inducible cytochrome P450 'b' form.

3) The reason why transplacental induction does not cause a precocious development of cytochrome P450 'c' and 'd' isoenzymes.

4) The ontogeny of cytochrome P450 mRNA's.

5) Investigation of the hydroxylated stereoisomers of DPH by a more extensive and precise structure activity study.

Further experimentation is also required to elucidate the role of metabolism in the bioactivation of DPH this could be achieved by:-

1) Comparison of the metabolites produced by rat embryo cells IN VITRO to those produced by a DPH-sensitive species, i.e. mouse LB cells.

2) Comparison of the IC50 values of DPH and its metabolites IN VITRO in a DPH-sensitive species (i.e. mouse LB cells to rat LB cells).

3) Coincubating serum from DPH pretreated dams (both DPH sensitive and non-sensitive species) with embryo cells.

4) Incubating embryo cells with DPH in the presence of maternal liver microsomes (induced with a variety of inducers).
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IN VITRO METABOLISM OF TERATOGENS BY DIFFERENTIATING RAT EMBRYO CELLS

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Abstract—Rapid and accurate prediction of teratogenic hazard had been achieved using cultures of differentiating limb mesenchyme (LB) and midbrain (CNS) cells from 13-day-old rat embryos. In this study we have used these cultures to examine the role of metabolism in the in vitro teratogenic activity of diphenylhydantoin (DPH) and cyclophosphamide (CPA). Two approaches were used. The first involved modulation of cytochrome P-450 activity by co-incubation in vitro with a variety of inhibitors at concentrations that were non-cytotoxic to the cells. This enhanced the toxicity of DPH by 13–82% in LB and by 3–52% in CNS cells. Benzimidazole and ellipticine caused the greatest enhancement and SKF 525A the least. DPH appears to be the proximate teratogen and there appear to be embryo-tissue cytochrome P-450s that assist in its detoxification. Following prior transplacental induction, CPA was toxic in vitro to LB cells from β-naphthoflavone-pretreated mothers. CPA was non-toxic in cells of control, phenobarbitone- or 3-methylcholanthrene-treated embryos. Thus there appear to be inducible levels of cytochrome P-448 in embryo cells. In the second approach, positive immunocytochemical staining of the cells with both monoclonal and polyclonal P-450 antibodies identified phenobarbitone, β-naphthoflavone- and 3-methylcholanthrene-inducible cytochrome P-450s at a constitutive level. Cytochromes P-448 (β-naphthoflavone type) and P-450 (phenobarbitone type, PB3 fraction) were inducible, confirming that cytochrome P-450s are in fact present in the embryo cells.

Introduction

Well-designed animal tests should detect the majority of potential human drug teratogens but they are resource intensive, requiring large numbers of animals and skilled staff to analyse the foetuses. The demand on these resources would be diminished if there were a simple rapid and highly predictive in vitro system for hazard assessment early in the screening programme for new drugs. One such screen is the micromass teratogen test which uses primary cultures of differentiating rat embryo limb bud (LB) and midbrain (CNS) cells (Flint, 1980; Flint & Orton, 1984; Flint, Orton & Ferguson, 1984) and which has an accuracy greater than 90%.

A major problem with any in vitro test for teratogens is that foetal-placental-maternal interactions are not available (see also Flint, 1986). Since some teratogens may require bioactivation, the inclusion of a reliable metabo1izing system or source of metabolites may be important. There are several ways of doing this:

(i) Use of serum from whole animals pretreated with the test compound (Chatot, Klein, Plenefisch, Carey et al. 1982).

(ii) Induction of xenobiotic-metabolizing enzymes (cytochrome P-450) in the cultured cells by treating the embryo with classical P-450 inducers in utero (via the dam) prior to removal for culture. Limb-bud organ cultures from mice pretreated with Aroclor 1254 bioactivate the pro-teratogen cyclophosphamide while uninduced controls show no effect (Neubert & Bluth, 1981).

(iii) Co-culture with isolated hepatocytes, which are capable of both phase I and II metabolism (Manson & Simons, 1979).

(iv) Co-incubation with hepatic microsomes plus co-factors (S-9 mix) from Aroclor 1254-induced male rodents or rabbits (Flint, 1986; Flint & Orton, 1984; Kitchin, Schmid & Sanyal, 1981).

In addition we suggest the following methods that might be experimentally useful but that have not yet been reported in the literature:

(i) Addition of purified components of the mixed-function oxidase (MFO) system.

(ii) Use of S-9 fractions from pregnant females pretreated with MFO inducers.

(iii) Incubation of the compound with an S-9 mix and then addition of the filtrate to the in vitro test system.

Many teratogens that are well metabolized in vivo, such as diphenylhydantoin (DPH; Wells & Harbison, 1980), aspirin (Greenaway, Bark & Juchau, 1984) and diazepam (Idanpaan-Heikkila, Jouppila, Poulakka & Pierro, 1980; Klein, Plenefisch, Carey et al. 1982).

Abbreviations: CNS = central nervous system cultures (i.e. midbrain cells); DPH = diphenylhydantoin; DMSO = dimethylsulphoxide; EBSS = Earle's balanced salt solution; HPLC = high-pressure liquid chromatography; LB = limb bud cells; MFO = mixed-function oxidase; TCPO = trichloropropene oxide; TLC = thin-layer chromatography.

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Vorne, 1971) are, however, directly toxic in the micromass culture test in the absence of added microsomal metabolizing enzymes (Flint & Orton, 1984). Cyclophosphamide is the notable exception; this compound requires bioactivation by S-9 mix in order to become teratogenic (Flint & Orton, 1984; Hales, 1981; Hill, Laster & Struck, 1972).

In this study we have used the differentiating rat embryo cells in vitro to examine the contribution of metabolism by the cells themselves to the teratogenic activity of DPH. Two approaches were used—modulation of the cytochrome P-450 activity of the cultured cells either by co-incubation of the test compound with non-cytotoxic concentrations of a variety of inhibitors of cytochrome P-450 or by using cells exposed transplacentally to cytochrome P-450 inducers, and secondly identification of cytochrome P-450 isozymes in the cultured cell by immunocytochemical staining with both monoclonal and polyclonal P-450 antibodies.

Experimental

Compounds. Test compounds of the highest purity commercially available were used. DPH, ellipicine, saccharin, cimetidine, aspirin, inosine, 3-methylcholanthrene, phenobarbital and β-naphthoflavone were obtained from Sigma Chemical Co., Poole, Dorset, benzimidazole and α-naphthoflavone from Fluka AG, Buchs, Switzerland, and m-hydroxydiphenylhydantoin, p-hydroxydiphenylhydantoin and tri-chloropropene oxide (TCPO) from Aldrich Chemical Co. Ltd, Poole, Dorset. SKF 525A and ranitidine hydrochloride were gifts from Smith, Kline and French Laboratories Ltd, Saffron Walden, Essex. In LB cultures only the differentiated darkly stained foci of chondrocytes were counted and in CNS cultures only the darkly stained foci of neurons were counted.

Graphs of the number of differentiated foci per cell island against the concentration of the compound (μg/ml) were plotted. The highest concentrations inhibiting differentiation by 50% of the control values (IC50) were determined from the graphs.

Exposure of cultures to test substance or vehicle

DPH, m-hydroxyDPH and DPH acid were dissolved in ethanol and added at 1.0%, v/v, to the culture medium. p-HydroxyDPH dissolved in dimethylsulphoxide (DMSO) was incorporated at 0.5%, v/v, in the culture medium, and cyclophosphamide in EBSS was incorporated at (1.0%, v/v). The test compounds in the appropriate solvent vehicle were added directly to the culture medium at the beginning of the 5 days of culture. Exposure was therefore effectively over a 5-day period in vitro.

Concentration-response curves of the various cytochrome P-450 inhibitors were obtained and the inhibitors were added at their highest non-toxic concentrations in combination with DPH at the start of the culture period.

Immunocytochemical staining of cytochrome P-450 in LB and CNS cultures

The peroxidase-antiperoxidase technique described by Wolf, Moll, Friedberg et al. (1984) was used on 5-day-old cultures of LB and CNS. Fresh cultures were stained against a variety of P-450 antibodies. Polyclonal phenobarbitone-raised P-450, β-naphthoflavone-raised P-448 and clofibrate-raised P-452 were provided by Dr G. Gibson. The monoclonal β-naphthoflavone-raised antibody 4:2 was a gift from the ICI Central Toxicology Laboratory and Hammersmith Hospital, London. The polyclonal 3-methylcholanthrene-raised P-448 and phenobarbitone-raised P-450 antibodies were gifts from Dr C. R. Wolf, Pharmacology Department, Western General Hospital, Edinburgh.
Metabolism of teratogens by rat embryo cells

Transplacental induction of metabolizing enzymes in the embryo

Dams were dosed in vivo and the embryos were subsequently removed on day 13 of gestation. Cultures were prepared as described above. The dosing schedules used were as follows: 3-methylcholanthrene (in arachis oil), 25 mg/kg given ip on days 10–12 of gestation; β-naphthoflavone (in arachis oil), 35 mg/kg given ip on days 10–12 of gestation; 0.1% sodium phenobarbitone, supplied ad lib. in the drinking-water on days 5–13 of gestation.

Results

Modulation of cytochrome P-450 activity

Effect of DPH and its major metabolites. DPH and its hydroxy metabolites appear to be approximately equitoxic when CNS and LB cultures were considered separately (Table 1) but DPH and its major hydroxy metabolite (the para form) were significantly more toxic to CNS than to LB cultures. The ring-opened acid metabolite was non-toxic up to its maximum solubility and is thus a possible detoxification route in vivo.

Co-treatment with cytochrome P-450 inhibitors in vitro. Co-incubation of DPH with a variety of cytochrome P-450 inhibitors in vitro (at their non-toxic concentrations) increased DPH toxicity to both LB and CNS cultures (Table 2). DPH toxicity was enhanced to a greater extent in LB than in CNS cultures by all the inhibitors except α-naphthoflavone. Benzimidazole, ellipticine, aspirin and metyrapone were the most effective (67–82% increases in toxicity in LB and 41–52% in CNS cultures). SKF 525A was the least effective with a 13% increase in LB and 3% in CNS cultures.

Ranitidine has the same pharmacological properties as cimetidine (both are H2 antagonists) and is structurally similar. It is not, however, an inhibitor of cytochrome P-450 activity (Bell, Gower, Martin et al. 1981) and did not alter the IC50 of DPH when co-incubated at non-toxic concentrations. Saccharin and inosine are both non-teratogens, with no inhibitory effect relative to the IC50 of DPH alone (see Table 1).

Effect of in vivo pretreatment. Following in vivo transplacental exposure to cytochrome P-450 inducers and subsequent culture of the cells, it was found that only LB cells from β-naphthoflavone-pretreated dams could activate the pro-teratogen cyclophosphamide (Fig. 1); 3-methylcholanthrene and phenobarbitone pretreatments had no effect on either type of cell. Addition of the inducers directly to the culture at non-cytotoxic concentrations had no effect (data not shown).

Identification of cytochrome P-450 isoenzymes in LB and CNS cultures

Constitutive levels of cytochromes P-450 (phenobarbitone-inducible) and P-448 (3-methylcholanthrene-inducible) were detected in 5-day-old cultures of both LB and CNS cells, with generally higher levels in LB than in CNS (Table 3). PB1 was the prominent form of phenobarbitone-inducible P-450. Phenobarbitone induced a P-450 isoenzyme in CNS cultures but not in LB (PB2 type; Wolf et al. 1984). None of the other PB forms were induced by transplacental exposure to phenobarbitone. Transplacental exposure to β-naphthoflavone, but interestingly not to 3-methylcholanthrene, induced a P-448 type of isoenzyme in both LB and CNS cultures, but to a greater extent in LB cultures. 3-Methylcholanthrene

Table 1. Comparative effects of diphenylhydantoin (DPH) and its major metabolites on CNS and LB cultures in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>CNS cultures</th>
<th>LB cultures</th>
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<tbody>
<tr>
<td></td>
<td>IC50 values (µg/ml)</td>
<td>IC50 values (µg/ml)</td>
</tr>
<tr>
<td></td>
<td>CNS cultures</td>
<td>LB cultures</td>
</tr>
<tr>
<td>DPH</td>
<td>67 ± 18**</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>p-HydroxyDPH</td>
<td>54 ± 11*</td>
<td>80 ± 9</td>
</tr>
<tr>
<td>m-HydroxyDPH</td>
<td>79 ± 8</td>
<td>84 ± 9</td>
</tr>
<tr>
<td>DPH acid</td>
<td>&gt;250†</td>
<td>&gt;250</td>
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</tbody>
</table>

CNS = Midbrain cells LB = Limb bud cells

Table 2. Modulation of diphenylhydantoin (DPH) toxicity in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>Non-cytotoxic level of compound (µg/ml)</th>
<th>IC50 of DPH + compound (µg/ml)*</th>
<th>Increase in toxicity (%)</th>
<th>Non-cytotoxic compound (µg/ml)</th>
<th>IC50 of DPH + compound (µg/ml)*</th>
<th>Increase in toxicity (%)</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td>SKF 525A</td>
<td>3</td>
<td>64 ± 2</td>
<td>3 ± 1</td>
<td>1</td>
<td>79 ± 1</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>4</td>
<td>42 ± 5</td>
<td>37 ± 5</td>
<td>4</td>
<td>71 ± 9</td>
<td>22 ± 10</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>400</td>
<td>57 ± 9</td>
<td>15 ± 10</td>
<td>400</td>
<td>47 ± 3</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>250</td>
<td>39 ± 8</td>
<td>41 ± 8</td>
<td>10</td>
<td>30 ± 4</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Aspirin</td>
<td>50</td>
<td>35 ± 6</td>
<td>47 ± 8</td>
<td>100</td>
<td>28 ± 6</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>200</td>
<td>32 ± 2</td>
<td>52 ± 12</td>
<td>0.001</td>
<td>27 ± 2</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>Benznidazole</td>
<td>200</td>
<td>39 ± 6</td>
<td>42 ± 6</td>
<td>200</td>
<td>16 ± 6</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>TCPO</td>
<td>1</td>
<td>63 ± 7</td>
<td>5 ± 7</td>
<td>1</td>
<td>90 ± 4</td>
<td>1 ± 3</td>
</tr>
<tr>
<td>Saccharin</td>
<td>600</td>
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<td>3 ± 4</td>
<td>600</td>
<td>90 ± 3</td>
<td>1 ± 3</td>
</tr>
<tr>
<td>Inosine</td>
<td>500</td>
<td>66 ± 6</td>
<td>1 ± 6</td>
<td>500</td>
<td>89 ± 5</td>
<td>2 ± 5</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>250</td>
<td>65 ± 6</td>
<td>3 ± 6</td>
<td>250</td>
<td>93 ± 2</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

*Values are means ± SD for at least three determinations. There were no significant differences between the IC50 values for DPH and its major hydroxy metabolites. Values marked with asterisks differ significantly from the corresponding value for LB cultures: *P < 0.01; **P > 0.001.
Fig. 1. Activation of cyclophosphamide (CPA) to a toxic metabolite by cultured rat embryo limb bud cells following in vivo transplacental induction of the embryos with β-naphthoflavone (βNF). The control line is from cultures of cells not pretreated with βNF in vivo and the dotted lines indicate the calculation of the IC₅₀ (44 μg/ml).

did not appear to induce any forms of cytochrome P-450.

Discussion

In vitro modulation of DPH toxicity (increased effect) by a variety of cytochrome P-450/P-448 inhibitors implies that the cells themselves have the ability to metabolize DPH and that DPH is the ultimate teratogen. Assuming that metabolism in vitro follows a similar pattern to that found in vivo (Fig. 2), co-incubation with cytochrome P-450 inhibitors influences ring hydroxylation. (It is not known whether hydroxylation proceeds by direct insertion or via the arene oxide.)

We have also shown that the embryo cells possess cytochrome P-450 isoenzymes. Immunocytochemical identification of cytochromes P-450 has shown that both LB and CNS cells have a constitutive level of both cytochromes P-450 and P-448, and that there is selective transplacental induction. The P-448 inducer β-naphthoflavone induced P-448 in LB cells and this was associated with an ability to activate the proteratogen cyclophosphamide. P-448 was also induced

Table 3. Immunocytochemical identification of cytochrome P-450 in LB and CNS cultures

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Untreated cultures</th>
<th>Cultures treated with:</th>
<th>3MC</th>
<th>PB</th>
<th>βNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LB</td>
<td>CNS</td>
<td>LB</td>
<td>CNS</td>
<td>LB</td>
</tr>
<tr>
<td>Gibson/polyclonal.</td>
<td>Anti-PB-raised P-450</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CTL/Hammersmith</td>
<td>monoclonal 4:2.</td>
<td>Anti-βNF-raised P-448</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wolf/polyclonal.</td>
<td>Anti-PB-raised</td>
<td>P-450 PB1</td>
<td>(+)</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Anti-PB-raised</td>
<td>P-450 PB2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Anti-PB-raised</td>
<td>P-450 PB3</td>
<td>(+)</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Anti-3MC-raised</td>
<td>P-448 3MC</td>
<td>+</td>
<td>(+)</td>
<td>NT</td>
</tr>
<tr>
<td>Wolf/polyclonal.</td>
<td>Anti-3MC-raised</td>
<td>P-448 3MC, βNF</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

3MC = 3-Methylcholanthrene  PB = Phenobarbitone  βNF = β-Naphthoflavone  LB = Limb bud cells  CNS = Midbrain cells  NT = Not tested

*Grading of response: 0 = absent; (+) = less than minimal, but present; + = minimal; ++ = moderate; +++ = very strong.
FURTHER METABOLISM & EXCRETION

Fig. 2. Probable routes of metabolism of diphenylhydantoin (DPH) in vivo.

in CNS cells (as gauged by a qualitative increase in staining) but these cells were unable to activate cyclophosphamide. Whole rat embryos cultured in the presence of cyclophosphamide have also been shown to be mainly susceptible to P-448 inducers (Faustman-Watts, Giachelli, Greenaway et al. 1984; Juchau, Giachelli, Pantel et al. 1985; Pelkonen, 1980); immunocytochemical identification of these enzymes was not undertaken by these authors. This is the first report identifying cytochromes P-450 in differentiating embryo cells. In addition we observed induction of the PB, form of the phenobarbitone-induced isozyme in CNS cultures.

Whether DPH or an arene oxide intermediate is the ultimate teratogen has been widely debated in the literature. Evidence in support of DPH as the teratogenic species includes the findings that the teratogenic response induced by the major metabolites at equimolar doses in mice in vivo was only 10% of that caused by DPH itself (Harbison & Becker, 1974), that DPH teratogenicity was increased threefold in mice fed a purified diet and was associated with raised blood levels of DPH due to depressed MFO activity (McClain & Rohrs, 1985), and that SKF 525A pretreatment increased DPH teratogenicity in the mouse in vivo and was associated with raised levels of DPH in the blood. Conversely phenobarbitone pretreatment decreased DPH teratogenicity and decreased blood concentrations of DPH (Harbison & Becker, 1970 & 1974).

Evidence for the arene oxide intermediate of DPH metabolism as the ultimate teratogen includes in vitro studies on covalent binding, presumably of the reactive arene oxide to foetal tissue (Blake & Martz, 1980; Martz, Fallinger & Blake, 1977; Milton, Hansen & Billings, 1985), showing a correlation between the teratogenic effect and the amount of covalently bound material in the gestational tissue of the mouse following administration of [14C]DPH. Furthermore, co-administration of TCPO (an epoxide hydratase inhibitor) and DPH doubled the incidence of terata and covalent binding to gestational tissue (Martz et al. 1977). This contrasted with our findings (Table 2) that co-incubation of TCPO and DPH in vitro did not alter DPH toxicity.

The evidence we present argues strongly that DPH itself is the ultimate and most potent teratogen (at least in vitro) and that embryo cells contain inducible cytochromes P-450 capable of metabolizing DPH, probably to its hydroxy metabolites. DPH and its p- and m-hydroxy metabolites appear to be equitoxic (Table 1). The cytochrome P-450 inhibitor studies, however, show DPH to be more toxic. The hydroxy metabolites still represent a substantial teratogenic hazard. That hydroxylation is not a completely detoxifying pathway may explain the apparent disagreement in the literature about the nature of the ultimate teratogen. The only completely detoxifying pathway available appears to be ring opening with the formation of diphenylhydantoic acid (a non-P-450-mediated reaction). Confirmation of the actual metabolic pathways followed in vitro awaits TLC and HPLC assessment of the metabolites formed.

Acknowledgements—We should like to thank Dr J. Foster, CTL, ICI plc, Macclesfield, for his help in the immunocytochemical staining of the cultures.
REFERENCES


METABOLISM OF TERATOGENS BY MAMMALIAN EMBRYOS; 
AN IN VITRO INVESTIGATION

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SUMMARY

Using differentiating rat embryo (13 days post coitum) limb cells in vitro it has been shown that 1) the toxicity of diphenylhydantoin to the differentiating cells is increased in the presence of a variety of inhibitors of cytochrome P450, 2) following transplacental induction by β-naphthoflavone the cells can activate cyclophosphamide (a proximate teratogen) to toxic reactive metabolite(s), 3) structure-toxicity relationships with substituted triazoles indicate toxicity is mediated by metabolism, and 4) it is possible to identify both constitutive and inducible levels of cytochrome P450 isozymes in the cultured cells by staining with specific monoclonal and polyclonal antibodies. Thus, four independent lines of evidence argue strongly that rat embryo cells (during organogenesis) are capable of xenobiotic metabolism.

INTRODUCTION

The complexity of the embryo's relationship with maternal drug pharmacokinetics and metabolism in teratogenicity is exemplified by cyclophosphamide. Co-administration during organogenesis of the cytochrome P450 inducer phenobarbital either reduced in the mouse or increased in the rat the number of observed fetal abnormalities. Although this may in part be due to differences of route, dose and duration of administration it is difficult to avoid the perplexing conclusion that in one species cyclophosphamide can act as a proteratogen and in another as an ultimate teratogen.

In vitro experiments using either the whole rat embryo in culture or cultures of differentiating rat embryo mid-brain and limb cells provide unequivocal evidence (the necessity for an external hepatic metabolising system—S9 mix) that cyclophosphamide requires metabolism to a reactive metabolite to express its embryotoxicity. Thus the confounding factor in the in vivo experiments must be the rate and extent to which maternal metabolism detoxifies the reactive metabolite.
The difficulty of understanding teratogenic mechanisms by in vivo experiment alone is further exemplified by diphenylhydantoin. As with cyclophosphamide different experiments argue, with equal force, that diphenylhydantoin is either a proteratogen or an ultimate teratogen.

Diphenylhydantoin as proteratogen:

1. The incidence of cleft lip and palate in Swiss ICR mice following a single dose (50mg/kg) of diphenylhydantoin on day 11 is doubled (as is covalent binding of the compound to the embryo cells) following co-treatment with the epoxide hydratase inhibitor trichloropropene oxide. A toxic arene oxide intermediate appears to be the ultimate teratogen.

2. The incidence of diphenylhydantoin induced abnormalities is inversely proportional to maternal circulating glutathione levels. This would follow if a toxic arene oxide intermediate were being conjugated (detoxified) with glutathione.

Diphenylhydantoin as ultimate teratogen:

1. Pretreatment of Swiss Webster mice with phenobarbitone enhances the metabolism of diphenylhydantoin to the p-hydroxy derivative, presumably via arene oxide formation, yet teratogenicity is significantly decreased.

2. If maternal basal drug metabolising activity is lowered (purified diet study or inter-strain differences of metabolism) the incidence of diphenylhydantoin related abnormalities is increased.

In vitro studies with the whole mouse embryo or cultures of differentiating rat embryo mid brain and limb cells appear to indicate that diphenylhydantoin is a direct acting teratogen not requiring metabolic activation by medium supplements of metabolising enzymes (prepared from homogenates of adult rat liver - S9 fraction). But direct toxicity of diphenylhydantoin in vitro does not confirm the absence of metabolic activation, since the cultured embryo or its
dissociated cells, may metabolise the compound. Some of the conflicting in vivo data might be explained in terms of the amount of unchanged diphenylhydantoin or reactive metabolite that reaches the embryo. The question of whether diphenylhydantoin is a proteratogen or ultimate teratogen remains unanswered on the basis of the in vivo data alone and will only be fully answered by additional studies with cultures of embryos or embryo cells.

Evidence has only recently begun to accumulate that, during organogenesis, the mammalian embryo is capable of drug metabolism.\textsuperscript{11,12} We present here four new lines of evidence using cell cultures which confirm that rat embryo limb buds are able to metabolise drugs. We also show that diphenylhydantoin is metabolised by the embryo cells in vitro but this appears to be a partial detoxification.

METHODS

Compounds: Test compounds of the highest purity commercially available were used. Diphenylhydantoin, ellipticine, cimetidine, 3-methylcholanthrene, phenobarbitone and β-napthoflavone were obtained from Sigma Chemical Company, Poole, Dorset, U.K. Benzimidazole was obtained from Fluka, A.G. Buchs, Switzerland. m-hydroxydiphenylhydantoin, p-hydroxydiphenylhydantoin and trichloropropene oxide were obtained from Aldrich Chemical Company Ltd., Dorset, U.K. Ranitidine hydrochloride was a gift from Glaxo Research Group, Ware, Herts, U.K. and diphenylhydantoic acid was synthesised by the Organic Chemistry Department at ICI, Alderley Park. Culture media and sera were obtained from Gibco Biocult, Paisley, Scotland.

Preparation of rat limb cell cultures: The technique has been previously described in detail by Flint and co-workers.\textsuperscript{4,13,14} Essentially, embryos of 34-36 somites are collected from pregnant female rats approximately 13 days after conception. Forelimbs are dissected free and following enzymatic dissociation, single cell suspensions are prepared in culture medium (Ham's F12 plus 10% fetal
Cell suspensions are adjusted to give $2 \times 10^7$ limb cells per ml. Ten or 20 ul drops are delivered to culture dishes (normally 5 drops per 35 mm diam. culture dish).

Sufficient replicate dishes are prepared for all subsequent treatments. The cells in each drop are allowed to settle and adhere to the dish over two hours at 37°C. The dish is then flooded with excess culture medium which may be supplemented with the test substance or vehicle control. The adhering cells form separate micromass islands of from 6-8 mm diameter. These are cultured for 5 days at 37°C and 5% CO$_2$ in air.

During the five days of culture a proportion of the cells in each micromass island differentiate to form small foci of chondrocytes. After fixation these foci are preferentially stained by alcian blue becoming regions of high contrast easily detectable with an automated image analyser. Counts of differentiated foci are compared in test substance exposed and control cultures. A concentration dependent inhibition profile is prepared and from this the concentration (IC50) inhibiting differentiation by 50% control values is estimated.

Immunocytochemical staining of cytochrome P450 in limb cultures: The peroxidase anti-peroxidase technique described by Wolf et al. was used on cultures of limb cells after 5 days incubation. Fresh cultures were stained against a variety of anti-rat cytochrome P450 antibodies. Polyclonal phenobarbitone raised P450, β-naphthoflavone raised P448 and clofibrate raised P452 were a gift from Dr G Gibson, Biochemistry Department, University of Surrey. The monoclonal β-naphthoflavone raised antibody 3:4:2 was a gift from Central Toxicology, Alderley Park, Macclesfield Cheshire and Hammersmith Hospital, London. The polyclonal 3-methylcholanthrene raised P448 and phenobarbitone raised P450 antibodies were a gift from Dr C R Wolf, Pharmacology Department, Western General Hospital, Edinburgh.

Transplacental Induction of Embryo Metabolising Enzymes:
Dams were dosed in vivo and the embryos subsequently removed at day 13 of gestation. Cultures were prepared as described. The dosing schedules were: 3 methylcholanthrene (in arachis oil) 25 mg/kg, i.p., days 10-12 gestation; β-napthoflavone (in arachis oil) 35 mg/kg, i.p. days 10-12 gestation; 0.1% sodium phenobarbitone was supplied ad libitum in the drinking water, days 6-13 gestation. These dosing regimes were known to raise hepatic cytochrome P450 levels in the dam.

RESULTS

Activation of cyclophosphamide by rat embryo cells in vitro:
Cyclophosphamide has no inhibitory effect on limb cell differentiation (number of differentiated foci) unless a preparation of arochlor 1254 induced actively metabolising adult rat liver enzymes (S9 plus NADPH) is present (Fig 1). Thus cyclophosphamide is an excellent positive control for bioactivation studies. Following transplacental induction by β-napthoflavone differentiation was inhibited by cyclophosphamide in limb cell cultures (Fig 2) suggesting the presence of (functional and) inducible cytochromes P450. Neither transplacental exposure to 3-methylcholanthrene nor phenobarbitone affected cyclophosphamide toxicity.

Identification of cytochrome P450 isozymes in limb cultures:
Constitutive levels of cytochrome P450 (phenobarbitone type inducible) and P448 (3-methylcholanthrene type inducible) were detected in 5 day cultures (Table 1). Interestingly, transplacental induction was only observed with β-napthoflavone (inducing a P448 type of isozyme). Neither 3-methylcholanthrene nor phenobarbitone induced isozyme levels above the constitutive. These observations are in close agreement with the ability of these inducers (see above) to provoke cyclophosphamide metabolism by the embryo cells. The possibility that 3-methylcholanthrene and phenobarbitone do not reach the embryo in sufficient quantities is being investigated.

Modulation of cytochrome P450 activity in vitro using diphenylhydantoin as a model teratogen: Diphenylhydantoin appears to be equitoxic to rat limb cells (IC50: 91 ± 4 ug/ml) with its major p.hydroxy (IC50: 80 ± 9 ug/ml) and minor m.hydroxy (IC50: 84 ± 9 ug/ml) metabolites.
The ring opened diphenylhydantoic acid is non toxic up to its maximum solubility in culture medium (250 ug/ml) and may represent a detoxification pathway. The acid is excreted in limited quantities in the urine of laboratory animals and man but certain esters of the acid formed in vivo can undergo rapid ring closure at body temperature and physiological pH to form diphenylhydantoin, thus giving a false impression that the acid is a minor metabolite.

A number of inhibitors of cytochromes P448/P450, including cimetidine (Fig 3), increased the toxicity of diphenylhydantoin in vitro when co-incubated at concentrations previously determined to be non cytotoxic (Table 2).

The increase in toxicity appeared to be specifically caused by inhibition of (cytochrome P450 mediated) metabolism of diphenylhydantoin since cimetidine had no effect on the toxicity of the major metabolite p-hydroxydiphenylhydantoin (Fig 4).

Ranitidine, a non cytochrome P450 inhibiting pharmacological analogue of the inhibitor cimetidine did not alter the toxicity of diphenylhydantoin confirming that increase in toxicity is not a non specific effect.

Trichloropropene oxide was non inhibiting thus arguing against arene oxide formation being responsible for toxicity (Table 2) and suggesting, also that if the hydroxy metabolites are formed in vitro it is by direct ring hydroxylation.

Since the p-hydroxy metabolite is toxic in vitro it cannot be argued that diphenylhydantoin is the only potential ultimate teratogen but on the evidence of the inhibitor studies it may actually be the most toxic entity. Direct ring hydroxylation in vitro however probably forms the still toxic p-hydroxy metabolite. The toxicity of both the parent compound and its major metabolite may go some way towards explaining apparently conflicting in vivo studies of teratogenicity and diphenylhydantoin metabolism (see Introduction).
Structure-activity relationships among triazole antifungals: Some triazole antifungals (basic structure in Tables 3,4) are teratogenic.\(^{17}\) Toxicity appears to be directly related to both lipophilicity and the type of substituent in the phenyl rings. Substitution at the 4-position with metabolically stable and/or electron withdrawing groups reduces triazole toxicity in vitro apparently by protecting the phenyl ring from metabolic attack (Table 3). Compared to the 2-position the exposed 4-position appears to be most vulnerable to metabolic attack, unless protected by a suitable substituent (Table 4). The data suggest that toxicity in vitro is mediated by the formation of a toxic intermediate (quinone or arene oxide) possibly via metabolic attack on the 4 position in the phenyl ring.

DISCUSSION AND CONCLUSIONS

In view of the potential for embryonic xenobiotic metabolism during organogenesis results from in vivo studies into the mechanisms of teratogenesis by co-administration of metabolic modulators with the teratogen can often provide confusing results. Two factors need to be determined: i) the concentration of the compound in the embryo and ii) the distinction between the contribution of maternal and embryonic metabolism. The relevance of these two factors is best studied under in vitro conditions with either cultures of whole embryos or embryo cells.

Flint and Orton\(^4\) found that 24 out of 25 teratogens screened in vitro directly inhibited rat embryo cell differentiation implying that most teratogens are direct acting and that metabolism is not generally required for teratogenic toxicity. This impression must in at least some cases not be substantiated given the evidence of the present study that embryo cells themselves have the potential to metabolise xenobiotics. Many teratogens (e.g. aldrin, diazepam, diethylstilboestrol) are in fact extensively metabolised in vivo. All of the possible drug metabolising enzymes cannot be provided in vitro, but in vitro tests, particularly the bacterial tests for detecting mutagens, have routinely used supernatants prepared from homogenates of adult male rat liver plus the necessary cofactors (S9-mix). We found S9-mix to be toxic to eukaryotic mammalian cells,\(^4\) permitting two hours exposure to 50-100mu/S9 mix/ml culture medium or 5 days exposure to 3 ul S9 mix/ml culture medium. Clearly this is an
inadequate model of chronic drug exposure to pregnant female animals.

We are now investigating routine transplacental induction of embryo cytochrome P450 by phenobarbitone, β-naphthoflavone or aroclor 1254 as an alternative method of activating test compounds. The possibility that the dam detoxifies the compound prior to reaching the embryo may in some cases limit the predictivity of the wholly in vitro test. An ex vivo test, culturing embryo cells following exposure to test compound in vivo, has been proposed as a suitable method of accounting for this aspect of maternal metabolism.

The authors are grateful to Dr T C Orton for his constructive advice during this project.


### Table 1. Immunocytochemical Identification of Cytochrome P450 in Cultures of Rat Embryo Limb Cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Untreated</th>
<th>3-Methylcholanthrene</th>
<th>Phenobarbital</th>
<th>B-Naphthoflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wolf/polyclonal Anti-3MC raised P448 (3MC1) (Levin form d)</td>
<td>+</td>
<td>(+)</td>
<td>NT</td>
<td>++</td>
</tr>
<tr>
<td>Wolf/polyclonal Anti-3MC raised P448 (3MC2) (Levin form c)</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>++</td>
</tr>
<tr>
<td>CTL/Hammersmith monoclonal 3:4:2 Anti-PNF raised P448</td>
<td>+</td>
<td>+</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
</tr>
<tr>
<td>Gibson/polyclonal Anti-PB raised P450</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Wolf/polyclonal Anti-PB raised P450 (PB1)</td>
<td>(+)</td>
<td>NT</td>
<td>(+)</td>
<td>NT</td>
</tr>
<tr>
<td>Wolf/polyclonal Anti-PB raised P450 (PB2)</td>
<td>++</td>
<td>NT</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>Wolf/polyclonal Anti-PB raised P450 (PB3) (Levin form b)</td>
<td>(+)</td>
<td>NT</td>
<td>(+)</td>
<td>NT</td>
</tr>
</tbody>
</table>

a. After five days of culture. Peroxidase stain was particularly associated with differentiating cells at the edges of chondrogenic foci.

b. 0 = absent, (+) = less than minimal, but present, + = minimal, ++ = moderate, +++ = very strong.

c. NT = not tested
<table>
<thead>
<tr>
<th>Modulator</th>
<th>Concentration(^a) (ug/ml culture medium)</th>
<th>IC50(^b) of Diphenylhydantoin (ug/ml culture medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>91 ± 4 ug/ml</td>
</tr>
<tr>
<td>Cimetidine (mainly P450 inhibitor)</td>
<td>400</td>
<td>47 ± 3*</td>
</tr>
<tr>
<td>Ranitidine (non P450 inhibiting analogue of cimetidine)</td>
<td>250</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>Metyrapone (P450 inhibitor)</td>
<td>10</td>
<td>30 ± 4*</td>
</tr>
<tr>
<td>Benzimidazole (broad spectrum P450 inhibitor)</td>
<td>200</td>
<td>16 ± 6*</td>
</tr>
<tr>
<td>Ellipticine (P448 inhibitor)</td>
<td>0.001</td>
<td>27 ± 2*</td>
</tr>
<tr>
<td>Trichloropropene oxide (Epoxide hydratase inhibitor)</td>
<td>1</td>
<td>90 ± 4</td>
</tr>
</tbody>
</table>

\(^a\) Highest concentration having no effect on limb cell growth or differentiation in vitro.

\(^b\) Concentration causing 50% reduction from control values in number of differentiated foci. *:Inhibition was significant with p < 0.5 (Student's t-test).

**TABLE 2. EFFECT OF COINCUBATION WITH SOME CYTOCHROME P450 INHIBITORS AND OTHER COMPOUNDS ON TOXICITY OF DIPHENYLDANTOIN.**
### Table 3:
Effect of substitution in antifungal triazole compounds with groups of increasing metabolic stability and/or electron withdrawing character on inhibition of differentiation in cultures of rat embryo limb cells. Inhibition is measured as the IC50 (concentration inhibiting differentiation by 50% control value).
Bistriazole

<table>
<thead>
<tr>
<th>Substituent ($R_2$)</th>
<th>IC50 (nMoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2Cl</td>
<td>85.11</td>
</tr>
<tr>
<td>4Cl</td>
<td>131.83</td>
</tr>
<tr>
<td>2CH₃</td>
<td>58.88</td>
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<tr>
<td>4CH₃</td>
<td>354.81</td>
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<tr>
<td>2CF₃</td>
<td>89.12</td>
</tr>
<tr>
<td>4CF₃</td>
<td>416.87</td>
</tr>
<tr>
<td>2CF₃,4F</td>
<td>177.83</td>
</tr>
<tr>
<td>2F,4CF₃</td>
<td>616.59</td>
</tr>
</tbody>
</table>

Table 4:
Effect of ortho compared to para substituents in antifungal triazole compounds on inhibition of differentiation in cultures of rat embryo limb cells (IC50: see table 3).
Fig 1. Differentiation in cultures of rat embryo limb cells following exposure to cyclophosphamide in the presence of metabolising enzymes (S9-mix) prepared from adult rat liver homogenate. 3 ul of complete S9-mix was added to each ml of culture medium. S9-mix was S9-fraction and phosphate buffer (0.07 M, pH 7.4) in a ratio of 3 : 7 v/v, with or without 10 mM NADPH (prepared in the buffer).

Fig 2. Activation of cyclophosphamide to a toxic metabolite by cultured rat embryo limb cells following transplacental induction of the embryos with β-naphtoflavone (see Methods).

Fig 3. Inhibition of differentiation in cultures of rat embryo limb cells following exposure to increasing concentrations of diphenylhydantoin in the presence or absence of the cytochrome P450 inhibitor cimetidine.

Fig 4. Inhibition of differentiation in cultures of rat embryo limb cells following exposure to the major metabolite of diphenylhydantoin, p-hydroxydiphenylhydantoin, in the presence or absence of the cytochrome P540 inhibitor cimetidine.
Number of differentiated foci

- S9 - NADPH
- S9 + NADPH

µg cyclophosphamide / ml culture medium
Control cultures not pretreated with βNF in vivo

IC50 = 44 μg/ml

μg cyclophosphamide/ml culture medium

Number of differentiated foci

150 75 0
Number of differentiated foci

- + cimetidine (400 μg/ml)
- cimetidine

IC50: 39 μg/ml
IC50: 87 μg/ml

μg diphenylhydantoin / ml culture medium
Number of differentiated foci

IC50: 79 μg/ml
IC50: 77 μg/ml

μg p-hydroxydiphenylhydantoin / ml culture medium
RAT EMBRYO LIMB CELLS IN CULTURE: ABILITY TO METABOLISE XENOBIOTICS

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INTRODUCTION

One significant problem in determining the mechanism of teratogenesis is the nature of the ultimate toxin; whether it is a metabolite or the compound itself.

Coadministration to the dam of the teratogen and various inhibitors or inducers of drug metabolism followed by examination of the fetuses for changes in the incidence of terata suggests that in some cases metabolic activation might be important. For example the epoxide hydratase inhibitor trichloropropene oxide (TCP0) doubles the incidence of cleft lip and palate in Swiss ICR mice following a single dose (50mg/kg) of diphenylhydantoin. A toxic arene oxide intermediate appears to be the ultimate teratogen. On the other hand if the basal activity of maternal cytochrome P450 is enhanced by the inducer phenobarbitone or diminished by purified diet diphenylhydantoin related abnormalities are correspondingly reduced or increased. These experiments suggest that a metabolite is not involved; diphenylhydantoin appears to be the ultimate teratogen.

These conflicting conclusions result from an interpretation which assumes the dominance of maternal metabolism. The embryo is seen as inertly subject to maternally determined changes in the level of compound and metabolite. The embryo is not however metabolically inert, and metabolism of xenobiotics by the embryo cells themselves may be of profound importance in teratogenesis.

One way of separating the influence of maternal from embryo metabolism is to maintain the embryo or its component cells in culture. The micromass technique is a method of culturing rat embryo limb or midbrain cells so that many aspects of cell differentiation related to normal embryogenesis are maintained in vitro. This paper describes preliminary experiments aimed at defining the cytochromes which are metabolically active in limb micromass cultures. Analysis of the in vitro toxicity of vitamin A and some related compounds, or of the glycol ethers suggests that there are limits to the ability of embryo cells to metabolise xenobiotics.
METHODS

Compounds: Test compounds of the highest purity commercially available were used. The glycol ethers and their metabolites (alkoxyacids) were a gift of Dr Nigel Brown, MRC, Carshalton, Surrey.

Preparation of rat limb cell cultures: The technique has been previously described in detail by Flint and co-workers.\textsuperscript{10,11} Essentially, embryos of 34-36 somites are collected from pregnant female rats approximately 13 days after conception. Forelimbs are dissected free and following enzymatic dissociation, single cell suspensions are prepared in culture medium (Ham's F12 plus 10% fetal calf serum). Cell suspensions are adjusted to give $2 \times 10^7$ limb cells per ml. Ten or 20 ul drops are delivered to culture dishes (normally 5 drops per 35 mm diam. culture dish).

Sufficient replicate dishes are prepared for all subsequent treatments. The cells in each drop are allowed to settle and adhere to the dish over two hours at 37°C. The dish is then flooded with excess culture medium which may be supplemented with the test substance or vehicle control. The adhering cells form separate micromass islands of from 6-8 mm diameter. These are cultured for 5 days at 37°C and 5% CO\textsubscript{2} in air.

During the five days of culture a proportion of the cells in each micromass island differentiates to form small foci of chondrocytes. After fixation these foci are preferentially stained by alcian blue becoming regions of high contrast easily detectable with an automated image analyser. Counts of differentiated foci are compared in test substance exposed and control cultures. A concentration dependent inhibition profile is prepared and from this the concentration (IC\textsubscript{50}) inhibiting differentiation by 50% control values is estimated.

Cell survival is also estimated spectrophotometrically as the non specific uptake by all surviving cells of the dye neutral red. An IC\textsubscript{50} is also estimated from the concentration dependent survival curve.

Prediction of teratogenic hazard in vitro

We have found the following guidelines useful for the interpretation of
IC50 values whenever a concentration response curve indicates toxicity of the test compound.

1. Potent teratogens (e.g. aldrin, azaguanine, colchicine, cyloheximide, diethylstilbestrol, 5 flourouracil) all inhibit differentiation with an IC50 below 10 ug/ml. Weak or poorly substantiated teratogens (e.g. aspirin, diazepam, sulfisoxazole) have IC50's greater than 50 ug/ml. As a general rule IC50's less than 100 ug/ml are indicative of teratogenic hazard, becoming strongly indicative if less than 50 ug/ml.

2. The IC50 of the model teratogen vitamin A in limb cultures (Table 2) for cell survival (3.5 ug/ml) is 500 fold higher than the IC50 for cell differentiation (0.007 ug/ml) suggesting highly selective toxicity for cell differentiation. This sign of teratogenic hazard may be particularly indicative of those teratogens which will cause fetal abnormalities at doses which may not be significantly toxic to the mother, and is therefore considered to be an important criterion.

3. Teratogens are characterised by their selective toxicity for different organs (e.g. hydrocortisone and diphenylhydantoin - orofacial clefts; thalidomide - limb reduction). Therefore a difference of IC50 between midbrain and limb cultures may be taken as a further indication of teratogenic hazard.

Immunocytochemical staining of cytochrome P450 in limb cultures: The peroxidase anti-peroxidase technique described by Wolf et al. was used. Fresh cultures were stained against a variety of anti-rat cytochrome P450 antibodies. Polyclonal phenobarbitone raised P450, and napthoflavone raised P448 were a gift from Dr Gibson, Biochemistry Department, University of Surrey. The monoclonal β-napthflavone raised antibody 3:4:2 was a gift from Central Toxicology, Alderley Park, Macclesfield, Cheshire and Hammersmith Hospital, London. The polyclonal 3-methylcholanthrene raised P448 and phenobarbitone raised P450 antibodies were a gift from Dr C R Wolf, Pharmacology Department, Western General Hospital, Edinburgh.
Transplacental Induction of Embryo Metabolising Enzymes: Dams were dosed with inducers in vivo and the embryos subsequently removed at day 13 of gestation. Cultures were prepared as described. The dosing schedules were: 3-methylcholanthrene (in arachis oil) 25 mg/kg, i.p., days 10-12 gestation; β-naphthoflavone (in arachis oil) 35 mg/kg, i.p. days 10-12 gestation; 0.1% sodium phenobarbitone was supplied ad libitum in the drinking water, days 6-13 gestation. These dosing regimes were known to raise hepatic cytochrome P450 levels in the dam.

RESULTS

Localisation of cytochromes P450/P448 by antibody staining: Staining of 5 day cultures of rat embryo midbrain and limb cells with antibodies to cytochromes P450 and P448 reveals constitutive levels of both isozymes. Following transplacental exposure 3-methylcholanthrene (3MC) slightly induces the P448 isozyme. β-napthoflavone (BNF) is a much stonger transplacental inducer of the embryo cytochrome P448. The cytochrome P450 isozyme is not inducible by any of the inducers to which the embryo was exposed. Isoenzymes which were inducible transplacentally were also directly inducible in vitro but to a lesser extent, as gauged by the intensity of anti-peroxidase stain.

Further studies with these antibodies now reveals that the cytochrome P450 antigen is present at constitutive levels from day 1-2 of culture but that cytochrome P448 only appears by day 3-4 of culture. Staining against the cytochrome P448 antigen is most intense around the periphery of the chondrogenic foci in limb cultures.

Drug metabolism in vitro by rat embryo cytochromes P450/P448: The proteratogen cyclophosphamide is not toxic to induced cultured cells unless a preparation of rat liver metabolising enzymes is present. Transplacently induced cells (BNF only, not 3MC or phenobarbitone-PB) are however able to metabolise cyclophosphamide to its toxic metabolite suggesting that the induction not only elevates the levels of cytochrome P448 but induces its synthesis at an earlier stage in culture than day 3-4. Antibody staining of induced cultures is underway to demonstrate the precocious appearance of cytochrome P448. There appear to be differences between the enzyme antigenically defined as cytochrome P448 in the embryo and that in the adult, because metabolism of
cyclophosphamide is normally associated with cytochrome P450 isoenzymes. The toxicity of diphenylhydantoin (another teratogen) is also increased following transplacental exposure to 3MC and to BNF, but not to PB (Table 1).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Limb culture IC50 ug/ml</th>
<th>S\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>None\textsuperscript{b}</td>
<td>91 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>Phenobarbitone\textsuperscript{c}</td>
<td>90 ± 17</td>
<td>-</td>
</tr>
<tr>
<td>3-methylcholanthrene</td>
<td>73 ± 6</td>
<td>*</td>
</tr>
<tr>
<td>Beta naphthoflavane</td>
<td>64 ± 14</td>
<td>*</td>
</tr>
</tbody>
</table>

a. Statistical significance (t test): * = \( p < 0.05 \).
b. Brown et al, 1986 (average of eight measurements).\textsuperscript{6}
c. IC50 for each inducer is the average of three experiments.

TABLE 1. IC50 of rat embryo limb cultures exposed to diphenylhydantoin following transplacental exposure to a variety of cytochrome P450/P448 inducers.

This data strongly suggests that diphenylhydantoin toxicity in vitro may in part be mediated by a toxic metabolite following induction by 3MC/BNF but not PB. In fact the major p-hydroxy metabolite is at least equitoxic in vitro with the parent compound\textsuperscript{6}. A toxic epoxide intermediate may not be involved, because the epoxide hydratase inhibitor TCPO added in vitro does not effect the IC50 of diphenylhydantoin even though covalent binding to limb cell protein appears to increase substantially (10-20 fold) in the presence of TCPO (unpublished results) indicating activity of the enzyme in the embryo. In addition glutathione and n-acetyl cysteine which would be expected to react non-enzymatically with a DPH epoxide do not affect toxicity in vitro (unpublished results).

A variety of inhibitors of cytochrome P450/P448 metabolism (benzimidazole, cimetidine, metyrapone, ellipticine) however, increase the toxicity of diphenylhydantoin when coincubated in vitro\textsuperscript{7}, suggesting that the parent compound is the ultimate toxin.

This apparently conflicting evidence may be resolved by the hypothesis (Fig 1) that both diphenylhydantoin and its major metabolite are
responsible for toxicity in vitro but that toxicity is diminished because of the loss of intermediate reactive metabolites (probably epoxides) which bind to non essential proteins or form a non toxic dihydrodiol. Induction of cytochrome P450 increases the rate of production of the toxic p-hydroxy metabolite, whereas inhibition increases steady state levels of the toxic parent compound; thus in both cases toxicity will increase. TCPO causes more epoxide to be lost by binding to non essential proteins but does not effect an accumulation of diphenylhydantoin so that toxicity does not change.

The embryo itself may thus modulate the toxicity of diphenylhydantoin. The degree of teratogenicity in vivo must in addition depend on maternal detoxification and elimination; i.e. the amount of either parent compound or unchanged p-hydroxy metabolite which reach the embryo. This may go some way to explaining the apparently contradictory outcome of experiments (outlined in the introduction) where diphenylhydantoin was coadministered with inhibitors or inducers of maternal cytochrome P450 activity or the inhibitor of epoxide hydratase, TCPO. Clearly inhibiting the activity of cytochrome P450 will lead to increased amounts of diphenylhydantoin reaching the embryo and thus increase the teratogenicity. Inducers, such as phenobarbitone essentially promote detoxification decreasing teratogenicity because less parent compound reaches the embryo. The increased incidence of the teratogenicity in the presence of TCPO suggests teratogenicity via a toxic arene oxide intermediate, but this intermediate has not been detected and must be so short lived that unless it is generated in the embryo its toxic effect will not be observed. An epoxide intermediate generated by the embryo cells themselves does not appear to lead to increased toxicity (see above, results with TCPO in vitro). It can only be speculated that the effect of TCPO in vivo is to inhibit maternal detoxification via dihydrodiol formation leading to increased toxicity because of increased levels of parent compound (or even possibly the p-hydroxy metabolite following direct ring hydroxylation).

Limitations to xenobiotic metabolism with cultured rat embryo cells:

1. Synthetic retinoids, such as etretinate have recently been used with
some success in the treatment of non-malignant dermatological disease in man. Vitamin A itself is a model teratogen as is etretinate and therefore the teratogenicity of all compounds related to vitamin A has come into question. \( \beta \)-carotene (Fig 2) a major dietary source of vitamin A, is a symmetrical molecule which is broken down in vivo from two molecules of vitamin A. \( \beta \)-carotene is without toxicity in vitro, though vitamin A itself is a potent inhibitor of limb cell differentiation in vitro at concentrations one thousand fold lower than those killing cells (Table 2). Thus the cells do not appear to possess the \( \beta \)-carotene 15,15' oxygenase which in vivo would metabolise \( \beta \)-carotene to the toxic vitamin A. This enzyme is found in the adult only in the intestine and the liver, and in agreement with our data, has not been reported in early embryonic tissue.

Vitamin A (acid or alcohol) consists of a cyclohexene ring attached to a polyene fatty acid or alcohol side chain (Fig 2). Modifications to the side chain such as an amide rather than an acid or alcohol termination appear (at least in the hamster) to reduce or eliminate teratogenicity. It is thus pertinent to ask about the contribution of the fatty acid/alcohol side chain and the cyclohexene ring to the teratogenicity of the vitamin A molecule. The ring itself with a greatly truncated side chain (\( \beta \)-ionone, Fig 2) is without teratogenic hazard in vitro (Table 2). Crocetin is a symmetrical polyene dicarboxylic fatty acid which is essentially \( \beta \)-carotene without the cyclohexene rings (Fig 2). Crocetin is toxic in vitro but at higher concentrations than vitamin A and with no specific toxicity for cell differentiation (Table 2). Thus specific inhibition of differentiation in vitamin A only arises when the fatty acid or alcohol is linked to the cyclohexene ring. Vitamin A alcohol or acid exert their physiological effects on the cell following binding to intracellular retinol or retinoic acid binding proteins. This binding is of course conformationally specific to the vitamin A molecule and would not be expected for a polyene fatty acid such as crocetin. Though the toxicity of vitamin A appears to be related to the structure of its side chain this toxicity does not appear to be revealed (at least as a specific teratogenic hazard in vitro) unless promoted by binding of the molecule to a retinol binding protein.
This protein appears to ubiquitously present in vivo and in many cultured cell lines.\(^{19}\)

<table>
<thead>
<tr>
<th>IC50 (ug/ml)</th>
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<tr>
<td>CELL DIFFERENTIATION(^a)</td>
</tr>
<tr>
<td>(\beta)-Carotene</td>
</tr>
<tr>
<td>Vitamin A, alcohol</td>
</tr>
<tr>
<td>(\beta)-ionone</td>
</tr>
<tr>
<td>Crocetin</td>
</tr>
</tbody>
</table>

\(a\). Number of differentiated chondrogenic foci per micromass island. \\
\(b\). Neutral red spectrophotometric estimation of surviving cells. \\
\(c\). No effect to maximum concentration on cell differentiation or cell survival.

Table 2. Effect of vitamin A and related compounds on cell differentiation and cell survival in rat embryo limb cells after five days in culture.

2. Methoxy ethanol (ME) is teratogenic in the rat\(^{20}\). It is however without effect on the cultured rat embryo limb cells up to 5000 ug/ml (Table 3). Methoxyacetic acid (MA) which is generated in vivo by alcohol dehydrogenase from methoxy ethanol\(^ {21,24}\) is toxic in vitro (Table 3) specifically inhibiting cell differentiation, confirming the observation that in vivo teratogenicity is probably mediated by this metabolite\(^ {22}\). As the chain length of the principle metabolite increases up to butyl (Table 3) so the concentration needed to inhibit cell differentiation increases. This appears to be a relationship that is limited to cell differentiation because evidence on the in vitro cytolethality\(^ {23}\) of the glycol ethers suggests that toxicity increases with chain length (ethoxyacetate is more cytotoxic than methoxyacetate, Table 3).

In other words the toxicity we observe in vitro is not simply the result of cell killing but probably related specifically to the teratogenicity of those compounds. Propoxyethanol\(^ {25}\) and butoxyethanol\(^ {26}\) are not teratogenic in vivo. This is clearly
supported by the lack of in vitro toxicity of the major acid metabolites (Table 3).

The in vitro toxicity of ethoxyethanol (Table 3) at first suggests the possible generation of its toxic metabolite by endogenous alcohol dehydrogenase. This however, is unlikely to be the case because there is no preferential inhibition of cell differentiation as with the acid metabolite. In addition the IC50 of the parent compound is three fold greater at 7.4 mM, a concentration which is unlikely to occur in vivo since serum concentrations appear to saturate around 5 mM (P. Foster - Personal communication).

The in vitro data demonstrates that the embryo cells are not equipped with alcohol dehydrogenase. The acid metabolites are long lived (i.e. can be detected in the urine) and the teratogenicity of the glycol ethers in vivo may thus not be mediated by embryo metabolism. One final point arising out of this data is that though usually IC50's less than 100 ug/ml may be indicative of teratogenic hazard (see methods) this should not be taken as a hard and fast rule. With some compounds, such as the glycol ethers, far greater concentrations are achievable in vivo. The most important criterion appears to be preferential inhibition of cell differentiation.

CONCLUSION

Rat embryo limb cells in vitro contain at least one if not more functional and inducible isozymes of cytochrome P448 which are capable of metabolising xenobiotics to toxic metabolites. Experiments with both diphenylhydantoin and cyclophosphamide strongly suggest that rat embryo limb cytochromes P448 inducible by 3MC and BNF are functional xenobiotic metabolising enzymes. Flint and Orton10 found that of 27 teratogens most of which are actively metabolised in vitro 25 were directly toxic to the cultured cells, and thus metabolism did not appear to be a significant factor. This conclusion may have to be revised if the embryo cells themselves are potent sources of functional metabolising enzymes.

Not all teratogenicity appears to be mediated by cytochrome P450 metabolism. Maternal enzymes responsible in vivo for metabolism of beta carotene or methoxyethanol to the teratogenic metabolites vitamin A or methoxyacetic acid are absent from the embryo cells. The in vitro
results with micromass cultures highlight the separation of non toxic parent compound from the toxic metabolite in a simple way which would be difficult to assess in vivo. It should be possible to use these cultures to investigate problems of metabolic activation of teratogens in a more systematic way.

The authors would like to thank Mrs K Jones for her technical assistance, Dr Nigel Brown for alkoxy alcohols and metabolites and Dr T C Orton for helpful discussions of the manuscript.
<table>
<thead>
<tr>
<th></th>
<th>Limb IC50 (mM)</th>
<th>Max Conc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Whole embryo culture&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell Differentiation</td>
<td>Cell Survival</td>
<td>(mM)</td>
</tr>
<tr>
<td>2 Methoxyethanol (CH₃OCH₂CH₂OH)</td>
<td>NE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NE</td>
<td>65.7</td>
</tr>
<tr>
<td>2 Ethoxyethanol (CH₃CH₂OCH₂CH₂OH)</td>
<td>7.4</td>
<td>9.9</td>
<td>55.5</td>
</tr>
<tr>
<td>Methoxyacetate (CH₃OCH₂COOH)</td>
<td>2.5</td>
<td>18.2</td>
<td>55.5</td>
</tr>
<tr>
<td>Ethoxyacetate (CH₃CH₂OCH₂COOH)</td>
<td>3.6</td>
<td>13.8</td>
<td>48.0</td>
</tr>
<tr>
<td>Propoxyacetate (CH₃CH₂CH₂OCH₂COOH)</td>
<td>NE</td>
<td>NE</td>
<td>15.1</td>
</tr>
<tr>
<td>Butoxyacetate (CH₃CH₂CH₂CH₂OCH₂COOH)</td>
<td>NE</td>
<td>NE</td>
<td>16.9</td>
</tr>
</tbody>
</table>

- Maximum concentration tested in HAM's F12 Culture Medium (10% fetal calf serum) - equivalent to maximum solubility for metabolites.
- Results for whole embryo culture taken from Rawlings et al. Toxicology Letters 28, 49-58, 1985.
- No effect to maximum concentration used (5000 ug/ml)
- No published data available.

Table 3. Effect of glycol ethers (alkoxy alcohols) and alkoxy acid metabolites on cell differentiation and survival in cultured rat embryo limb cells.
REFERENCES


FIG 1 In vitro metabolism of diphenylhydantoin
In Vitro Metabolism of Diphenylhydantoin

**DIPHENYLHYDANTOIN**

P448 Inducers — increase toxicity
P448 Inhibitors — increase toxicity

Cytochrome P450

**EPOXIDE**

Non Specific Binding

Detoxification

TCPO — No effect on toxicity
- 10 - 20 fold increase in protein binding.

Epoxide Hydratase

Dihydrodiol

pOH—DIPHENYLHYDANTOIN
In Vitro Metabolism of Diphenylhydantoin

DIPHENYLHYDANTOIN

P448 Inducers — increase toxicity
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Non Specific Binding

Detoxification

Epoxide Hydratase

Dihydrodiol

TCPO — No effect on toxicity
— 10 - 20 fold increase in protein binding.

pOH—DIPHENYLHYDANTOIN
FIG 2  Metabolic pathway by which β-Carotene is converted in vivo into two molecules of vitamin A alcohol. The figure includes structures of β-Ionone and Crocetin
\[ \beta\text{-ionone} \xrightarrow{\text{Crocin}} \beta\text{-carotene 15,15'-oxygenase} \]
\[ \text{retinaldehyde reductase (+NADH)} \]
\[ \text{alcohol dehydrogenase} \]

Intestine, liver, eye

Retinol

\[ \text{CHO} \]

Intestine, liver

\[ \text{CH}_2\text{OH} \]
CHEMICAL TERATOGENESIS: TESTING METHODS
AND THE ROLE OF METABOLISM

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Key words: Teratogenesis - Testing Methods - Metabolism - Bioactivation

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1. INTRODUCTION

Several lines of evidence substantiate the key role of metabolism in chemical teratogenesis. Metabolic studies with inducers and inhibitors of the maternal mixed-function oxidase system (the cytochrome P-450 system) have shown that for some teratogens, metabolism plays a key role in the eventual teratogenic effect (1-3), and that inclusion of a metabolic activating system to in vitro teratogenicity tests is very important in order to reflect the in vivo situation (4,5). In addition it is possible to demonstrate that rodent (and probably human) embryos possess low levels of at least some of the drug metabolising enzymes, and that some of these enzymes are inducible by xenobiotics (6,7).

In addition, the role of maternal and embryonic activating reactions on the teratogenic outcome in vivo depends on several factors including the half life of the active metabolite, the fetal genotype, and the maternal genotype.

In this review, a brief outline of the in vivo and in vitro teratogen tests is described, followed by examples of the key pieces of evidence to demonstrate the role of metabolism in bioactivation of teratogens.
1.1 TESTING METHODS FOR TERATOGENICITY

1.1.1 In Vivo:

The aim of in vivo test procedures is to assess the effects of chemicals on reproduction using common laboratory species as models. Testing has a number of common requirements:

(i) The chemical has to be tested for effects on general reproductive performance as well as embryo development.

(ii) Preferred species are the rat, mouse and rabbit. Other species may be used when differences in metabolism or toxicokinetics between the preferred species and man are known, or to clarify an ambiguous result.

(iii) Sufficient numbers of animals to permit statistical evaluation.

(iv) The route of administration should parallel the route by which man is to be exposed.

(v) Chemicals administered at several dose levels (usually three).

(vi) The lowest dose should produce no observable effects and the highest dose should be the maximum maternal tolerated dose (MTD).

(vii) Adequate concurrent control groups.

Detailed protocols from various countries and leading regulatory agencies can be found in references 8-13.

Chernoff and Kavlock (14) have proposed an in vivo screen (in the mouse) in which compounds are administered throughout organogenesis at, or near MTD, and the newborn are examined for number and weight gain both at birth and three days after parturition. The rationale is that prenatal insults
will manifest themselves postnatally as reduced viability and/or impaired growth up to 250 days post-partum (15). There was a good correlation with known teratogens (100%, 15 out of 15), but also a high incidence of false positive results with non-teratogens (33%, 3 out of 9). A recent report (16) suggests that maternal toxicity itself may result in embryotoxicity, and since the Chernoff and Kavlock screen uses doses at or near the MTD this could account for the high percentage of false positives reported. This latter test is more expensive and time consuming than the in vitro screens described below, but is not necessarily more predictive.

1.1.2 In vitro screens for teratogens and role of metabolism:-

In the quest for new, more pharmacologically active drugs, the need for early hazard assessment in the screening programme is essential. The answer would be an ideal in vitro test system that would be simple, rapid, yield a large number of interpretable and reproducible results (giving few 'false negatives' and 'false positives') and retain some relevance to mechanisms of teratogenesis. In vitro teratogen tests have problems that are not found with other in vitro toxicity tests. Firstly, the toxicological target is the embryo, a mass of rapidly dividing cells, simultaneously differentiating along divergent lines. As far as possible the test should reproduce these aspects of embryogenesis. Secondly, both maternal metabolism of xenobiotics and fetal-placental-maternal interactions are usually not present in vitro. Since some teratogens may require bioactivation to the active form, the inclusion of a reliable metabolising system or source of metabolites in the screen would extend the utility of the test procedure. This has been achieved as follows:

(i) Incorporation of serum from chemical-exposed animals. Serum has been obtained from animals administered the test compound and then added to the culture medium for whole embryo cultures and limb bud
organ cultures. Whole rat embryo cultures were unaffected by the
direct addition of cyclophosphamide (CPA) up to 800 µg/ml culture
medium, although serum from CPA pretreated (180 mg/kg) animals
was embryo-lethal after only a 1 hr incubation period (17). Similarly
culture medium supplemented with serum from procarbazine
pretreated rats (but not the compound added directly to the culture
medium) showed a response to the drug (18). Serum from human
patients receiving anticonvulsant therapy or chemotherapy has also
been included in the culture medium of the whole rat embryo culture
system and the induced teratogenic responses compared to control
serum (19,20). These examples suggest that serum provides a
satisfactory method of including metabolites into in vitro culture
systems, however it is not routinely used because of the added expense
in dosing and preparing heat inactivated serum.

(ii) Induction of maternal cytochrome P-450 isoenzymes. For example
whole rat embryos explanted from dams pretreated with
3-methylcholanthrene and subsequently cultured could bioactivate
2-acetylaminoﬂuorene to a teratogenic metabolite whereas uninduced
controls could not (7,21). Limb bud organ cultures from mice
pretreated in utero with Aroclor 1254 could bioactivate CPA, whereas
(non-treated) controls were refractory to the toxicity (22). Results
from our own laboratories have shown that cultures of rat embryo limb
bud cells from β-naphthoflavone pretreated dams could bioactivate
CPA to a species which inhibited differentiation (6), whereas
phenobarbitone or 3-methylcholanthrene pretreatment and control
tissue showed no metabolic activation of the proteratogen.

(iii) Tissues or target cells with an in vitro activation system. CPA was
activated to teratogenic metabolites by both rabbit and hamster
hepatocytes when co-cultured with whole rat embryos (23). Hamster
embryo cells (capable of both phase I and II metabolism) were successful in activating CPA to a toxic metabolite when co-incubated with mouse limb bud cultures (4).

Co-incubation with hepatic microsomes or S9 liver fractions plus cofactors, usually from Aroclor 1254-induced male rodents, has been widely used, resulting in the successful activation of various compounds including CPA, 2-acetylaminofluorene, 5-flurouracil, diphenylhydantoin and thalidomide in a number of in vitro screens including whole embryo culture (24-28), embryonic limb bud organ culture (29), differentiating embryo cells (30-33), inhibition of mouse ascite tumour cell attachment to lectin-coated surfaces (34-36) and differentiating human embryonic palatal mesenchymal cells (37).

The major disadvantages of the use of S9 fractions and cofactors as a source of metabolites are 1) the toxicity of the S9 mix to embryo tissue/cells in vitro; 2) the limited period of activity of the added activation system.

In addition we suggest the following methods that might be experimentally relevant but which have not yet been reported in the literature.

(i) In vitro exposure to inducers given that the embryo cells themselves contain inducible forms of the bioactivation enzymes (38).

(ii) Addition of S9 fractions from pregnant females, both control and preinduced.

(iii) Incubation of the test compound with an S9 fraction, then addition of the filtrate to the in vitro test system, thus circumventing the problem of the toxicity of S9 fractions in vitro.

(iv) Addition of purified enzyme components of the drug metabolising system.
The most satisfactory way of taking the fetal-maternal-placental unit into account is to dose the mother with the test compound, prior to removing the embryos for culture (39-41). This would then constitute a combined in vivo/in vitro approach. Beaudoin and Fisher (41) dosed pregnant rats either 24 hrs or 4 hrs prior to the recovery of day 10 embryos for in vitro culture and all compounds they tested inhibited the rate of development in vitro. Embryos from the 24 hr (prior to explant) dosed dams were more severely affected than those from the 4 hr dosed dams. Similarly, Flint et al. dosed pregnant rats 18 hrs prior to the recovery of day 13 embryos for the micromass cell culture assay and found that the 18 teratogens tested had significantly increased inhibition of differentiation in vitro compared to the 13 non-teratogens tested (39).

Several reviews on the subject of teratogen testing exist, covering the advantages, disadvantages and experimental techniques of all the major categories of in vitro screens (29,32,42-51). In general it appears that in vitro screens lack a reliable drug metabolizing/activating system. It has been suggested (50) that 59 hepatic fractions from Arochlor-1254 induced male Sprague-Dawley rats should be included in in vitro tests to standardise the activation system. Recently, the consensus workshop on in vitro teratogenicity testing recommended that test validation would be facilitated by testing specific xenobiotics with defined teratogenicity. Smith et al. (52) list 47 compounds which is intended to serve as a basis for in vitro screens in the hope of identifying systems which will contribute to a more effective test programme.
2. FACTORS INFLUENCING THE EXPRESSION OF A TERATOGENIC INSULT:

The expression of teratogenesis is influenced by several factors, including genetic, sex and interspecific differences in metabolic capability.

(i) Genetic: The Ah locus is a cluster of genes thought to be the centre of the genetic regulation of some of the hepatic cytochrome P450 dependent mono-oxygenases (53,54), metabolising aryl hydrocarbons. Benzo(a)pyrene is metabolized by cytochrome P450 mono-oxygenases under the control of the Ah locus and the degree of responsiveness/susceptibility to both benzo(a)pyrene teratogenesis and enzyme induction differs in two mice strains (C57/BL6 = responsive strain, AKR = non-responsive strain) (55,56). Responsive mice were found to have 50 times more hepatic cytosolic receptor (a protein controlling induction) than the non-responsive strain (57). Teratogens that show a correlation between strain sensitivity and increased Ah receptors in mice include 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (54,58,59), diazepam (60), glucocorticoids (58,61-64) diphenylhydantoin (58,65-70) and acetazolamide (71-73). However, caution is required in the interpretation of the apparent correlation between teratogenic susceptibility and Ah responsiveness, as the strains involved vary at innumerable loci in addition to the Ah locus. A further example of genetic variability was the case study of the discordant expression of the fetal hydantoin syndrome in heteropaternal dizygotic twins (74). The mother, an epileptic receiving diphenylhydantoin therapy, the heteropaternal twins despite the same in utero exposure discordantly expressed the fetal hydantoin syndrome.

(ii) Sex: The sex of the animal also has an influence on susceptibility to teratogenesis. Methylmercury is an established in vivo teratogen, and additionally decreases hepatic mono-oxygenase activity in the male
offspring only (75). In contrast, acetazolamide-induced teratogenicity seems to affect a greater percentage of female offspring (76). Similarly the synthetic oestrogen, diethylstilbestrol, therapeutically used between 1950-1970 in order to prevent threatened miscarriage during the first trimester, caused the female off-spring to develop vaginal adenocarcinomas (77,78), during sexual maturation (14-22 yrs old). In addition, male offspring similarly exposed pre-natally, have been reported to have reduced sperm counts and a high incidence of sterility (79), but no effect on hormonally sensitive tissue.

(iii) Species differences in teratogenesis and metabolism: Table 1 shows the inter-species variations between the minimum teratogenic doses of some selected compounds. There are clear differences for example, between the mouse and rat in that the mouse appears more sensitive to the teratogenic effects of diazepam, 2,5-diaminotoluene, caffeine, diphenylhydantoin and aspirin. In contrast, the rat is more sensitive to the effects of L-dopa. The interspecies variations are not entirely due to differences in placentation (because the mouse and rat are similar) but may well be due to differences in either xenobiotic biotransformation or the pharmacokinetic profile of the chemical.
3. PHARMACOKINETIC PHENOMENA IN PREGNANT ANIMALS

During pregnancy there are increases in the clearance of orally administered drugs whose excretion is predominantly renal and related to creatinine clearance, but no change in the clearance of oral drugs that are extensively metabolized by the liver, i.e. have a high hepatic extraction ratio (120). Processes which affect the kinetics of clearance (renal plasma flow and glomerular filtration rate) are usually increased during pregnancy (121). The apparent volume of distribution is also altered due to increased plasma volume, an increase in body fat and a decrease in the concentration of plasma proteins. The net result is a tendency towards prolonged drug half-life (122). In addition, delayed gastric emptying has been noted during pregnancy (approximately twice as long as compared to non-pregnant women, 123).

Most drugs are partially bound to plasma proteins, predominantly albumin. The albumin concentration falls in the first 4 months of pregnancy which reduces the binding capacity of a given volume of plasma, but since the plasma volume increases during pregnancy the total mass of albumin effectively remains constant. For highly bound drugs, the hypoalbuminaemia of pregnancy will produce a lower total plasma concentration, associated with a decrease of the bound drug fraction and a corresponding increase of the free drug fraction in the plasma (124,125). In contrast to albumin, the concentration of most globulin fractions rise during pregnancy. The total effect of changing patterns of plasma proteins and of physiological competitors for their binding sites is complex and varies from compound to compound (123). Altered pharmacokinetics during pregnancy is therefore a well documented area in both humans and laboratory animals (121,126-131).

In humans there is little evidence of changes in drug metabolite profiles during pregnancy, or liver size/weight changes (129). However, the liver of
the pregnant rat has been reported to increase by up to 40% of the normal weight and it has been suggested that this increase is an adaptive response to accommodate hydroxylation of the rising tide of sex hormones (132). Interestingly, the same group also noted a decrease (53-73%) in cytochrome P450-mediated reactions accompanied by liver weight changes prior to parturition (132).
4. **ENZYMOLGY OF TERATOGEN ACTIVATION**

Studies of the interaction of chemicals with biological systems have shown that in many cases, the parent compound is not the chemical species responsible for the observed toxic effects. Rather, the parent compound is biotransformed and converted to a toxic reactive intermediate or metabolite. These reactions have been studied in maternal hepatic tissue and in tissues of importance to teratogenesis, including the placenta and fetal liver.

4.1 Drug metabolism by the placenta:

The main role of placental cytochrome P450 appears to be in cholesterol side chain cleavage and biosynthesis of estrogens from C19 endogenous steroids (133-138). Most of the drug biotransformation reactions present in the liver have also been reported in the placenta (133). Examples are cytochrome P450 dependent reactions (134), glucuronidation, sulphation, glutathione conjugation, epoxide hydratase, catechol-O-methyltransferase and monoamine oxidase (133). Human placental cytochrome P450 concentration is approximately 20% of that found in maternal liver (135,136), with the placental mitochondrial cytochrome P450 concentration being several times greater than in the microsomal fraction (137).

There is some evidence for the multiplicity of cytochrome P450 isoenzymes in the placenta (137-139) and placental cytochrome P450 appears to be inducible only by 3-methylcholanthrene (3MC) and related inducers (137). The aryl hydrocarbon hydroxylase system (i.e. the polycyclic hydrocarbon metabolising system) in the placenta appears to have a naturally high basal activity compared to other types of MFO reactions (140) and is highly inducible, especially by benzo(a)pyrene or cigarette smoking (138,139,141,142). However, the detoxication of benzo(a)pyrene metabolites by phase II glutathione conjugation does not appear to be similarly induced (143).
4.2. Drug metabolism by the fetus:

The fetal liver of primates possess a well-developed complement of xenobiotic metabolising enzymes (albeit less active than the adult), in contrast to the fetuses of many laboratory animals (rat, mouse, guinea pig, rabbit and hamster) which are deficient in the microsomal cytochrome P450 system. The latter appears to be due to the late development of the smooth endoplasmic reticulum (144) and hence the associated development of cytochrome P-450 (145,146).

a) Tissue sub-fractions:

Studies on the stump-tail monkey (147) and marmoset (148) have shown that their xenobiotic metabolizing capacity at mid-gestation resembles closely the human fetal capacity (147). Human fetal microsomes as early as the 13th week of gestation have the capability of metabolizing the teratogens aldrin, benzo(a)pyrene, diphenylhydantoin and diazepam (134,145,149,150).

Classical metabolism studies using model substrate have shown that the liver of laboratory animals, with few exceptions, have a low capacity to oxidise xenobiotics until after birth. The appearance of oxidative enzymes in the liver are first detectable close to birth, and the adult levels are attained in a few days (as in the guinea pig) or a few weeks (as in the rat) (151). There also appears to be a sex difference in time of attainment of adult levels in the rat; the female requires 30 days and the male 40 days (152). For a more comprehensive analysis of the above aspect of developmental pharmacology than is possible here, the numerous reviews and articles on the subject should be consulted (145-157).
b) Isolated cells and organs:

Human fetal hepatocytes do have phase I metabolizing enzyme activity albeit less than adult levels. For example, the cytochrome P-450 content of fetal livers (gestational ages 9-15 weeks) was 50% that of the adult (0.2-0.4 nmol/mg microsomal protein compared to 0.4-0.8 nmol/mg microsomal protein in the adult liver, 145). In fetal livers of gestational age range 15-24 weeks, epoxide hydrolase activity was 25% of adult livers (158) and glutathione-S-transferase activity 40% of adult livers (158). Table 2 shows the metabolism of some teratogens in the fetus relative to the adult.

Recently using molecular biology techniques, the work of Nebert et al. (161), has shown the presence of mRNA for cytochrome P-450/P-448 as early as day 13 of gestation in the mouse. Such evidence for the presence of the enzyme at so early a stage in development contrasts sharply with the lack of metabolic capability (except for benzo(a)pyrene epoxidation and hydroxylation (57,162)). It may be that the use of model drug substrates is too insensitive to detect metabolism which may be confined to specific cells (e.g. those of the differentiating embryo).

In vitro studies with cultured fetal rat hepatocytes have shown that their ability to metabolize certain xenobiotics only appeared after day 18 of gestation (163,164). Other studies with cultured mouse embryos (during early development) have however indicated that the onset of polycyclic aromatic hydrocarbon activation occurs very early coinciding with blastocyte formation (162), and that by day 12 of gestation, benzo(a)pyrene hydroxylation is easily detectable (57). In vivo pretreatment of the mothers, or in vitro exposure to inducers have shown that certain cytochrome P-450 isoenzymes in fetal hepatocytes can be induced (6,161-166). Mouse limb bud organ cultures for example can activate the proteratogen cyclophosphamide
following 3 days of Aroclor 1234 induction in utero (22), without the need for an external activating source. In utero or in vitro exposure to either 3-methylcholanthrene or β-naphthoflavone can induce a form of cytochrome P-448 detected immunocytochemically, in limb bud cultures from rat embryos (167). It appears that fetal/embryo cytochrome P-450 is only inducible by certain inducers (6,7,21,164-168).
5. BIOACTIVATION OF TERATOGENS

5.1 Compounds that require bioactivation:

a) Cyclophosphamide

The bioactivation of cyclophosphamide (CPA) has been most extensively studied. In 1961, Foley et al. (169) demonstrated that CPA required hepatic activation before (growth) inhibition of tumour cells could be observed, and it is now widely accepted that CPA-induced teratogenesis involves metabolic activation. The maternal liver is the site of bioactivation (170-172) via the cytochrome P450 dependent monoxygenases (14,28,173-176). This has been further confirmed by in vitro studies with inducers and inhibitors of cytochrome P450-mediated metabolism (Table 3).

In vivo studies: In the rat, phenobarbitone pretreatment of the pregnant female increased CPA teratogenicity; pretreatment with β-naphthoflavone however, had either no effect or decreased the teratogenicity (171). In the mouse, phenobarbitone again increased teratogenicity and SKF 525A decreased the response; in these animals, phenobarbitone decreased and SKF 5225A increased the fraction of unchanged drug in maternal plasma and the embryos (1,2), indicating that a CPA metabolite is the ultimate teratogen. In contrast, acute or chronic administration of Aroclor 1254 (a polychlorinated biphenyl mixture) attenuated CPA embryotoxicity and teratogenicity; the kinetics and placental transfer of total radioactivity following $^{14}$C-CPA administration was unaffected by Aroclor 1254 pretreatment (177).

In vitro studies: Incubation of whole rat embryos in vitro with CPA had no effect on embryotoxicity or teratogenicity (178,179); a 5 hr exposure in the culture medium with a metabolising system (S9 mix) is however sufficient to elicit the teratogenic effect (180). The embryotoxic and teratogenic
effect of CPA was increased in rat embryo culture with liver S9 fraction from phenobarbitone (7) or Aroclor 1254 pretreated male rats (7,181); β-naphthoflavone or 3-methylcholanthrene pretreatment had no effect (7,181). Interestingly, it was not possible to induce malformations in embryos transplacentally pretreated with phenobarbitone (or 3 methylcholanthrene) and then exposed to CPA in vitro (6,7), however β-naphthoflavone could activate CPA in vitro with limb bud cells from differentiating rat embryos (6).

The major reactive (teratogenic) metabolites of CPA are acrolein and phosphoramid mustard (Figure 1). Phosphoramid mustard is a potent alkylating agent (172,182), and is presumed to be the ultimate teratogen of CPA (170,183). In vivo, acrolein, has been reported to be embryolethal but not teratogenic (170,184). However, embryonic abnormalities are observed in cultured whole rat embryos exposed to acrolein in serum free medium (185), suggesting that its extensive binding to protein may play an important role in reducing the teratogenic effect in vivo. Co-administration of CPA with thiol compounds (cysteine, glutathione and S-methylglutathione) in vivo to rats causes a decrease in CPA teratogenicity, whereas co-administration with diethyl maleate (DEM, a thiol depleting agent), causes an increase in teratogenic effects (186). 4-Hydroperoxy CPA, which decomposes to phosphoramid mustard and acrolein (170,182,184), causes dysmorphogenesis in mouse limb-bud cultures (187) similar to that observed with CPA in vivo.

b) 2-Acetylaminofluorene

In vitro studies: 2-acetylaminofluorene (2AAF) is teratogenic to whole rat embryos in vitro only in the presence of a hepatic S9 mix from Aroclor or 3-methylcholanthrene pretreated male rat (188). N-hydroxy AAF, a metabolite of 2AAF, was found to be directly teratogenic in vitro (188) giving
the prosencephalic defects observed with 2AAF following in vitro activation. Embryos transplacentally exposed to 3-methylcholanthrene and subsequently treated during culture with 2AAF in the absence of S9 mix showed increases in malformation incidence; transplacental exposure to phenobarbitone had no effect (7). Oxidative metabolites of 2AAF were detected during incubation of 2AAF with homogenates of day 10 embryos transplacentally exposed to 3-methylcholanthrene (7).

c) Benzhydrylpiperazine derivatives

The benzhydrylpiperazine antihistaminic drugs, chlorcyclizine, cyclizine, bucyclizine, meclizine, hydroxyzine and norcyclizine are teratogenic in rats and mice (189-191). Cytochrome P450 mediated N-dealkylation of chlorcyclizine and meclizine forms a common metabolite, norchlorcyclizine (192). In the rat, the incidence of cleft palate induced by chlorcyclizine, but not norchlorocyclizine, was reduced by SKF 525A administration. This response was associated with an increased fetal concentration of chlorcycline (6-12 fold) and a decrease in norchlorcyclizine, indicating that norchlorcyclizine is the ultimate teratogen (3).

d) Benzo(a)pyrene

The Ah locus encodes for a cytosolic receptor which regulates the expression of particular isozymes of cytochrome P-450 (161,193-197). This cytosolic receptor is highly inducible by polycyclic aromatic compounds in some mouse strains; \( \text{Ah}^b \) denotes the responsive and dominant allele and \( \text{Ah}^d \) the non-responsive and recessive allele (161,193-197). The work of Nebert et al. (161,193-197) illustrates that benzo(a)pyrene (BP) teratogenesis is due to a metabolite and that several other factors govern the teratogenic susceptibility to BP including the maternal genotype, fetal genotype and route of BP administration.
When BP is administrated intraperitoneally to pregnant female non-responsive mice (e.g. C57BL strain Ah\textsuperscript{d}/Ah\textsuperscript{d}) that had been mated with responsive (Ah\textsuperscript{b}/Ah\textsuperscript{b} DBA) male mice, the embryos with Ah\textsuperscript{b}/Ah\textsuperscript{d} phenotype were more susceptible to intrauterine toxicity than embryos of the phenotype Ah\textsuperscript{d}/Ah\textsuperscript{d} (196). This was due to induction of cytochrome P-450 isoenzymes and aryl hydrocarbon hydroxylase activity in the embryo and presumably a higher steady state of toxic BP metabolites. All off-spring from responsive Ah\textsuperscript{b}/Ah\textsuperscript{d} mothers regardless of the paternal genotype did not exhibit genetic differences in embryo-toxicity because of the higher amounts of P-450 isoenzymes in the mother and hence metabolites available for placental transfer.

However, when BP was given orally to Ah\textsuperscript{d}/Ah\textsuperscript{d} mothers mated to responsive males the homozygous (Ah\textsuperscript{d}/Ah\textsuperscript{d}) off-spring were more affected than the heterozygous off-spring. The explanation is the heterozygous off-spring appear to detoxify BP more rapidly and presumably transfer metabolites into the amniotic sac or back across the placenta while Ah\textsuperscript{d}/Ah\textsuperscript{d} off-spring have higher levels of BP and metabolites trapped within proximal embryonic tissues (197). If BP is given orally to pregnant Ah\textsuperscript{b}/Ah\textsuperscript{d} responsive females then the result is a reduction in embryotoxic effects. Genetic differences in susceptibility between the embryos are obliterated due to high maternal P-448 levels and hence high levels of circulating toxic metabolites for placental transfer.

e) Rifampin

Rifampin, an antibiotic, causes neural defects in the rat (198), however, in vitro it causes rat embryo growth retardation but no increases in neural tube defects. The addition of a liver microsomal preparation and cofactors to embryos cultured in the presence of rifampin resulted in an increased
neural tube defect which could be related to the abnormalities observed in fetuses exposed \textit{in utero} (199). These results indicate that an unidentified metabolite, probably produced by a cytochrome P450-mediated reaction, is the ultimate teratogen.

f) Procarbazine

Procarbazine is teratogenic in rats (200) however it is not teratogenic to whole cultured rat embryos \textit{in vitro} unless the serum from pretreated animals or an S9 mix is added to the culture medium (18), again emphasising the importance of metabolism in teratogen bioactivation.

5.2 Compounds that may or may not require bioactivation:

a) Aldrin

\textit{In vivo} studies: Literature reports indicate that both aldrin and its major metabolite dieldrin are teratogenic, but induce different types of malformation (201-202). Studies on the teratogenic effects of aldrin and its major metabolites at equimolar doses did not elucidate whether or not the parent compound was the more teratogenic entity (203).

\textit{In vitro} studies: Aldrin (33) and dieldrin (39) inhibit differentiation of rat embryo limb bud and CNS cells in the absence of a metabolising system.

b) Diethylstilbestrol

\textit{In vivo} studies: Diethylstilbestrol (DES) is a transplacental carcinogen and teratogen. The effect of this compound appears to be dependent on the amount of unchanged drug reaching the fetus (204,205) although it has been recently reported that a metabolite may be the active species (204-208).
c) Thalidomide

The main transformation of thalidomide in the body is generally considered to be spontaneous non-enzymic hydrolysis (112-113). However in tissues of a variety of experimental animals, the turnover rates in plasma and muscle tissue are surprisingly constant, whereas the rates in the liver vary considerably. At present, it has never been clearly defined if thalidomide does or does not require metabolic activation to express its teratogenicity.

In vitro studies: Differentiation of rat limb bud cells, cultured at low density was inhibited by thalidomide in the absence of a metabolising system (33). Metabolism has been shown however to play a role in thalidomide cytotoxicity. Gordon et al. (209) reported that only microsomes from a thalidomide sensitive species were capable of producing toxic metabolites (arene oxide?) in an in vitro lymphocyte system. Braun et al measured adhesion of mouse ovarian ascites tumour cells and showed that thalidomide toxicity requires activation by hepatic microsomes, an NADPH-generating system and molecular oxygen (36,210).

d) Diphenylhydantoin

Diphenylhydantoin (DPH) is used in the treatment of epilepsy despite a syndrome of drug related teratogenic effects described in humans (91,92,211). A dichotomy exists between the belief that DPH itself (93,212-216) or a metabolically derived arene oxide intermediate is the ultimate teratogen. (217-226) (Figure 2).

I) Evidence that an arene oxide intermediate of DPH metabolism is the ultimate teratogen.

1. In common with other aromatic hydroxylations (222,224) it has been proposed that the hydroxylation of DPH to 4-(4-hydroxyphenyl)-5-phenylhydantoin, observed in rat and
man, proceeds via an arene oxide intermediate. In vitro studies have shown that an oxidative metabolite of DPH (ie NADPH/O₂ dependent) irreversibly binds to rat liver microsomes; this binding could be increased by co-incubation with trichloropropene oxide, an epoxide hydratase inhibitor, and reduced by glutathione (220-222), suggestive of formation of a reactive arene oxide. Similar findings were reported using a human leucocyte system (223).

2. There is a correlation between the teratogenic effect and amount of covalently bound material in fetal tissue of the mouse following administration of ¹⁴C-DPH. Coadministration of trichloropropene oxide and DPH to the mouse doubled the incidence of cleft palate and the extent of covalent binding (220). This experiment can not be regarded as conclusive evidence of the role of the reactive metabolite, as the dihydrodiol metabolite was detected in maternal fluid samples indicating ineffective inhibition of epoxide hydratase.

II) Evidence that DPH is teratogenic per se.

1. Studies on the teratogenic response induced by the major metabolites of DPH at equimolar doses in mouse embryos in vitro show that the maximum response of any of the metabolites was only 10% of that caused by DPH itself (212). However this evidence cannot be regarded as conclusive as the dihydrodiol metabolite (or the arene oxide intermediate) have not been tested.
2. DPH is directly inhibitory in the rat micromass cell culture system (33).

3. DPH toxicity in in vitro micromass cell culture was increased by between 13-80% above control levels by co-incubation with a variety of cytochrome P450 inhibitors (6).

4. DPH teratogenicity was increased over 3-fold in A/J mice fed a purified diet compared to standard rodent chow. This difference is associated with an increased concentration of DPH in maternal blood (227).

5. SKF 525A pre-treatment increased DPH teratogenicity in the mouse. In this experiment, metabolism of DPH was inhibited as indicated by a raised concentration of DPH and a lowered concentration p-hydroxy DPH in maternal plasma, but parent and metabolite concentrations in (whole) fetuses were however unaffected. In contrast, phenobarbitone pretreatment of the mother decreased DPH teratogenicity and this was associated with lowered concentration of DPH and raised concentration of p-hydroxy DPH in the fetus (215).

Other theories as to the ultimate teratogenic species of DPH include a DPH-protein complex/modified serum protein (20,228) or the catechol metabolite (229,230).

e) Chlorambucil

Chlorambucil is a bifunctional alkylating agent (231) and is teratogenic when added directly to cultures of whole rat embryos (232). The addition of
an S9 mix enhances the effects, although cytochrome P450 was shown not to be involved by selective inhibitor studies (232). It has been postulated that chlorambucil and/or its phenylacetic acid metabolite are responsible for the teratogenic effects (232).

f) Adriamycin

Adriamycin is a glycosidic anthracycline antibiotic commonly used in the treatment of a variety of tumours. The compound is teratogenic in the rat (233) but not in the rabbit. Embryotoxicity was observed when cultured rat embryos were directly exposed to adriamycin. This response was enhanced when cultures were incubated with liver S9 fraction prepared from rats pre-treated with 3 methylcholanthrene or Aroclor 1254; the addition of S9 from control or phenobarbitone treated rats had no effect (234). The data suggests adriamycin is metabolically activated, although the failure of carbon monoxide to inhibit the response with an S9 fraction from 3-methylcholanthrene pretreated animals argues against the involvement of a cytochrome P448-dependent oxygenation, leading to activation.

5.3 Compounds not requiring bioactivation:

Direct acting teratogens include:

a) the metals which form protein complexes that directly interfere with cell proliferation and organogenesis (235).

b) androgens, estrogens, glucocorticoids and synthetic steroids which bind to cytosolic receptors and are translocated as complexes into the nucleus where the complex binds to chromatin and ultimately disrupts protein biosynthesis (53,58,65,204,236).

c) D-oxo-norleucine which is thought to act through binding to a protein receptor (65).
d) vitamin A which is more teratogenic than any of its metabolites (35, 237). When vitamin A was given at both teratogenic and non-teratogenic doses, the amount of retinol-binding protein transferred to mice fetuses was found to be directly proportional to the maternal dose of vitamin A (238), strongly suggesting a role for receptors rather than bioactivation.

e) Ethylenethiourea is teratogenic both orally and subcutaneously in rats and hamsters (239-241). In both species, metabolic modulation studies showed that phenobarbitone pretreatment had no effect on the teratogenicity while SKF525A increased the incidence of the malformations. The increased teratogenicity with SKF525A suggests the parent compound is the ultimate teragen.

f) Salicyclic acid and none of its putative metabolites cause dysmorphogenesis in cultured rat embryos (242, 243). In the same series of experiments, pre-incubation of salicyclic acid with a variety of biotransforming systems failed to generate a more toxic metabolite.

g) Cytochalasin D is directly embryotoxic in the rat embryo culture system but the effect is greatly reduced by incubation with an Arochlor 1254 pretreated rat liver enzyme preparation (39, 244), presumably due to metabolism (and hence inactivation) of the active parent compound.

h) Ethanol but not its principle metabolite, acetaldehyde, causes fetal malformations in vivo in the mouse (106). Further, co-administration of ethanol with the alcohol
dehydrogenase inhibitor, 4-methylpyrazole, causes an increase in malformations (106). Malformations in the cultured rat embryo are found on direct exposure to ethanol, confirming that the alcohol is very likely the ultimate teratogen (245,246).

i) Acetazolamide is probably a direct acting teratogen as either intra-amniotic or intra-uterine injection of the compound give rise to the typical pattern of limb abnormalities observed following oral administration (247-248). However, this conclusion assumes that the developing embryo/fetus lacks the activation enzymes.

j) Caffeine is a direct acting teratogen as pretreatment with β-naphthoflavone to cytochrome P450-inducible and non-inducible strains of mice showed that increased drug metabolism was concomitant with a decrease in caffeine teratogenicity (249).

k) OTHER COMPOUNDS

There remain a group of compounds which are teratogenic because of their effects on the mother or the extra-embryonic tissues rather than the embryo itself. One example is Mirex which causes a significant decrease in blood flow to the uterus and placenta and this is related to its embryotoxicity (250). Trypan-blue which does not cross into the embryo (251) inhibits visceral yolk sac lysosomal enzymes, effectively starving the embryo of essential nutrients (252).

The teratogenic activity of several compounds can be accounted for by the maternal toxicity caused at doses where teratogenicity was observed (16,253,254). When these compounds (insulin, tolbutamide, 6-amino-nicotinamide, morphine sulphate, dinoseb, zinc-1, 10-phenanthroline complex,
maytansine, vincristine, nickel chloride, indole-3-acetic acid, di-(2-ethylhexyl)-phthalate, phencyclidine, 2-nitro-p-phenylenediamine, ethylene glycol monomethyl ether and N-phthaloyl-L-glutamic acid) were administered at non maternally toxic doses, no teratogenicity was observed.
6. BIOACTIVATION OF TERATOGENS BY RAT EMBRYO CELLS IN VITRO

6.1. Preparation of micromass cultures:

The preparation and use of rat limb cell cultures in teratogenicity studies has been previously described in detail by Flint and co-workers (6,32,33,38). Essentially, embryos of 34-36 somites are collected from pregnant female rats approximately 13 days post conception. Forelimbs are dissected free and following enzymatic dissociation, single cell suspensions are prepared in culture medium (Ham's F12 plus 10% fetal calf serum). Cell suspensions are adjusted to give $2 \times 10^7$ limb cells per ml. 10 or 20 µl Drops are delivered to culture dishes and the cells in each drop are allowed to settle and adhere to the dish over 2 hrs at 37°C. The dish is then flooded with excess culture medium which may be supplemented with the test substance or vehicle control. The adhering cells form separate micromass islands of 6-8 mm diameter, and are continuously cultured for 5 days at 37°C, 5% CO₂ in air. During the five days of culture a proportion of the cells in each micromass island differentiate to form small foci of chondrocytes. After fixation these foci are preferentially stained by alcian blue, becoming regions of high contrast easily detectable with an automated image analyser. Counts of differentiated foci are compared in test substance-exposed and control cultures. A concentration dependent inhibition profile is prepared and from this the concentration ($IC_{50}$) inhibiting differentiation by 50% of the control values is estimated. Thus, the lower the $IC_{50}$ value, then the more potent is the chemical.

This is a highly predictive test. In a validation study 24 out of 25 (> 93%) teratogens tested directly inhibited differentiation (33), with very few false positive or false negative results.
6.2. Evidence for functional cytochrome P-450's in rat embryo cells:

Inhibitor studies:

(i) Co-incubation with a variety of P-450 inhibitors (at concentrations that were non-cytotoxic) increased the toxicity of diphenylhydantoin (DPH) in vitro by between 13-80% compared to control IC\textsubscript{50} values. SKF525A was the least effective and ellipticine and benzimidazole the most effective (6,38,255). No modulation of the toxicity of the p-hydroxy metabolite in vitro was observed when co-incubated with any of the inhibitors. Cimetidine increased DPH toxicity by 47%, however a structurally and pharmacologically similar compound, ranitidine, which is not a cytochrome P-450 inhibitor, had no effect on DPH toxicity when co-incubated in vitro.

These results imply that the increase in toxicity appeared to be specifically caused by inhibition of the cytochrome P-450 mediated metabolism of DPH.

Structure/Activity Relationships:

(ii) Structure-activity relationships among triazole antifungals showed that toxicity appears to be directly related to both hydrophobicity and the type of substituent in the phenyl rings. Substitution at the 4-position with metabolically stable and/or electron withdrawing groups reduces triazole toxicity in vitro apparently by protecting the phenyl ring from metabolic attack. Compared to the 2-position, the exposed 4-position appears to be most vulnerable to metabolic attack, unless protected by a suitable substituent (255). The data suggests that toxicity in vitro is mediated by the formation of a toxic intermediate (a quinone or arene oxide) possibly via metabolic attack on the 4-position on the phenyl ring.
Induction and Functional Activity of Embryonic Cytochrome P-450:

(iii) Transplacental induction of embryo xenobiotic metabolising enzymes was achieved by dosing the dams in vivo and subsequently removing the embryos at day 13 of gestation.

Cyclophosphamide (CPA) has no inhibitory effect on limb cell differentiation (number of differentiated foci) unless a preparation of Aroclor 1254 induced, actively metabolising adult rat liver enzymes (S9 plus NADPH) is present (33). Thus CPA is an excellent positive control for bioactivation studies. Following transplacental induction by β-naphthoflavone, differentiation was inhibited by CPA suggesting the presence of a functional and inducible cytochrome P-450 isoenzyme. Neither transplacental exposure to 3-methylcholanthrene nor phenobarbital induced significant CPA teratogenesis (6,38,255).

Immunocytochemical Identification of Cytochrome P-450:

(iv) Immunocytochemical staining of cytochrome P-450 by the antiperoxidase technique described by Wolf et al. (256) was used on the cultures after 5 days incubation. Fresh cultures were stained against a variety of cytochrome P-450 antibodies.

Our recent results showed a constitutive level of cytochrome P-450 (a phenobarbitone-inducible form) and P-448 (a 3-methylcholanthrene-inducible form) were detected in 5 day old cultures (6,38,167,255). However, trans-placental induction was only observed with β-naphthoflavone with phenobarbitone having no effect. These observations are in close agreement with the ability of the inducers to induce CPA metabolism.

The evidence presented here indicates strongly that rat embryo cells in vitro not only have constitutive levels of cytochrome P-450's but that some are inducible and catalytically competent. Most teratogens that
are well metabolised in vivo (33) were direct acting in vitro. In the light of evidence presented in this section we should now consider the possibility that the embryo cells themselves metabolise these teratogens, forming toxic reactive intermediates. The cultures thus open up the possibility of investigating the role of embryo metabolism as opposed to the maternal metabolism in teratogenesis.
7. EPILOGUE

Like all new sciences, the study of teratology has, until recently, been largely phenomenological. The relative importance of the role of maternal and embryonic metabolism has yet to be fully established, but we have tried to illustrate some of the pharmacokinetic and metabolic influences in the action of teratogens which should, in the future, receive more attention. Problems of metabolism in teratology are almost certainly more easily approachable with current techniques than the far more intractable problems surrounding the actual mechanisms of dysmorphogenesis. This latter problem will have to await a more comprehensive understanding of the molecular basis of developmental cell biology.
8. REFERENCES


TABLE 1: INTERSPECIES DIFFERENCES BETWEEN THE LOWEST TERATOGENIC DOSE

The following compounds have been given for between 3 and 6 days of the most sensitive phase of organogenesis for each species. The minimum teratogenic dose is the lowest maternal dose at which terata are observed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Species</th>
<th>Route of administration</th>
<th>Minimum teratogenic dose (mg/kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorocholine chloride</td>
<td>Hamster</td>
<td>Oral</td>
<td>300-400</td>
<td>(80)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>500</td>
<td>(81)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Human</td>
<td>Oral</td>
<td>?</td>
<td>(82)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>300</td>
<td>(83)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>50-75</td>
<td>(83)</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Rabbit</td>
<td>Oral</td>
<td>450</td>
<td>(84)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>10</td>
<td>(85)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>10</td>
<td>(86)</td>
</tr>
<tr>
<td>Dichlorophen-oxyacetic acid</td>
<td>Hamster</td>
<td>Oral</td>
<td>100</td>
<td>(87)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>25,50-100</td>
<td>(88,89)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>177-221</td>
<td>(90)</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>Human</td>
<td>Oral</td>
<td>?</td>
<td>(91-93)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>100</td>
<td>(94)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>45</td>
<td>(95)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Human</td>
<td>Oral</td>
<td>?</td>
<td>(96-98)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>200</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>100</td>
<td>(95)</td>
</tr>
<tr>
<td></td>
<td>Guinea pig</td>
<td>Oral</td>
<td>280</td>
<td>(100)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Rat</td>
<td>Oral</td>
<td>400</td>
<td>(101)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>15</td>
<td>(102)</td>
</tr>
<tr>
<td></td>
<td>Cat</td>
<td>Oral</td>
<td>25</td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>Oral</td>
<td>400</td>
<td>(104)</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Oral</td>
<td>250</td>
<td>(105)</td>
</tr>
<tr>
<td>L-Dopa</td>
<td>Rat</td>
<td>Oral</td>
<td>10</td>
<td>(106)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>500</td>
<td>(107)</td>
</tr>
<tr>
<td>Compound</td>
<td>Species</td>
<td>Route of administration</td>
<td>Minimum teratogenic dose (mg/kg)</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------</td>
<td>---------</td>
<td>-------------------------</td>
<td>----------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>2,5-Diamino-toluene</td>
<td>Rat</td>
<td>Sub-cutaneous</td>
<td>-</td>
<td>(108)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Sub-cutaneous</td>
<td>50</td>
<td>(109)</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>Human</td>
<td>Oral</td>
<td>?</td>
<td>(110,111)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Oral</td>
<td>150</td>
<td>(111-115)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Oral</td>
<td>200</td>
<td>(115)</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>Oral</td>
<td>200</td>
<td>(116)</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Oral</td>
<td>25-200</td>
<td>(112,117)</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>Oral</td>
<td>10</td>
<td>(118)</td>
</tr>
</tbody>
</table>

(a) Thalidomide may produce a teratogenic effect if given at a high dose by intravenous administration (See References 114,119).
TABLE 2: TERATOGENS METABOLISED IN VITRO BY CULTURED FETAL HEPATOCYTES

<table>
<thead>
<tr>
<th>Teratogen</th>
<th>Species</th>
<th>Metabolic Reaction</th>
<th>% of Adult Liver Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo(a)pyrene</td>
<td>rat&lt;sup&gt;1&lt;/sup&gt;</td>
<td>hydroxylation</td>
<td>0.7</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>human&lt;sup&gt;2&lt;/sup&gt;</td>
<td>hydroxylation</td>
<td>2-4</td>
<td>134</td>
</tr>
<tr>
<td>Aldrin</td>
<td>rat&lt;sup&gt;3&lt;/sup&gt;</td>
<td>epoxidation</td>
<td>30-40</td>
<td>134</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>human&lt;sup&gt;2&lt;/sup&gt;</td>
<td>hydroxylation</td>
<td>detectable</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>epoxidation</td>
<td>detectable</td>
<td>145</td>
</tr>
<tr>
<td>Diazepam</td>
<td>rat&lt;sup&gt;3&lt;/sup&gt;</td>
<td>metabolite production</td>
<td>10%</td>
<td>160</td>
</tr>
</tbody>
</table>

1. - fetuses gestational age of 17-20 days.
2. - fetuses gestational age of 10-15 weeks.
3. - fetuses gestational age of 21 days.
**TABLE 3:**

**EFFECT OF MODULATING METABOLIC PATHWAYS INVOLVED IN TERATOGEN ACTIVATION**

<table>
<thead>
<tr>
<th>Teratogen</th>
<th>Test System</th>
<th>Effect of Modulators on the Teratogenic Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide (CPA)</td>
<td><em>In vivo</em>, mouse</td>
<td>PB increased and SKF 525 A reduced teratogenicity</td>
<td>(1,2)</td>
</tr>
<tr>
<td></td>
<td><em>In vivo</em>, rat</td>
<td>PB caused an increase and βNF had no effect</td>
<td>(171)</td>
</tr>
<tr>
<td></td>
<td>Thiol compounds reduced and thiol-depleting compounds increased teratogenicity</td>
<td>(186)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>In vitro</em>, whole rat embryo cultures</td>
<td>Co-incubation with either PB or Aroclor 1254 induced microsomes enhanced the teratogenic effect; βNF and 3MC induced microsomes had no effect.</td>
<td>(7, 181)</td>
</tr>
<tr>
<td></td>
<td><em>In vitro</em>, mouse limb bud cultures</td>
<td>After in utero pretreatment of the mothers with Aroclor 1254 the limb buds could activate CPA to a teratogenic metabolite.</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td><em>In vitro</em>, rat embryo micro-mass</td>
<td>βNF in utero exposure permitted limb bud cells to activate CPA to a toxic metabolite. PB and 3MC had no effect.</td>
<td>(6)</td>
</tr>
<tr>
<td>2-Acetylamino-fluorene (AAF)</td>
<td><em>In vitro</em>, whole rat embryos</td>
<td>3MC and Aroclor 1254 induced microsomes enhanced the teratogenic effect. In utero exposure to 3MC allowed AAF activation by cultures; PB had no effect.</td>
<td>(7, 118)</td>
</tr>
<tr>
<td>Chlorcyclizine</td>
<td><em>In vivo</em>, rat</td>
<td>SKF 525 A reduced the incidences of malformation</td>
<td>(3)</td>
</tr>
</tbody>
</table>

(Cont.)
<table>
<thead>
<tr>
<th>Substance</th>
<th>Assay Type</th>
<th>Effect Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diphenylhydantoin</td>
<td><em>In vivo</em>, mouse</td>
<td>PB decreased and SKF 525 A increased teratogenicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Co-administration with a thiol-depleting agent (diethylmaleate) increased teratogenicity. (222)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Co-administration with 1,2-exoxy-3,3,3-trichloropropane (an epoxide hydratase inhibitor) increased the effects. (222)</td>
</tr>
<tr>
<td><em>In vitro</em>, rat embryo micro-mass</td>
<td>Co-incubation with a variety of cytochrome P450 inhibitors increased the toxicity. (6)</td>
<td></td>
</tr>
<tr>
<td>Adriamycin</td>
<td><em>In vitro</em>, whole rat embryo</td>
<td>Co-incubation with either 3MC or Aroclor 1254 induced microsomes enhanced the effects; PB induced or control microsomes had no effect. (234)</td>
</tr>
<tr>
<td>Ethylenethiourea</td>
<td><em>In vivo</em>, rat in vivo, hamster</td>
<td>PB had no effect, and SKF 525 A increased teratogenicity. (239-241)</td>
</tr>
<tr>
<td>Ethanol</td>
<td><em>In vivo</em>, mouse</td>
<td>Co-administration with an alcohol dehydrogenase inhibitor (4-methylpyrazole) increased teratogenicity. (106)</td>
</tr>
<tr>
<td>Caffeine</td>
<td><em>In vivo</em>, mouse</td>
<td>βNF co-treatment decreased the effects. (249)</td>
</tr>
</tbody>
</table>

Abbreviations used are:  
PB : phenobarbitone  
βNF : β-naphthoflavone  
3MC : 3-methylcholanthrene
LEGENDS TO FIGURES

Figure 1: Role of metabolism in the teratogenic bioactivation of cyclophosphamide.

Figure 2: Metabolic fate of diphenylhydantoin.
cyclophosphamide → Liver microsomal oxidation (P-450) → 4 hydroxy cyclophosphamide → keto cyclophosphamide

aldophosphamide → aldehyde oxidase → phosphoramidase mustard (PAM) + acrolein

carboxyphosphamide → nitrogen mustard + HO-PO-NH₂
Diphenylhydantoin (DPH)

Diphenylhydantoic acid

Phenylglycine

Proposed arene oxide intermediate

Epoxide hydratase

p-hydroxy-DPH

m-hydroxy-DPH

Dihydrodiol

DPH-3,4 catechol

DPH-3-O-methyl catechol