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Biochemical Toxicology of Chemical Teratogenesis

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ABSTRACT: Although exposure during pregnancy to many drugs and environmental chemicals is known to cause *in utero* death of the embryo or fetus, or initiate birth defects (teratogenesis) in the surviving offspring, surprisingly little is known about the underlying biochemical and molecular mechanisms, or the determinants of teratological susceptibility, particularly in humans. *In vitro* and *in vivo* studies based primarily on rodent models suggest that many potential embryotoxic xenobiotics are actually proteratogens that must be bioactivated by enzymes such as the cytochromes P450 and peroxidases such as prostaglandin H synthase to teratogenic reactive intermediary metabolites. These reactive intermediates generally are electrophiles or free radicals that bind covalently (irreversibly) to, or directly or indirectly oxidize, embryonic cellular macromolecules such as DNA, protein, and lipid, irreversibly altering cellular function. Target oxidation, known as oxidative stress, often appears to be mediated by reactive oxygen species (ROS) such as hydroxyl radicals. The precise nature of the teratologically relevant molecular targets remains to be established, as do the relative contributions of the various types of macromolecular lesions. Teratological susceptibility appears to be determined in part by a balance among pathways of maternal xenobiotic elimination, embryonic xenobiotic bioactivation and detoxification of the xenobiotic reactive intermediate, direct and indirect pathways for the detoxification of ROS (cytoprotection), and repair of macromolecular lesions. Due largely to immature or otherwise compromised embryonic pathways for detoxification, cytoprotection, and repair, the embryo is relatively susceptible to reactive intermediates, and teratogenesis via this mechanism can occur from exposure to therapeutic concentrations of drugs, or supposedly safe concentrations of environmental chemicals. Greater insight into the mechanisms involved in human reactive intermediate-mediated teratogenicity, and the determinants of individual teratological susceptibility, will be necessary to reduce the unwarranted embryonic attrition from xenobiotic exposure.

KEY WORDS: electrophiles teratogenesis, chemical developmental toxicology, reactive intermediates, reactive oxygen species, oxidative stress, benzo[*a*]pyrene, phenytoin, thalidomide, free radicals, cytochromes P450, prostaglandin H synthase, elimination, cytoprotection, molecular targets, repair.

ABBREVIATIONS

ASA, acetylsalicylic acid (aspirin); **BCNU,** 1,3-*bis*(2-chloroethyl)-1-nitrosourea; **CYP,** cytochrome P450; **ETYA,** eicosatetraynoic acid; **GSH,** glutathione; **GST,** glutathione *S*-transferase; **H_2O_2,** hydrogen peroxide; **LOOH,** lipid hydroperoxide; **LPO,** lipoxygenase; **P450,** cytochromes P450; **PBN,** *a*-phenyl-*N*-*t*-butylnitrone; **pHPPH,** 5-(*p*-hydroxyphenyl)-5-phenylhydantoin; **PHS,** prostaglandin H synthase; **ROS,** reactive oxygen species; **TCPO,** trichloropropylene oxide; **UGT,** UDP-glucuronosyltransferase.

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I. INTRODUCTION

Many drugs and environmental chemicals, collectively termed xenobiotics, are thought to cause toxicity in the embryo or fetus via a reactive intermediate. In this case, the parent compound, called a proteratogen, is relatively nontoxic, but can be converted *in vivo* to a short-lived and highly toxic reactive intermediary metabolite (Figure 1). If not immediately detoxified, this reactive intermediate directly or indirectly will react irreversibly with cellular macromolecules such as DNA, protein, and lipid. When such molecular damage exceeds compensatory function and the capacity for repair, developmental processes are altered, resulting in *in utero* death or developmental anomalies, termed teratogenesis, in the surviving offspring. Xenobiotic-initiated teratologic anomalies include not only the commonly appreciated structural defects, such as cleft lip or absent arms, but also biochemical and functional abnormalities, including the development of cancer decades after birth, and intellectual dysfunction. A widespread awareness of the teratologic impact of xenobiotics dates back to the discovery of human thalidomide teratogenicity in the late 1950s (Lenz, 1961, 1962; McBride, 1961). It has been estimated that over 50%, and perhaps as many as 75%, of human fertilized eggs or embryos will die before birth (Roberts and Lowe, 1975; Rolfe, 1982), and of the children who survive to birth, at least 2 to 3% (Heinonen et al., 1977; Edmonds et al., 1981), and as many as 16% (Chung and Myrianthopoulos, 1975), will exhibit some teratologic abnormality. The contribution of xenobiotics to this developmental attrition has been said to approximate 5% (Wilson, 1973); however, this figure likely constitutes an underestimate due to such factors as unrecognized xenobiotic-initiated embryotoxicity early in pregnancy, individual enhanced susceptibility, and synergistic and additive effects of xenobiotics.

Reactive intermediate-mediated teratogenesis differs in a number of clinically relevant ways from that caused by the reversible binding of a xenobiotic to a receptor (Table 1). In the latter case, which accounts for the therapeutic effects of most drugs, the effect increases, peaks, and declines with or shortly after similar changes in the plasma drug concentration. Toxic effects, which often are predictable extensions of a drug's therapeutic effect, generally appear only if excessive plasma xenobiotic concentrations are achieved, and decline as the xenobiotic is eliminated. Such reversible, receptor-mediated interactions and toxicities generally are not cumulative. In contrast, imbalances among various pathways for xenobiotic elimination, formation (bioactivation) and detoxification of reactive intermediates, and/or pathways of cytoprotection and macromolecular repair, can result in reactive intermediate-mediated toxicity at therapeutic concentrations of drugs, or at presumed safe concentrations of environmental chemicals (Figure 1). This hazard is compounded by the irreversible nature of the interaction of a reactive intermediate with tissues, which allows molecular lesions to accumulate. With drugs, such effects are as unpredictable as those initiated by environmental chemicals because they are not mediated by the receptor interactions that are involved in the therapeutic effect of the drug. Another important distinction with respect to reactive intermediates is that the biochemical pathways involved in the formation and detoxification of the reactive intermediate, albeit the critical determinants of toxicologic expression, often are quantitatively minor, amounting in some cases to only 1 to 10% of the total metabolism of that xenobiotic. Accordingly, substantial differences in these pathways

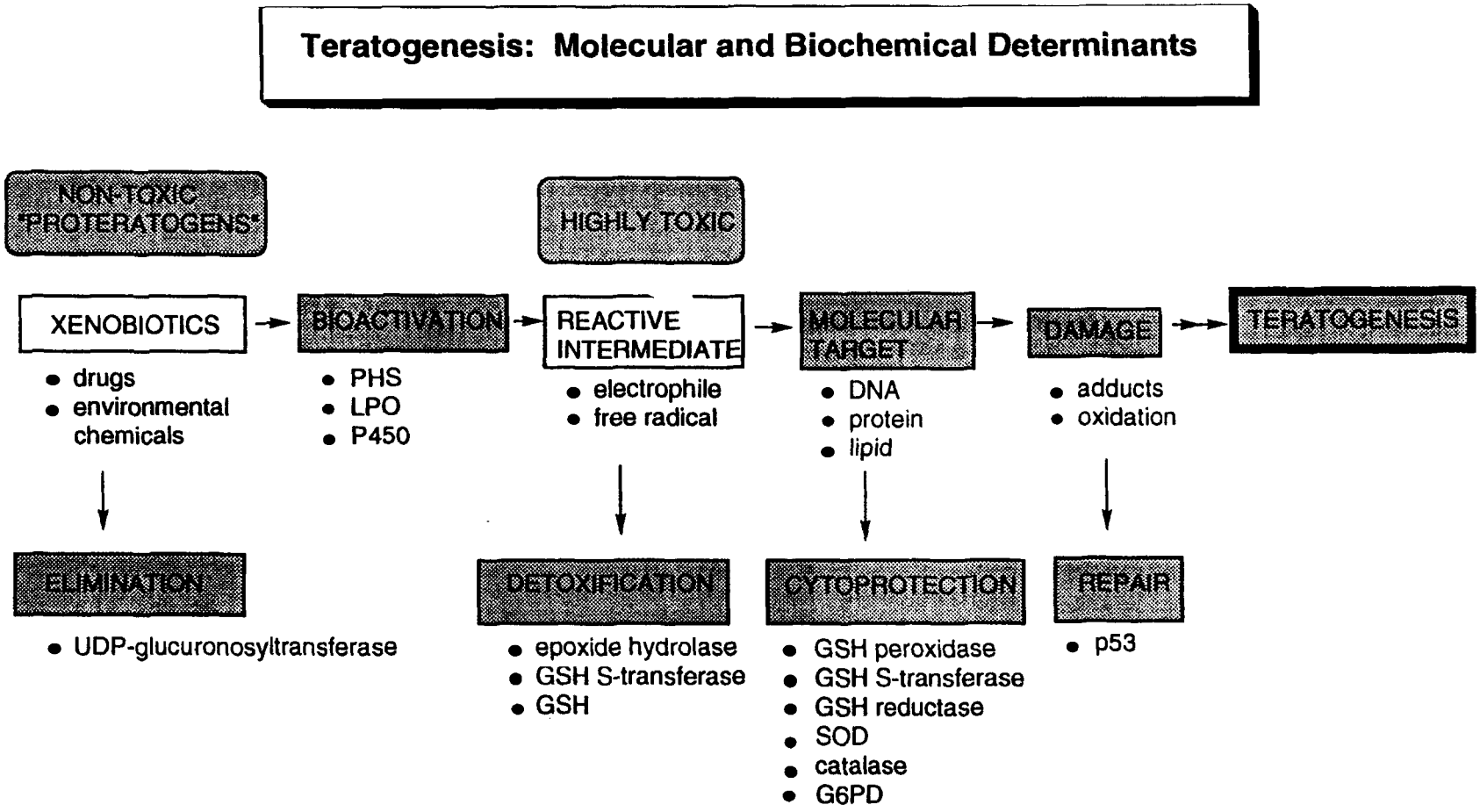

FIGURE 1. Postulated molecular and biochemical framework for understanding chemical teratogenesis and individual predisposition. (From Winn, L. M. and Wells, P. G. 1995a. *Eur. J. Neurol.* 2 (Suppl. 4): 5–29. With permission.)

TABLE 1
Characteristics of Xenobiotic Toxicity Initiated by Reactive Intermediates Compared with Reversible, Receptor-Mediated Interactions

	Mechanism of tissue interaction	
Characteristic	**Reactive intermediate**	**Receptor-mediated**
Initiating species	Reactive intermediary metabolite (highly unstable) Electrophile Free radical Often a minor metabolite amounting only to 1 to 10% of total xenobiotic/metabolites	Parent compound and/or a stable, major metabolite
Molecular target	Multiple sites within different cellular macromolecules (DNA, protein, lipid, and carbohydrate)	Specific receptor on one type of macromolecule (usually a protein)
Target interaction	Irreversible Covalent binding (arylation/alkylation) Oxidation	Reversible binding
Duration of target interaction	Cumulative	Transient
Toxic effects[a]	Unrelated to therapeutic effect[b]	Generally an extension of the therapeutic effect
Toxic dose/concentration	Toxicity can occur at therapeutic plasma drug concentrations or "safe" concentrations of environmental chemicals	Toxicity occurs when therapeutic or safe plasma concentrations are exceeded
Onset of toxicity	Toxicity occurs well after the time of the peak plasma xenobiotic concentration, and usually after the xenobiotic no longer is detectable in plasma or urine Depending upon both the xenobiotic and the toxicity, this delay can be hours, days, months, or years	Toxicity usually increases with rising plasma xenobiotic concentration, and decreases with or shortly after declining concentrations

[a] Effect in this case refers to the effect of therapeutic drugs, and is not relevant to environmental chemicals.
[b] There are some exceptions, such as alkylating anticancer drugs, where drug toxicity does result from the same mechanisms by which tumor cells are killed.

that render such individuals unusually sensitive to xenobiotic toxicity will not be reflected in measurable differences in plasma clearance or steady-state concentrations of the parent compound. This is particularly true in the embryo, in which teratologically important imbalances generally will not be reflected by alterations in the maternal plasma disposition of xenobiotics and their metabolites. Finally, unlike with reversible, receptor-mediated interactions, toxicities initiated by reactive intermediates may occur hours, weeks, months, years, and even decades after exposure. These contrasting features of reversible, receptor-mediated, and irreversible reactive intermediate-mediated toxicities reflect major differences in their molecular and biochemical basis, and generally require divergent approaches for clinical management. Reversible, receptor-mediated chemical teratogenesis has been reviewed elsewhere (Hansen, 1991; Juchau et al., 1992; Nau, 1994; Winn and Wells, 1995a). This review focuses upon reactive intermediate-mediated teratogenicity. Toxicological principles are illustrated using a limited selection of representative teratogens; in particular, the environmental polycyclic aromatic hydrocarbon benzo[*a*]pyrene, the anticonvulsant drug phenytoin (Dilantin®) and a number of structurally related anticonvulsant drugs, and the sedative/hypnotic drug thalidomide.

II. ELIMINATION

In general, the relative roles of maternal vs. embryonic drug metabolism with respect to reactive intermediates have yet to be established, particularly with regard to the formation and detoxification of reactive intermediates, as discussed below. However, maternal pathways of elimination, which may reduce the amount of a proteratogen that can reach the embryo as well as the amount available for bioactivation (Figure 1), are potentially important determinants of teratological susceptibility.

UDP-glucuronosyltransferases (UGTs) are a family of enzymes that catalyze the conjugation of most xenobiotics or their metabolites with UDP-glucuronic acid, thereby increasing their water solubility and facilitating elimination (Burchell and Coughtrie, 1989; Brierly and Burchell, 1993; Burchell et al., 1994). Because UGT-catalyzed glucuronidation often constitutes the major route of xenobiotic elimination, this pathway would be expected to be an important determinant of xenobiotic concentration in the embryo, and hence, teratogenicity. Hydroxylated metabolites of the carcinogen and teratogen benzo[*a*]pyrene are largely eliminated via glucuronidation, which avoids alternative bioactivation to toxic electrophilic and free radical reactive intermediates (Figure 2). Thus, maternal glucuronidation would be expected to reduce the amount of benzo[*a*]pyrene reaching the embryo, and possibly reduce the amount of benzo[*a*]pyrene bioactivated within the embryo. At a noncarcinogenic dose (25 mg/kg i.p.) that had no effect in pregnant UGT-normal Wistar controls, benzo[*a*]pyrene administered to UGT-deficient Gunn rats initiated a threefold increase in the incidence of fetal resorptions (*in utero* fetal death), suggesting that UGTs are important embryoprotective enzymes (Wells et al., 1989a). Maternal UGT-catalyzed elimination is particularly important given that UGTs are low or absent in fetal tissues (Dutton, 1971; Burchell, 1973) and, unlike adult UGTs, murine fetal UGTs are not phenobarbital inducible during organogenesis (Burchell and Dutton, 1975), the period of primary teratological susceptibility. It is likely that hereditary UGT deficiencies have broader teratological relevance. For example, both phenytoin and its major hy-

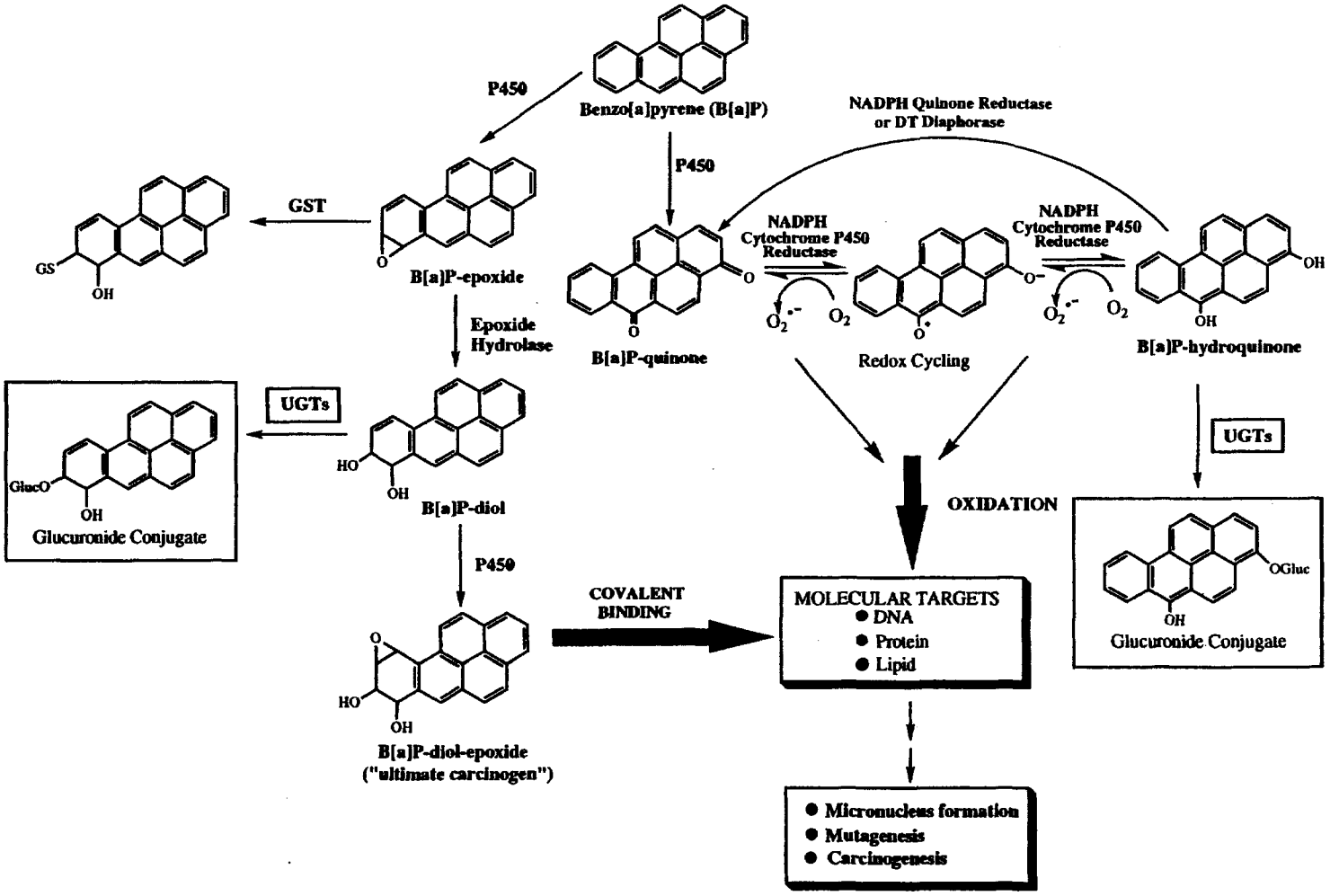

FIGURE 2. Potential cytoprotective role of elimination catalyzed by the UDP-glucuronosyltransferases (UGTs). As exemplified here for benzo[*a*]pyrene, UGT-catalyzed elimination may avoid xenobiotic bioactivation to a teratogenic reactive intermediate. (From Vienneau D. S., De Boni, U., and Wells, P. G. 1995. *Cancer Res.* 55: 1045–1051. With permission.)

droxylated metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (pHPPH), are embryopathic in mouse embryo culture, and both are glucuronidated, respectively, to *N*- (Smith et al., 1977; Kim et al., submitted) and *O*-glucuronides (Wells and Harbison, 1980). Using a rat skin fibroblast model, DNA-damaging activity reflected by micronucleus formation was higher for both phenytoin and pHPPH in cells from UGT-deficient animals (Wells and Kim, 1996; Kim et al., submitted), and teratological susceptibility may be similarly enhanced. Previous studies indicating that maternally administered pHPPH is not teratogenic (Harbison and Becker, 1974) likely resulted from maternal glucuronidation that prevented pHPPH from reaching the fetus, because pHPPH in clinically achievable concentrations initiates micronucleus formation only in UGT-deficient cells (Wells and Kim, 1996; Kim et al., submitted).

There is limited evidence for an influence of sulfation, another important pathway of xenobiotic elimination, upon chemical teratogenesis; however, it is known that human fetal lung can sulfate 1-naphthol and estrone (Jones et al., 1992).

Maternal cytochromes P450 (P450s) may be similarly important in reducing the amount of teratogen reaching the embryo. *In vivo* studies have shown that pretreatment of pregnant mice with P450 inducers such as phenobarbital can reduce the teratogenicity of the anticonvulsant drug phenytoin (Harbison and Becker, 1971) and the anticancer drug cyclophosphamide (Gibson and Becker, 1968), likely via increased maternal metabolism. Conversely, pretreatment with P450 inhibitors such as SKF 525A enhances the teratogenicity of phenytoin and cyclophosphamide, likely via inhibiting maternal metabolism and increasing the amount of drug transferred to the fetus. Interestingly, the antiepileptic drug stiripentol inhibits phenytoin teratogenicity in mice (Finnell et al., 1994), and it is reported to be a P450 inhibitor (Loiseau and Duche, 1989). However, most P450 inhibitors enhance phenytoin teratogenicity (Harbison and Becker, 1971; Wells and Gesicki, 1984), and stiripentol may exert its apparent protective effects via another mechanism. In contrast, Hales (1981a) found that pretreating rats with phenobarbital increased the teratogenicity of cyclophosphamide, and concluded that maternally produced reactive metabolites were stable enough to reach the fetus. There is support for this hypothesis in murine embryo culture studies, where cyclophosphamide is not embryopathic without the addition of maternal hepatocytes (Ozolins et al., 1995a) or microsomes and NADPH (Kitchin et al., 1981). These studies do not rule out the possibility that maternal metabolism also may produce a stable metabolite that readily crosses the placenta into the fetus, in which the metabolite is proximally bioactivated to a reactive intermediate, as discussed later (see Section III). This latter possibility may be exemplified by the phenytoin metabolite pHPPH, which, if not maternally glucuronidated, may cross the placenta and initiate teratogenesis, as discussed earlier. However, for many if not most xenobiotics thought to initiate teratogenicity via a reactive intermediate, embryopathy is demonstrable in embryo culture without supplemental sources of bioactivating enzymes, maternal or otherwise. *In vivo*, maternally produced reactive intermediates of most proteratogens may be too reactive to reach the fetus. The teratological importance of maternal metabolism also was illustrated with cytochrome P4501A1 (CYP1A1), comparing genetically inducible and noninducible pregnant mice pretreated with the P450 inducer 3-methylcholanthrene (Nebert, 1983). The CYP1A1-inducible dams were able to protect their susceptible embryos from benzo[*a*]pyrene-initiated teratogenicity, possibly via en-

hanced maternal metabolism and elimination of benzo[*a*]pyrene, reducing embryonic exposure. However, 3-methylcholanthrene also upregulates UGTs and glutathione *S*-transferases (Juchau et al., 1992), which may contribute to the observed maternal protection. Conversely, as discussed in the following section, CYP1A1-inducible pups from noninducible dams treated with benzo[*a*]pyrene had an increased incidence of teratological anomalies.

III. BIOACTIVATION AND DETOXIFICATION

A. Electrophiles

The teratogenicity of many xenobiotics has been postulated to be due to their oxidation to highly reactive, electron-deficient (electrophilic) intermediary metabolites, such as epoxides and arene oxides (Figure 3). Enzymes catalyzing this bioactivation vary with the substrate, and include P450s, prostaglandin H synthase (PHS), and possibly lipoxygenases (LPOs) (Table 2). If not immediately detoxified by enzymes such as epoxide hydrolase and glutathione *S*-transferase (GST), the electrophilic (positively charged) center of the xenobiotic reactive intermediate reacts with electron-rich groups, such as protein thiols, on cellular macromolecules, forming an irreversible, covalent bond. If not repaired, this covalent binding, or adduct formation, is thought to initiate a process that ultimately results in *in utero* death or teratogenesis.

Enhanced embryonic P450-catalyzed bioactivation may contribute to teratologic susceptibility. CYP1A1 is a nonconstitutive P450 that bioactivates the diol metabolite of the proteratogen and procarcinogen benzo[*a*]pyrene to its ultimate toxic intermediate, benzo[*a*]pyrene-7,8-diol-9,10-oxide (Figure 2). Embryonic bioactivation of benzo[*a*]-pyrene, attributed to CYP1A1, has been detected as early as the preimplantation stage in mice (gestational day 4) (Filler and Lew, 1981), well before organogenesis (days 8 to 15). In mouse embryos previously exposed *in vivo* to the CYP1A1-inducing agent 3-methylcholanthrene, the incidence of benzo[*a*]pyrene-initiated fetal anomalies was higher among embryos demonstrating substantial CYP1A1 induction compared with genetically noninducible littermates (Nebert, 1983). Similarly, in embryo culture, pretreatment of rats with 3-methylcholanthrene increased the embryotoxicity of 2-acetylaminofluorene, which was inhibited by the general P450 inhibitor carbon monoxide (Juchau et al., 1985a, b; Faustman-Watts et al., 1986). These results suggested that enhanced P450-catalyzed bioactivation of benzo[*a*]pyrene and 2-acetylaminofluorene in CYP1A1-induced embryos resulted in increased covalent binding and teratologic initiation. However, P450 activities are very low in rodent embryos, particularly until the end of the period of organogenesis (day 15) (Juchau, 1981; Juchau et al., 1992), and it is not clear whether even the elevated activities of those P450s that are inducible during this critical period are sufficient to catalyze teratologically relevant bioactivation. For some teratogens in rodents, an alternative mechanism may involve embryonic peroxidase-catalyzed bioactivation to a free radical intermediate, as discussed in the following section. P450s are more likely to play a role in human teratogenesis, where embryonic activities during organogenesis may achieve 10 to 40%, and for some P450s, as much as 70% or more of adult levels, depending upon the P450 species (Table 3) (Manson, 1986; Raucy and Carpenter, 1993; Kitada and Kamataki, 1994). Even higher P450 activities in human embryos occurring later in gestation, after the so-called

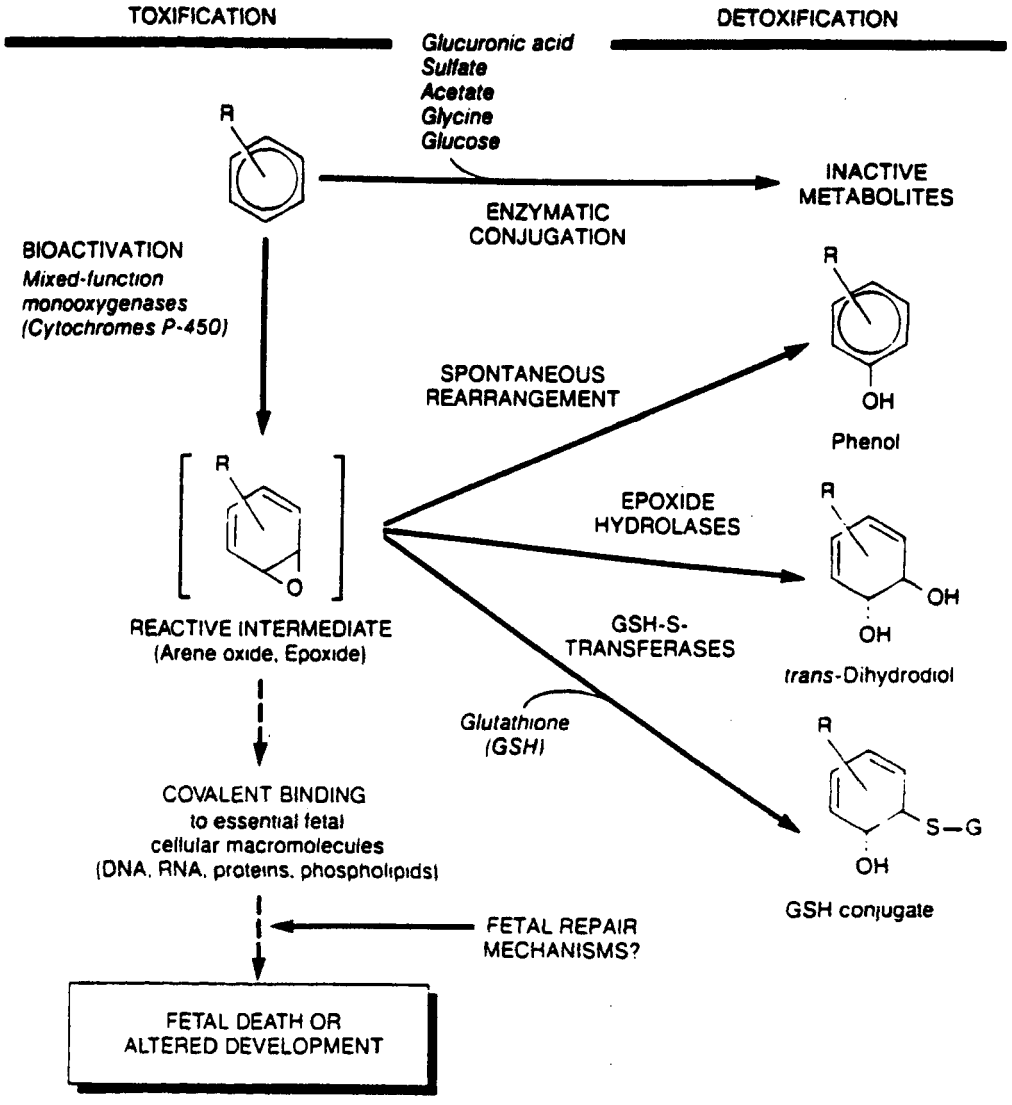

FIGURE 3. Postulated role of electrophilic reactive intermediates in chemical teratogenesis. (From Wells, P. G. 1989. In: *Principles of Medical Pharmacology,* 5th ed., pp. 644–657. (Kalant, H. and Roschlau, W. H. E., Eds.) Toronto: B. C. Decker. With permission.)

period of organogenesis, may be particularly relevant to intellectual dysfunction caused by teratogens such as ethanol (Carpenter and Raucy, 1995), because brain development continues throughout gestation and into the early postnatal period. Although over two thirds of the chemicals recognized as teratogens also are substrates for P450 (Juchau et al., 1992), there are no human studies directly examining the role of P450-catalyzed bioactivation as a determinant of teratologic susceptibility. However, when lymphocytes were obtained from children exposed *in utero* to the anticonvulsant drug phenytoin, coincubation with a P450 source (rodent liver microsomes) and the P450 cofactor NADPH was necessary for cytotoxicity, and the cytotoxicity was elevated in lymphocytes from those children who had a major malformation following *in utero*

TABLE 2
Enzymes Potentially Catalyzing the Bioactivation of Teratogenic Xenobiotics to Electrophilic Reactive Intermediates[a]

Enzyme[b]	Substrate[c]	Ref.[d]
Cytochromes P450[e]		
P450[f]	Phenytoin	Martz et al., 1977; Pantarotto et al., 1982; Roy and Snodgrass, 1990; Finnell et al., 1994
	Cyclophosphamide	Hales et al., 1981a; Fantel et al., 1979; Sanyal et al., 1979
	Thalidomide	Gordon et al., 1983; Braun et al., 1986
	2-Acetylaminofluorene	Faustmann-Watts et al., 1983, 1986; Juchau et al., 1985a, b
	Diethylstilbestrol	Balling et al., 1985
	Thiabendazole	Yoneyama and Ichikawa, 1986
	Acetaminophen	Harris et al., 1989
	Rifampin	Greenaway and Fantel, 1983
	N-Methyl-*N*-(7-propoxynaphthalene-2-ethylhydroxylamine	Terlouw et al., 1993
	Naphthaline	Iyer et al., 1991
CYP1A1	Benzo[*a*]pyrene	Filler and Lew, 1981; Shum et al., 1979
CYP1B1	Benzanthracene[g]	Pottenger et al., 1991; Savas et al., 1994; Otto et al., 1991, 1992
CYP2C3	Phenytoin	Doecke et al., 1991
CYP2C9	Phenytoin	Veronese et al., 1991
	Dextromethorphan	Raucy and Carpenter, 1993
CYP2D6	Trichloroethylene oxide	Fort et al., 1993
CYP2E1	Ethanol	Carpenter and Raucy, 1995
CYP3A3[h]	Aflatoxin B_1	Raucy and Carpenter, 1993
	Benzo[*a*]pyrene	
CYP3A4[h]	Benzo[*a*]pyrene-7,8-diol	Guengerich and Shimada, 1991
CYP3A5[h]	Aflatoxin B_1	Raucy and Carpenter, 1993
CYP3A7[h]	Warfarin	Yang et al., 1994
	Benzyloxyresorufin	
Prostaglandin H synthase (PHS)	Phenytoin	Kubow and Wells, 1986, 1989
	Benzo[*a*]pyrene	Marnett et al., 1975
	Benzo[*a*]pyrene-7,8-diol	Marnett et al., 1975; Byczkowski and Kulkarni, 1989
	2-Naphthylamine	Boyd and Eling, 1987
	2-Acetylaminofluorene	Boyd and Eling, 1984; Boyd et al., 1983

TABLE 2 (continued)
Enzymes Potentially Catalyzing the Bioactivation of Teratogenic Xenobiotics to Electrophilic Reactive Intermediates[a]

Enzyme[b]	Substrate[c]	Ref.[d]
Lipoxygenases (LPOs)	Phenytoin	Kubow and Wells, 1988; Yu and Wells, 1995
	Aflatoxin B_1	Datta and Kulkarni, 1994
	Benzo[*a*]pyrene-7,8-diol	Byczkowski and Kulkarni, 1989

[a] Electrophilic reactive intermediates are quantified by measuring covalent binding of the xenobiotic to target cellular macromolecules (usually DNA and/or protein). In some cases, covalent binding may result from free radical reactive intermediates, as discussed later.
[b] Abbreviations: P450, cytochromes P450; CYP, cytochrome P450; PHS, prostaglandin H synthase; LPO, lipoxygenase.
[c] Where available, teratogenic substrates are illustrated, but not all substrates are known teratogens (e.g., dextromethorphan).
[d] Citations here and throughout this review are only representative, and should not be considered to be comprehensive.
[e] In general, activities of most P450s (except 1B1) in rodent embryos during organogenesis are low to negligible compared with adult hepatic activities, while human fetal activities are substantially higher, constituting 20 to 40% or more of adult activities. The term P450 refers nonspecifically to activities for which the particular isoenzymes were not determined.
[f] In these studies, the P450 isoenzymes were not identified.
[g] Substrates include benzanthracene, but the teratological relevance is unknown.
[h] Fetal 3A enzymes have been identified in humans, but are reported not to be found in rodents and rabbits unless transplacentally induced (Wrighton and Stevens, 1992; Yang et al., 1994).

TABLE 3
Cytochromes P450-Dependent Reactions Catalyzed by Human Fetal Liver Preparations

Substrate	Metabolic reactions	Activity (% of adult liver)
Aminopyrine	*N*-Demethylation	30
Ethylmorphine	*N*-Demethylation	40–70
Dimethylnitrosamine	*N*-Demethylation	Present
Chlorpromazine	*N*-Demethylation;	Present
	aromatic hydroxylation	Present
Diazepam	Metabolites	10
Desmethylimipramine	Aromatic hydroxylation	Present
Aniline	Aromatic hydroxylation	30
Benzo[*a*]pyrene	Aromatic hydroxylation	2–4
Hexobarbital	Aliphatic hydroxylation	40

From Kitada, M. and Kamataki, T. 1994. *Drug Metab. Rev.* 26: 2305–2323. With permission.

phenytoin exposure (Strickler et al., 1985). These results suggested that a detoxifying pathway for a P450-generated phenytoin reactive intermediate was deficient in susceptible human embryos, although the pathway could not be identified. This lymphocyte model has demonstrated the potential for a similar P450-dependent mechanism in thalidomide teratogenicity, as discussed below.

The question of a maternal contribution to bioactivation and related pathways (see Section VI) remains unclear. For cyclophosphamide, this appears possible, if not probable. In the case of phenytoin, supplementation of murine embryo culture with an exogenous NADPH-dependent microsomal bioactivating system enhanced phenytoin covalent binding and embryopathy, indicating a potential maternal P450-dependent contribution to bioactivation (Shanks et al., 1989). Also, mouse embryos cocultured with hepatocytes from different species, including pregnant mice and rats, and male rabbits, were differentially susceptible to phenytoin embryopathy (Ozolins et al., 1995b). However, in the absence of any supplementation to the embryo culture system, a therapeutic concentration of phenytoin initiates molecular target damage and embryopathy (see below) (Shanks et al., 1989; Miranda et al., 1994; Winn and Wells, 1995b, 1996), indicating that maternal processes are not required for phenytoin bioactivation and teratogenicity.

A major pathway for detoxifying P450-generated electrophilic epoxides involves the insertion of a hydroxyl ion, catalyzed by epoxide hydrolases, forming a stable dihydrodiol metabolite. Epoxide hydrolase activities were found to be significantly lower in the amniocytes (fetal cells) of pregnancies in which children exposed *in utero* to phenytoin demonstrated a characteristic pattern of birth defects termed the fetal hydantoin syndrome (FHS), compared with similarly exposed children who developed normally (Buehler et al., 1990). Furthermore, epoxide hydrolase activity was measured in heteropaternal dizygotic twins exposed *in utero* to phenytoin (Buehler, 1984). These twins were discordant for the clinical features of FHS, and epoxide hydrolase activity was very low in the twin with FHS, but high in the unaffected twin. Thus, epoxide hydrolase may constitute an important detoxifying pathway protecting the embryo from chemical teratogenesis. These human results are consistent with studies in mice showing that pretreatment of pregnant dams with the epoxide hydrolase inhibitor trichloropropylene oxide (TCPO) enhanced the covalent binding of phenytoin to embryonic protein, and increased phenytoin teratogenicity (Martz et al., 1977; Harbison, 1978). Similarly with thalidomide, using an *in vitro* assay incubating human lymphocytes with homogenates from maternal rabbit liver, or from rabbit, monkey, or human fetuses, thalidomide caused NADPH-dependent cytotoxicity that was inhibited by TCPO or the addition of purified epoxide hydrolase (Gordon et al., 1981), implying bioactivation by P450s and detoxification of an arene oxide intermediate by epoxide hydrolase (Figure 3). Hepatic microsomes from rat, a species resistant to thalidomide teratogenicity, did not enhance thalidomide cytotoxicity in human lymphocytes. Similarly, the susceptibility of different strains of mice to phenytoin-initiated malformations correlated with their activities of epoxide hydrolase (Finnell and Chernoff, 1987). The apparent importance of epoxide hydrolases as determinants of teratologic susceptibility also implies a role for a P450-generated reactive intermediate, although epoxides can be generated by other mechanisms, as discussed below. It also is worth remembering at this point that, for TCPO and other chemical probes, as well as for nutritional modulations discussed in this review, unanticipated molecular and bio-

chemical effects may confound interpretation of the results, and a diversity of approaches may be required to achieve an acceptable level of confidence in a mechanism. In teratology, these interpretive problems are compounded by often substantial differences with respect to gestational timing, measurements *in vivo* compared with cultured intact embryos or embryonic cells, or homogenates, and maternal vs. embryonic activities and responses.

Another major pathway for the detoxification of electrophilic reactive intermediates is via conjugation with glutathione (GSH), catalyzed by GSTs. The teratological importance of thiols, and particularly GSH, in protecting the embryo from toxic reactive intermediates has been demonstrated for several teratogens *in vivo* and/or in embryo culture (Table 4). In these studies, xenobiotic teratogenicity usually is decreased by pretreatment with GSH or its precursors and enhanced by depletors of GSH or inhibitors of its synthesis. However, thiol rescue may not always be effective, despite a demonstrated embryoprotective role of GSH for a particular teratogen. For example, phenytoin teratogenicity was not reduced by *in vivo* pre- or posttreatment with the GSH precursor *N*-acetylcysteine (Wong and Wells, 1988), despite unequivocal evidence of enhancement of phenytoin embryopathy *in vivo* and in embryo culture by pretreatment with GSH depletors or inhibitors of GSH synthesis (Table 4). The failure of pretreatments with

TABLE 4
Embryoprotective Role of Glutathione (GSH) in Reactive Intermediate-Mediated Chemical Teratogenesis[a]

Pretreatment	Teratological consequence	Representative teratogens	Ref.
GSH and/or precursors[b]	Protection	Cyclophosphamide	Ashby et al., 1976; Hales, 1981b
		Acrolein	Slott and Hales, 1987
		Acetaminophen	Stark et al., 1987
		Cytochalasin D	Harris et al., 1988
		Methylmercury	Kromidas et al., 1990; Ornaghi et al., 1993
GSH depletors[c]	Enhancement	Cyclophosphamide	Hales, 1981b
		Phenytoin	Harbison, 1978; Lum and Wells, 1986; Wong et al., 1989; Miranda et al., 1994
		Acrolein	Slott and Hales, 1987
		Acetaminophen	Stark et al., 1987
		Cytochalasin D	Harris et al., 1988
		Acrylonitrile	Saillenfait et al., 1993

[a] Studies *in vivo* and/or in embryo culture.
[b] Precursors include cysteine, *N*-acetylcysteine, and methyl and ethyl esters of GSH.
[c] Includes GSH depletors (diethyl maleate, acetaminophen) and synthesis inhibitors (buthionine sulfoximine).

GSH in particular, and to a lesser extent with *N*-acetylcysteine, to inhibit reactive intermediate-mediated embryopathy may be due to inadequate uptake of these thiols into certain target tissues (Meister, 1983). In some cases, this artifact may be circumvented by the administration of methyl or ethyl esters of GSH, which readily enter all cell types, wherein they are cleaved by intracellular esterases, releasing GSH in increased or even supraphysiological intracellular concentrations (Anderson et al., 1985; Anderson and Meister, 1989). However, the respective methyl or ethyl alcohols concomitantly released may be embryotoxic and confound interpretation of the results (Winn and Wells, unpublished data). Alternatively, cellular penetration of GSH can be enhanced by administration in liposomes (Jurima-Romet and Shek, 1991; Suntres and Shek, 1994). *In vitro* subcellular models also have been employed to determine the potential teratologic relevance of GSH-dependent detoxification of reactive intermediates. For example, using rodent liver microsomes supplemented with the P450 cofactor NADPH, GSH and other thiols have been shown to reduce the covalent binding of phenytoin to microsomal protein (Pantarotto et al., 1982; Kubow and Wells, 1989; Roy and Snodgrass, 1990).

With respect to the catalytic enzyme for the conjugation of GSH to electrophilic reactive intermediates, hereditary deficiencies in some GSTs are common, and have been associated with enhanced susceptibility to chemical carcinogenesis (Sato, 1988; Gonzalez, 1995), which may be predictive of teratologic susceptibility. Despite the demonstrated importance of GSTs, there are no studies evaluating the teratologic relevance of this family of enzymes in detoxifying electrophiles, although there is presumptive evidence, discussed below, of a cytoprotective role for the GST isozyme that provides selenium-independent GSH peroxidase activity. The absence of information for GSTs in electrophile detoxification in teratogenesis may result from the normal embryonic activity of GST being too low to contribute to detoxification (Juchau, 1981), and/or the possibility that GST deficiencies are embryolethal and difficult to detect.

B. Free Radicals and Oxidative Stress

A number of xenobiotics are thought to initiate teratogenicity via bioactivation and the direct formation of a reactive free radical intermediate, and/or the subsequent indirect formation of reactive oxygen species (ROS) (Table 5). Several bioactivating enzymes, particularly peroxidases, have been postulated to catalyze the one-electron oxidase of xenobiotics (loss of one electron) to teratogenic free radical intermediates (Figure 4, Table 5). Peroxidases such as PHS (Marnett, 1990), and related enzymes such as LPOs, are particularly attractive as putative embryonic bioactivating enzymes because, unlike most P450s, they are present with high content (Hume, 1993; Wells et al., 1995; Winn and Wells, 1996) and activity (Table 5) in both rodent and human embryos during organogenesis. The xenobiotic free radicals and/or ROS can oxidize, as distinct from covalently binding to, molecular targets such as DNA, protein, and lipid in a process referred to as oxidative stress, which is thought to alter cellular function potentially resulting in *in utero* death or teratogenicity. In the case of phenytoin and related xenobiotics (Winn and Wells, 1995a), peroxidase-catalyzed bioactivation has been implicated by several lines of evidence. (1) in *in vitro* studies, purified PHS, LPOs, and/

TABLE 5
Enzymes That May Catalyze the Bioactivation of Xenobiotics to Teratogenic Free Radical Intermediates and/or the Subsequent Formation of Reactive Oxygen Species (ROS)

Enzyme	Substrate	Ref.
Prostaglandin H synthase (PHS)	Phenytoin	Kubow and Wells, 1986, 1989; Wells et al., 1989a; Liu and Wells, 1995b
	Mephenytoin	Liu and Wells, 1995b
	Nirvanol	Liu and Wells, 1995b
	Trimethadione	Wells et al., 1989b; Liu and Wells, 1995b
	Dimethadione	Wells et al., 1989b; Liu and Wells, 1995b
	Thalidomide	Arlen and Wells, 1989, 1996; Liu and Wells, 1995b
	Benzo[*a*]pyrene	Marnett et al., 1977, 1978; Winn and Wells, 1994
	13-*cis*-Retinoic acid (isotretinoin)	Samokyszyn et al., 1984; Kubow, 1992
	Diethylstilbestrol	Degen et al., 1982
	Cyclophosphamide	Kanekal and Kehrer, 1993
	Acetaminophen	Potter and Hinson, 1987; Keller and Hinson, 1991
	2-Naphthylamine	Boyd and Eling, 1987
	2-Acetylaminofluorene	Boyd and Eling, 1984
Lipoxygenases (LPOs)	Phenytoin	Kubow and Wells, 1988; Yu and Wells, 1995
	Cyclophosphamide	Kanekal and Kehrer, 1993
Thyroid peroxidase	Phenytoin	Kubow and Wells, 1989
Myeloperoxidase	Phenytoin	Uetrecht and Zahid, 1988
Horseradish peroxidase	Phenytoin	Kubow and Wells, 1989; Liu and Wells, 1995b
	2-Naphthylamine	Boyd and Eling, 1987
	2-Acetylaminofluorene	Boyd and Eling, 1984

or horseradish peroxidase produce cofactor-dependent bioactivation and free radical formation (Kubow and Wells, 1989; Parman et al., 1996), resulting in the covalent binding of phenytoin to protein (Kubow and Wells, 1989; Yu and Wells, 1995) and DNA (Liu and Wells, 1994a), as well as the oxidation of DNA, protein, and lipid by phenytoin and the structurally related xenobiotics trimethadione, dimethadione, mephenytoin, nirvanol, and thalidomide (Liu and Wells, 1995a, b). Furthermore, these effects are blocked by a number of inhibitors of PHS and/or LPOs, including indomethacin, acetylsalicylic acid (ASA, aspirin), and eicosatetraynoic acid (ETYA). Conversely, phenytoin teratogenicity is enhanced by diets that increase the membrane content of arachidonic acid (High and Kubow, 1994), which is the source of PGG_2

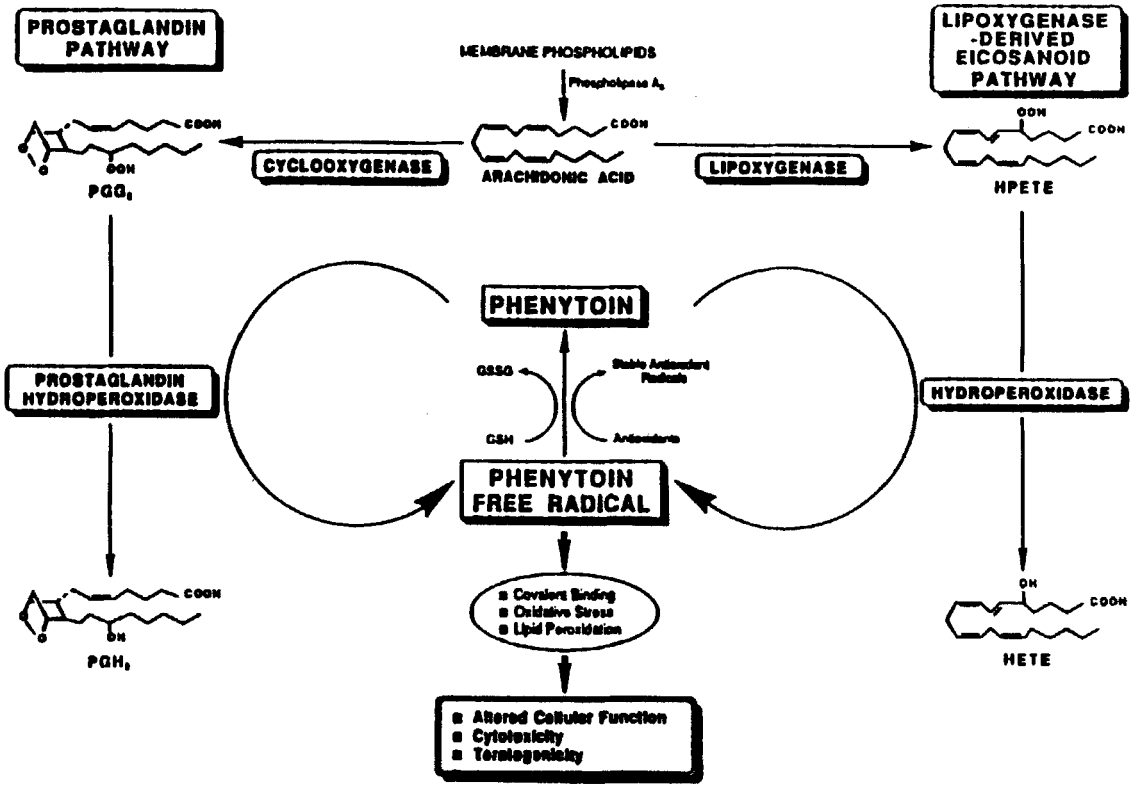

FIGURE 4. Postulated bioactivation of phenytoin to a teratogenic free radical intermediate by embryonic enzymes with peroxidase activity. Cyclooxygenase and hydroperoxidase are the components of prostaglandin H synthase (PHS). (From Yu, W. K. and Wells, P. G. 1995. *Toxicol. Appl. Pharmacol.* 131: 1–12. With permission.)

cosubstrate for reactions catalyzed by PHS and LPOs (Figure 4). (2) In embryo culture, substantial embryonic PHS content is detectable on gestational day 9 (Wells et al., 1995; Winn and Wells, 1996), and phenytoin embryopathy is blocked by the PHS/LPO inhibitor ETYA (Miranda et al., 1994). (3) In *in vivo* studies, the teratogenicity of phenytoin, trimethadione, and dimethadione in mice (Wells et al., 1989b, c; Yu and Wells, 1995), and thalidomide in rabbits (Arlen and Wells, 1989, 1996), is reduced by the respective PHS and PHS/LPO inhibitors ASA and ETYA, as are *in vivo* embryonic covalent binding of phenytoin to protein (Wells et al., 1989c) and phenytoin-initiated oxidation of embryonic protein (Liu and Wells, 1995b). As exemplified for benzo[*a*]pyrene (Figure 2), bioactivation may involve the direct production of a toxic reactive free radical intermediate, or the indirect, subsequent production of toxic ROS. In either case, xenobiotic free radicals can be directly reduced, and thereby detoxified, by reactions with GSH, in which case GSH is oxidized to its disulfide product, GSSG. Both phenytoin and thalidomide initiate thiol oxidation in rats, and thalidomide produces substantially more thiol oxidation in rabbits (Arlen and Wells, 1990, submitted), which, unlike rats, are highly susceptible to thalidomide teratogenicity. Similarly, embryonic GSH oxidation is enhanced in embryo culture by a therapeutic concentration of phenytoin (Wells and Williams, 1994; Winn and Wells, 1996). In addition to oxi-

dative stress, in some cases, xenobiotic free radical intermediates can form tissue adducts directly by abstracting an electron from a double bond, while in other cases, peroxyl radicals produced by PHS may epoxidize substrates, creating an electrophilic reactive intermediate that covalently binds to molecular targets, as has been shown for benzo[*a*]pyrene (Figure 5). *In vitro* covalent binding of phenytoin to protein has been shown to be catalyzed by purified PHS (Kubow and Wells, 1989) and LPO (Yu and Wells, 1995).

The detection of free radical intermediates of proteratogens has both mechanistic and clinical implications, as elaborated elsewhere (Wells et al., 1996). Most commonly, unstable free radicals can be "spin trapped" *in vitro* by reaction with chemicals such as *a*-phenyl-*N*-*t*-butylnitrone (PBN), forming a stable, xenobiotic-spin trap radical adduct that can be detected and characterized by electron spin resonance spectrometry (ESR). *In vitro* bioactivation of phenytoin by PHS produces a free radical intermediate, although the type of radical was not characterized (Kubow and Wells, 1989). For phenytoin and related xenobiotics, subsequent ESR studies using PBN suggest the formation of an initial C-centered xenobiotic free radical reactive intermediate that rapidly reacts to produce reactive oxygen species (Parman et al., 1996). Xenobiotic free radical intermediates often react directly or indirectly with molecular oxygen to produce ROS such as hydroxyl radicals (Figure 6) (Kappus, 1986; Halliwell and Gutteridge, 1989), the presence and teratological relevance of which often can be inferred by thiol oxidation (see above) and characteristic oxidative macromolecular lesions (see Section V below), as well as by the oxidation of probe substrates such as salicylate. *In vivo,* salicylate is hydroxylated by hydroxyl radicals at both the 3-C and 5-C positions, whereas enzymatic hydroxylation occurs only at the 5-carbon (Halliwell et al., 1991). Thus, administration of salicylate followed by plasma sampling over time permits an *in vivo* assessment of the xenobiotic-initiated endogenous production of potentially teratogenic ROS (Figure 7). In mice, phenytoin initiated substantial hydroxyl radical formation that varied remarkably among individual animals in both the amount and time of maximal formation (Kim and Wells, 1993, 1996). In addition to confirmation of chemical mechanisms, the determination of salicylate hydroxylation and similar approaches ultimately may prove useful in estimating individual teratologic susceptibility in humans.

Some xenobiotics can undergo reductive bioactivation (gain of one electron), catalyzed by enzymes such as P450 reductase (Table 6), followed by reoxidation by molecular oxygen, which also produces potentially teratogenic ROS (Figure 6). Such xenobiotics are known as "redox cyclers" and, in accordance with their redox potential, can accept electrons from a number of biological reducing agents, including reduced flavoproteins, reduced ferredoxin, NADPH, NADH, GSH and other thiols, and ascorbate (Figure 6) (Juchau et al., 1986). Some examples of redox cyclers are listed in Table 7 and are reviewed elsewhere (Juchau et al., 1986; Kappus, 1986).

Some teratogens may react directly with oxygen in biological systems to produce ROS. For example, the teratogenicity of hydroxyurea has been postulated to be mediated through the direct (nonenzymatic) formation of ROS (DeSesso, 1979; DeSesso et al., 1994), and antioxidants decrease hydroxyurea teratogenicity (DeSesso and Goeringer, 1990; DeSesso et al., 1994). Similarly, cocaine teratogenesis has been postulated to be mediated via ROS production caused by cocaine-initiated hypoxia/

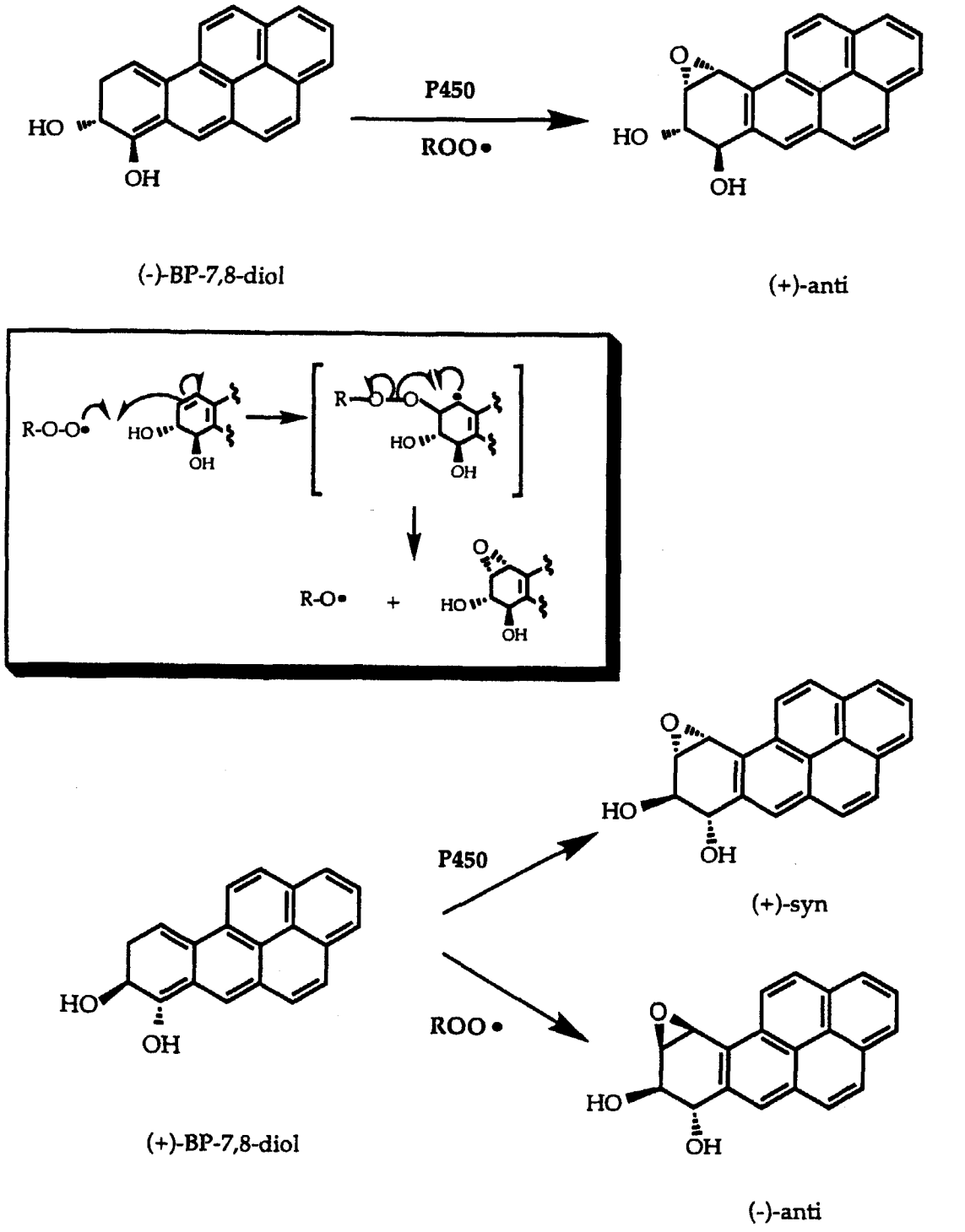

FIGURE 5. Role of prostaglandin H synthase in peroxyl radical-mediated epoxidation of benzo[*a*]pyrene to form B[*a*]P-7,8-diol epoxides. (Modified from Marnett, L. J. 1990. *Environ. Health Perspect.* 88: 5–12. With permission.)

reperfusion (Fantel et al., 1992). In support of this hypothesis, *in vivo* pretreatment with the free radical spin-trapping agent PBN or the antioxidant 2-oxothiazoiodine-4-carboxylate inhibited cocaine embryopathy (Zimmerman et al., 1994).

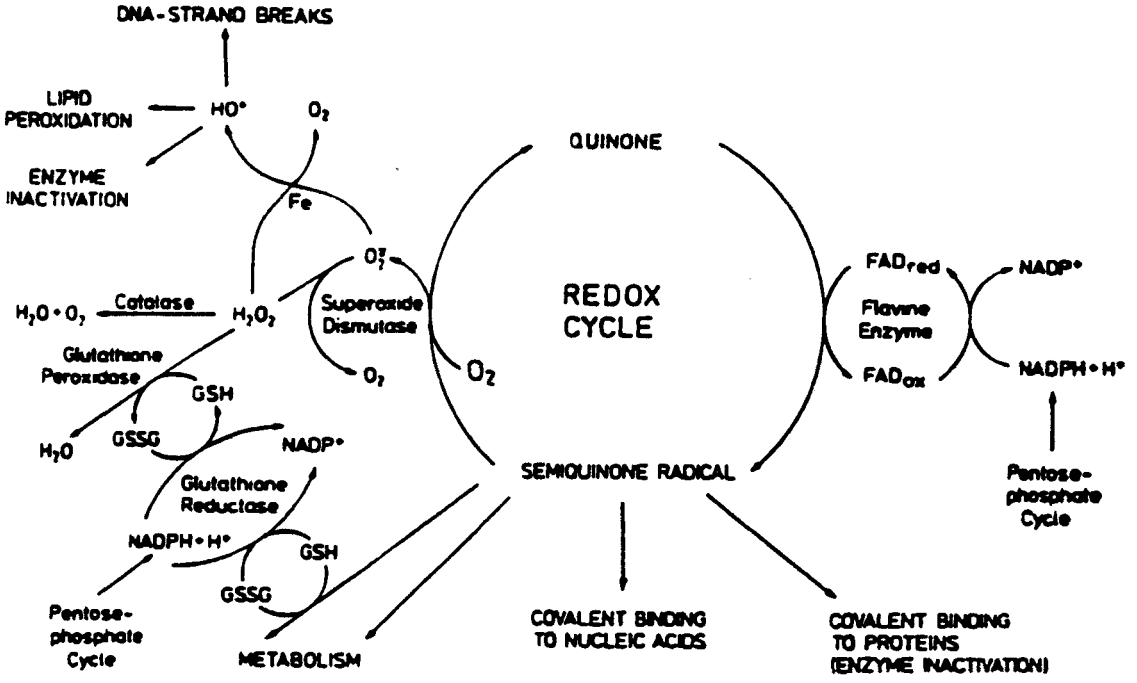

FIGURE 6. Redox cycling of teratogens and cytoprotective pathways potentially relevant to free radical-initiated lipid peroxidation and chemical teratogenesis. (From Kappus, H. 1986. *Biochem. Pharmacol.* 35: 1–6. With permission.)

IV. CYTOPROTECTION

A number of pathways are cytoprotective in that they protect the cell without directly detoxifying a xenobiotic reactive intermediate. Some so-called "phase II" pathways of elimination could be included here, and particularly the UGT-catalyzed conjugation of xenobiotics with glucuronic acid, as discussed above in Section II.

The potential teratological relevance of more commonly appreciated cytoprotective pathways (Table 8) (Halliwell and Gutteridge, 1989) is exemplified in the accompanying scheme depicting free radical-mediated initiation of lipid peroxidation and oxidation of DNA and protein (Figure 6). Selenium-dependent GSH peroxidase, using GSH as a cofactor, detoxifies hydrogen peroxide (H_2O_2) and lipid hydroperoxides (LOOH) (Figures 6 to 8). Pregnant CD-1 mice fed a selenium-deficient diet were more susceptible to phenytoin teratogenicity during the period of maximal depletion of selenium-dependent GSH peroxidase, and this teratological susceptibility was inhibited by selenite rescue, which restored GSH peroxidase activity (Ozolins et al., 1996). More prolonged selenium deprivation resulted in the induction of selenium-independent GSH peroxidase, which is a GST isoenzyme; pregnant mice treated at this time with phenytoin exhibited less teratogenicity, suggesting a potential embryoprotective role for GST.

The enzyme glutathione reductase is necessary to reduce GSSG formed during oxidative stress (Figure 6), hence maintaining the cellular concentration of GSH, which is necessary for the activity of GSH peroxidase, as well as many other enzymes and processes (Ziegler et al., 1983). The frequency and variability of glutathione reductase deficiencies in humans is quite high (Frischer et al., 1973) and may have signifi-

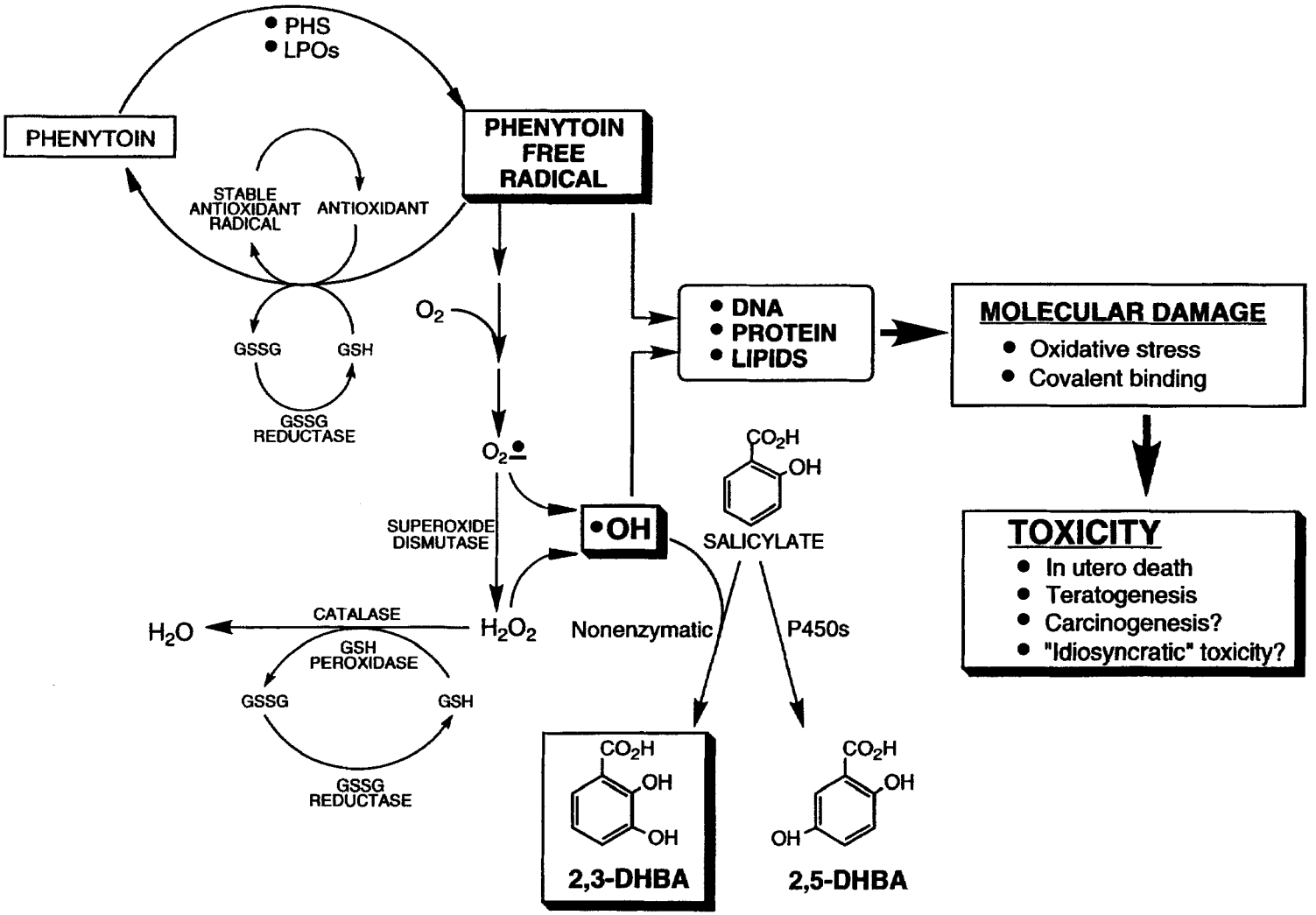

FIGURE 7. Rationale for the use of 3-hydroxylation of salicylate to determine *in vivo* phenytoin-initiated formation of potentially teratogenic reactive oxygen species (ROS) such as hydroxyl radicals (HO*). See text for discussion. The role of oxidative damage in phenytoin-initiated idiosyncratic drug reactions and reversible lymphoma is speculative. (From Kim, P. M. and Wells, P. G. 1996. *Mol. Pharmacol.* 49: 172–181. With permission.)

TABLE 6
Reducing Enzyme Systems

NADPH-cytochrome P450 reductase
(microsomal and nuclear)
Mitochondrial (NADH) dehydrogenase
Xanthine oxidase
Ferredoxin reductase
GSH reductase
Mitochondrial NAD(P)H nitro reductase

From Juchau, M. R., Lee, O. P., and Fantel, A. G. 1992. *Drug Metab. Rev.* 24: 195–238. With permission.

TABLE 7
Representative Teratogens or Potential Teratogens That Undergo Redox Cycling

Chemical	Teratogenic effects	Species
Quinones		
AZQ	?	?
Benzo[*a*]pyrene-3,6-quinone	?	?
Doxorubicin	Multiple	Rats (specific)
Mitomycin C	Skeletal	Rats, mice
1,4-Naphiloquinone	?	?
Streptonigrin	Eye, CNS, trunk, tail, limbs, etc.	Rats
Paraquat	Costal cartilage defects	Rats, hens
Aromatic nitro compounds		
Nitrofurazone	Limbs, digits, tail	Mice
Nitrofurantoin	Not defined	Mice
Metronidazole	?	?
Misonidazole	Limbs, eye, brain, palate	Mice
Nitrazepam	?	?
Nitrofen	Eye, kidney, diaphragm	Rats, mice
Nitrobenzene	Multiple	Rats
Azo compounds		
Trypan blue	Hydrocephaly, spina bifida	Several
Evans Blue	Brain, others	Rats
Congo Red	Hydronephrosis, microophthalmia, hydrocephalus	Rats
Aminoazobenzenes	Skeletal, cleft palate	Mice
Miscellaneous		
Bleomycin-Fe complex	Multiple	Rats, mice

From Juchau, M. R. et al. 1986. *Environ. Health Perspect.* 70: 131–136; Kappus, H. 1986. *Biochem. Pharmacol.* 35: 1–6. With permission.

cant toxicological relevance. Engineered Chinese hamster cells having only 50% glutathione reductase activity were more susceptible to diamide cytotoxicity compared with control cells (Tonoki, 1994). Pregnant CD-1 mice pretreated with nonteratogenic

TABLE 8
Cytoprotective Pathways for Oxidative Stress with Potential Teratological Relevance

Enzyme	Teratogen	Ref.
Glutathione peroxidase	Phenytoin	Ozolins et al., 1991, 1996
	Hyperglycemia	Eriksson and Borg, 1991
Glutathione S-transferase	Phenytoin	Ozolins et al., 1991, 1995c
	Cyclophosphamide[a]	Dirven et al., 1994
	Methylmercury	Nishikido et al., 1987
Superoxide dismutase	Phenytoin	Winn and Wells, 1995b
	Benzo[*a*]pyrene	Winn and Wells, 1994, 1996
	High oxygen tension	Noda et al., 1991
		Nonogaki et al., 1992
	Cyclophosphamide	Kanekal and Kehrer, 1993
	Hyperglycemia	Eriksson and Borg, 1991, 1993
Catalase	Phenytoin	Winn and Wells, 1995b
	Benzo[*a*]pyrene	Winn and Wells, 1994
	Hyperglycemia	Eriksson and Borg, 1991
Glutathione reductase	Phenytoin	Wong and Wells, 1989
Iron sequestration	Phenytoin	Wells et al., 1991
Glucose-6-phosphate dehydrogenase	Phenytoin	Nicol and Wells, 1996
Thioredoxin	High oxygen tension	Noda et al., 1989, 1991
γ-Glutamyl-cysteine synthase	Speculative	

[a] Study evaluated only metabolism.

doses of the GSH reductase inhibitor 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) were more susceptible to phenytoin teratogenicity (Wong and Wells, 1989). In embryo culture the embryopathy of tolbutamide was attributed to its ability to inhibit glutathione reductase and subsequently decrease GSH (Ziegler et al., 1993), although other effects of tolbutamide may be contributory, given that GSH depletion alone is not necessarily embryopathic (Lum and Wells, 1986; Wong et al., 1989; Miranda et al., 1994). The potentiation of phenytoin teratogenicity in mice by low-level exposure to nonteratogenic concentrations of methylmercury (Wells and Srivastava, 1992) may be due to the potent inhibition by methylmercury of cytoprotective enzymes such as GSH reductase and GST (Dierickx, 1982; Chung et al., 1982). This teratologic synergism illustrates the dual practical problems of assessing risk populations under conditions of multiple xenobiotic exposure and in establishing safe levels of exposure for environmental chemicals.

The potential teratological relevance of superoxide dismutase (SOD) and catalase, which, respectively, detoxify superoxide and H_2O_2, was observed in embryo culture, where the addition of either of these antioxidative enzymes increased embryonic activity (Eriksson and Borg, 1991, 1993; Winn and Wells, 1996) and blocked DNA and protein oxidation initiated by phenytoin (Winn and Wells, 1995), as well as protecting against the embryopathic effects of phenytoin (Winn and Wells, 1995b), benzo[*a*]pyrene (Winn and Wells,

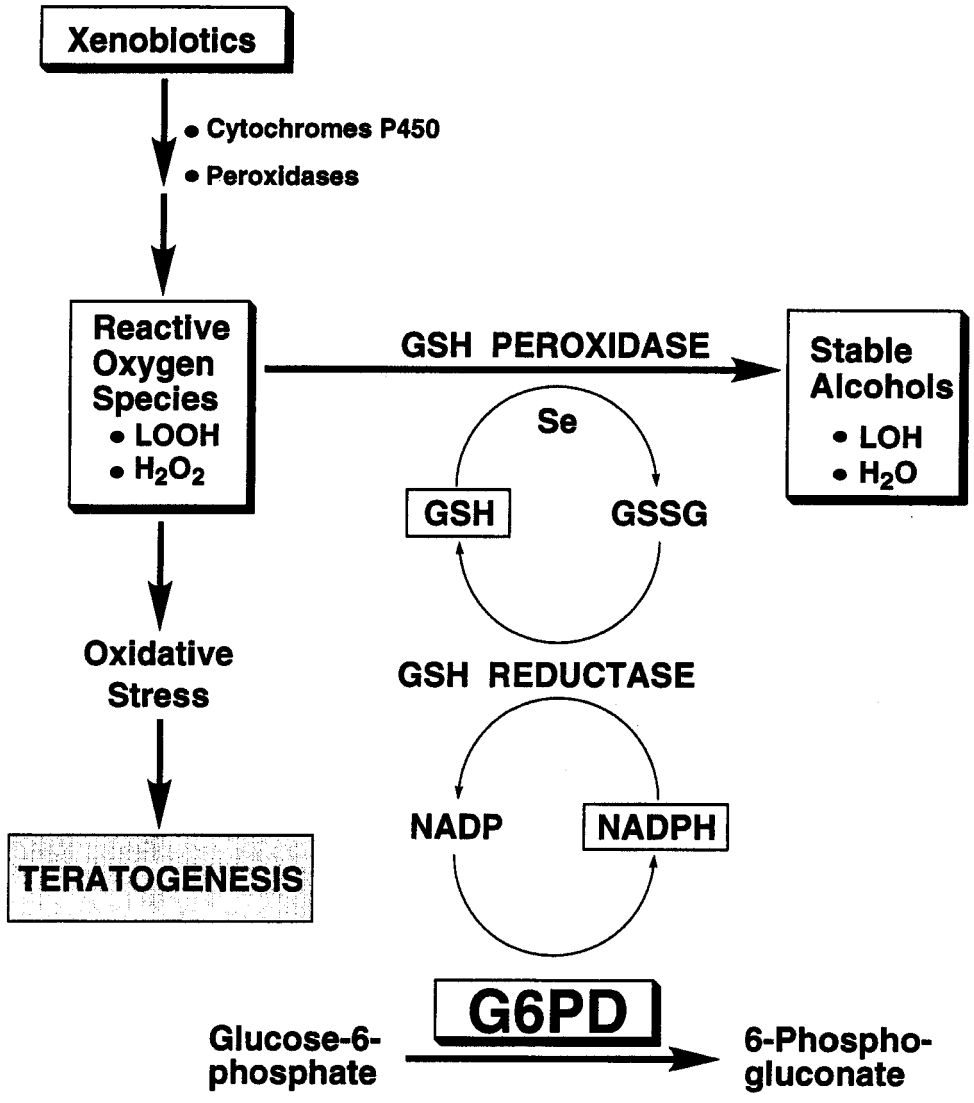

FIGURE 8. Postulated role of glucose-6-phosphate dehydrogenase (G6PD) in embryonic cytoprotection against oxidative teratogens. (From Nicol, C. G. and Wells, P. G. 1996. *Toxicologist.* 16: No. 1008. With permission.)

1996), and hyperglycemia (Eriksson and Borg, 1991, 1993). Conversely, the embryoprotective effects of these antioxidative enzymes provides strong evidence for the involvement of ROS in the molecular mechanism of teratogenesis for these teratogens and hyperglycemia.

Similarly, as discussed previously in part, cellular antioxidants such as GSH (cytosolic) and the vitamins C (cytosolic) and E (in membranes) are critical in the direct and/or indirect detoxification of xenobiotic free radicals and/or such potentially toxic free radical-initiated ROS as H_2O_2, hydroxyl radicals, and lipid peroxyl radicals (Figures 6 and 8). Phenytoin teratogenicity in mice is enhanced by depletors of GSH and inhibitors of GSH synthesis (Table 3) and is inhibited by pretreatment with the antioxidants caffeic acid (Wells et al., 1989c) and

vitamin E (Sanyal and Wells, 1993). In complementary *in vitro* studies, PHS-catalyzed phenytoin covalent binding to protein was inhibited by GSH and the antioxidant butylated hydroxyanisole (Kubow and Wells, 1989).

Free iron has been implicated in the generation of ROS (Figure 6) (Halliwell and Gutteridge, 1989), and pretreatment of pregnant mice with the iron chelator desferoxamine protected against phenytoin teratogenicity, suggesting that processes of embryonic iron sequestration may have a cytoprotective function (Wells et al., 1991).

Glucose-6-phosphate dehydrogenase (G-6-PD) is required for the maintenance of NADPH, and human hereditary deficiencies are common (Panich, 1986; Mehta, 1994). From a toxicological viewpoint, NADPH is particularly important as the cofactor for GSH reductase in maintaining GSH concentrations (Figure 8). While G-6-PD deficiencies generally are considered to be toxicologically relevant only in nonnucleated red blood cells that cannot synthesize new enzyme, it is possible that low embryonic activities for G-6-PD synthesis may render such cells similar in response to nonnucleated cells. Pregnant C3H mice with either a heterozygous or homozygous deficiency in G-6-PD were substantially more susceptible than G-6-PD-normal controls to phenytoin-initiated fetal resorptions (*in utero* death) and postpartum lethality (Nicol and Wells, 1996).

Thioredoxin is an enzyme that directly reduces disulfide bonds of proteins initiated by ROS (Goto et al., 1993). Addition of thioredoxin to embryo culture media was found to protect embryos against "*in vitro* mouse two-cell block", which occurs when cleavage in embryos is arrested at the first or second cell cycle in embryo culture (Noda et al., 1991; Nonogaki et al., 1992). Some baseline oxidative stress occurs in embryo culture, at least in part due to gassing conditions (18% O_2); however, this stress appears to be within physiologically tolerable limits, because the embryos develop normally (New, 1978; Fantel, 1982; Shanks et al., 1989; Miranda et al., 1994; Winn and Wells, 1995b).

More speculatively, hereditary deficiencies of the rate-limiting enzyme in GSH synthesis, γ-glutamyl-cysteine synthase, are rare, but render such individuals more susceptible to the toxicity of drugs bioactivated to a reactive intermediate (Jellum et al., 1983; Larsson and Hagenfeldt, 1983; Spielberg, 1985). While there is no information concerning the relevance of this enzyme to teratogenesis, it ought to be critically important, and it is quite possible that such a deficiency is often embryolethal and therefore difficult to detect.

V. MOLECULAR DAMAGE

With DNA, protein, and lipid as potential targets, in general, the types of xenobiotic-initiated molecular damage include (1) covalent binding of primarily electrophilic, but in some cases free radical, reactive intermediates, and (2) free radical-initiated oxidative stress resulting in target oxidation (Table 9). While inferences can be drawn from analogies to chemical carcinogenesis, little is known about the specific molecular damage underlying teratologic initiation. This is particularly noteworthy given that the teratogenicity of drugs such as thalidomide has been known since the late 1950s. The complexity of this problem can be illustrated with phenytoin, which has been shown to (1) covalently bind to both protein (Martz et al., 1977) and DNA (Liu and Wells, 1994a), as well as (2) oxidize protein (Liu and Wells, 1994b, 1995a; Wells et al., 1995) and GSH (Arlen and Wells, 1990; Wells and Williams, 1994),

TABLE 9
Molecular Damage of Potential Relevance to Chemical Teratogenesis

Molecular damage	Target	Substrate	Ref.
Covalent binding	Protein	Phenytoin	Martz et al., 1977
		Benzo[*a*]pyrene	Shum et al., 1979
		Thiabendazole	Yoneyama et al., 1985, 1986
		2-Acetylaminofluorene	Roy and Kulkarni, 1991
		Benzo[*a*]pyrene-7,8-diol	Marnett et al., 1975
	DNA	Phenytoin	Liu and Wells, 1995b; Winn and Wells, 1995b
		Benzo[*a*]pyrene	Shum et al., 1979; Wang and Lu, 1990; Lu et al., 1993
		2-Acetylaminofluorene	Roy and Kulkarni, 1991
		2-Naphthylamine	Boyd and Eling, 1987
		Benzo[*a*]pyrene-7,8-diol	Marnett et al., 1975
		Aflatoxin B_1	Hsieh and Hsieh, 1993
		Cyclophosphamide	Benson et al., 1988
		N-Methyl-*N*-nitrosourea	Platzek et al., 1988; Frank et al., 1993
		N-Acetoxy-2-acetylaminofluorene	Mirkes et al., 1991
		Ethylmethanesulfonate	Platzek et al., 1994a
		Acetoxymethyl-methylnitrosamine	Platzek et al., 1983
		6-Mercaptopurine riboside	Platzek et al., 1994b
Oxidation	Protein/ glutathione	Phenytoin	Liu and Wells, 1994b, 1995a; Wells and Williams, 1994; Wells et al., 1995
		Thalidomide	Arlen and Wells, 1990
		Diamide	Hiranruengchok and Harris, 1993
	DNA	Phenytoin	Liu and Wells, 1995b
		Mephenytoin	Liu and Wells, 1995b
		Nirvanol	Liu and Wells, 1995b
		Trimethadione	Liu and Wells, 1995b
		Thalidomide	Liu and Wells, 1995b
		Benzo[*a*]pyrene	Winn and Wells, 1994
		Cyclophosphamide (strand breaks)	Pillans et al., 1989
		Phosphoramide mustard (strand breaks)	Little and Mirkes, 1987
	Lipid	Phenytoin	Liu and Wells, 1994b, 1995a
		Cocaine	Zimmerman et al., 1994
		Cyclophosphamide	Lear et al., 1992

DNA (Liu and Wells, 1995b; Winn and Wells, 1995b), and lipid (Liu and Wells, 1994b, 1995a). The relative teratologic contributions of xenobiotic covalent binding to, and xenobiotic-initiated oxidation of, embryonic cellular macromolecules have yet to be determined, and likely vary with the chemistry of the teratogen, as well as with such factors as the target tissue, type of macromolecular target, and stage of conceptal development. In embryo culture, the complete embryopathic spectrum of phenytoin is abolished by addition of either of the antioxidative enzymes superoxide dismutase (SOD) or catalase (Winn and Wells, 1995b) (see Section IV above), indicating a critical role for ROS, and suggesting that oxidation rather than arylation is providing the critical teratological insult in the case of phenytoin. However, it remains to be determined whether similar results are observed *in vivo* and whether other embryopathic efforts of phenytoin that cannot be assessed in embryo culture, such as cleft palate and central nervous system dysfunction, follow a similar pattern. Such knowledge would be of considerable clinical importance, because it would provide a basis for developing therapeutic measures to avoid or reduce unwarranted *in utero* death and teratogenicity. The specific molecular targets also remain to be characterized, let alone individually analyzed in relation to teratologic outcome. The potential interrelationship among these pathways and their relevance to chemical teratogenesis is shown in Figure 9.

Given its role in chemical carcinogenesis (Williams and Weisburger, 1991), DNA is an attractive candidate for a teratologically relevant target. The fundamental importance of DNA lesions in mediating chemical teratogenesis is suggested by the observation that pregnant transgenic mice with a hereditary deficiency in the p53 tumor suppressor gene, which is necessary for DNA repair, are more susceptible to the teratogenicity of both benzo[*a*]pyrene (Nicol et al., 1995) and phenytoin (Laposa and Wells, 1995; 1996). The role of particular DNA adducts and target genes in chemical teratogenesis have not been determined, and constitutes an important outstanding question. Similarly, oxidation of embryonic DNA *in vivo* and/or in embryo culture has been identified only recently for phenytoin (Winn and Wells, 1995b; Liu and Wells, 1995b) and benzo[*a*]pyrene (Winn and Wells, 1994, 1996), and the teratologically relevant target genes remain to be characterized. Among phenytoin and structurally related teratogens, their potency for DNA oxidation in an *in vitro* peroxidase bioactivating system with calf thymus DNA correlated well with their *in vivo* teratologic potency in mice (Liu and Wells, 1995b). Furthermore, *in vivo* embryonic DNA oxidation initiated by phenytoin was blocked by doses of the PHS inhibitor ASA and the free radical spin-trapping agent PBN that inhibit phenytoin teratogenicity (Wells et al., 1989c; Liu and Wells, 1995b).

Alkylation/arylation and/or oxidation of proteins and lipids also likely contribute to mechanisms of chemical teratogenesis. Pretreatment of pregnant mice with chemical modulators that enhance or inhibit the teratogenicity of drugs like phenytoin also, respectively, enhance or inhibit both its covalent binding to, and oxidation of, protein and lipid (Liu and Wells, 1994a, b, 1995a). Only a few preliminary studies have reported a rudimentary characterization of oxidized protein targets (Wells et al., 1995), and this will constitute a fertile area of future teratological research.

VI. MOLECULAR REPAIR

Virtually nothing is known about embryonic protein repair. On the other hand, there is a considerable and rapidly expand-

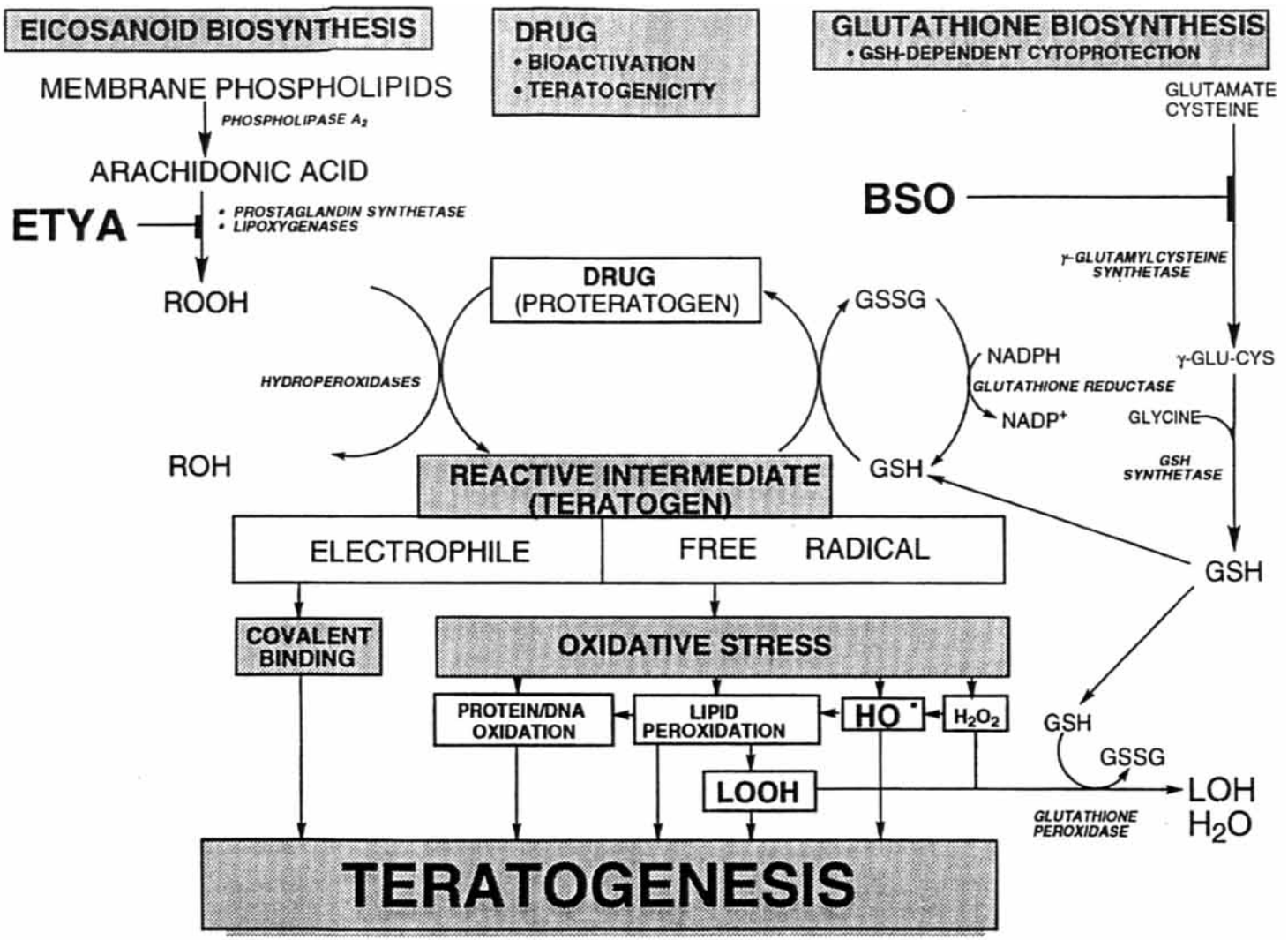

FIGURE 9. Postulated interrelationship among biochemical pathways relevant to chemical teratogenesis. (From Miranda, A. F., Wiley, M. J., and Wells, P. G. 1994. *Toxicol. Appl. Pharmacol.* 124: 230–241. With permission.)

ing understanding of DNA repair, particularly in relation to the field of cancer (Sancar, 1994; Ponz de Leon, 1994; Modrich, 1995; Radman et al., 1995). Embryos may have a substantial capacity for DNA repair, because mouse embryos appeared to remove oxidized guanosine bases at a rate similar to that for maternal liver following *in vivo* exposure to phenytoin (Liu and Wells, 1995b). Similarly, fetal monkey tissues were able to remove benzo[*a*]pyrene-DNA adducts (Lu et al., 1993), and mouse embryos exposed *in vivo* to methyl methane sulfonate and *N*-methyl-*N*-nitrosourea (MNU) demonstrated the removal of specific base substitutions from embryonic DNA (Bochert et al., 1978). It appears that DNA damage, via covalent binding and/or oxidation, may constitute an important mechanism of teratogenesis at least for benzo[*a*]pyrene (Nicol et al., 1995) and phenytoin (Laposa and Wells, 1995; Laposa et al., 1996) (Figure 10). Pregnant transgenic mice with a heterozygous deficiency in the p53 tumor suppressor gene, which directly and indirectly facilitates DNA repair, were more susceptible to *in utero* fetal death and teratogenicity initiated by both benzo[*a*]pyrene and phenytoin. Perhaps more importantly, PCR-based amplification and characterization of the embryonic genotype of fetal resorptions, the remnants of *in utero* death, showed for both benzo[*a*]pyrene and phenytoin that *in utero* deaths occurred predominantly in p53-deficient fetuses from p53-deficient dams (Nicol et al., 1995; Laposa et al., 1996). Interestingly, with benzo[*a*]pyrene, in contrast to most teratological anomalies, two defects occurred only in p53-normal dams (Figure 10), suggesting that a minority of fetal anomalies with this teratogen may be initiated via apoptotic mechanisms, some of which are p53 dependent. Such differential expression could be both target tissue-dependent and teratogen (mechanism) dependent, because phenytoin-initiated embryopathies all occurred more frequently in p53-deficient dams (Laposa and Wells, 1995), while in contrast, fetal anomalies initiated by 2-chloro-2′-deoxyadenosine occurred only in p53-normal dams (Wubah et al., 1996). Thus, for some DNA-damaging teratogens in developmental biology, at least some so-called tumor suppressor genes appear to serve as teratological suppressor genes. This might be expected, given that optimal reproduction has exerted a major evolutionary pressure over the millenia, while few during most of this period survived long enough to develop cancer.

VII. CONCLUSIONS

There is considerable evidence, primarily in rodent models, for the involvement of reactive intermediates in teratologic initiation by numerous xenobiotics. This hypothesis is consistent with the demonstrated roles of relevant pathways of elimination, bioactivation, detoxification, cytoprotection, and repair in modulating teratologic susceptibility. Nevertheless, the contribution of alternative mechanisms, and particularly receptor-mediated teratologic initiation, cannot be excluded for many such teratogens, and there is evidence for teratogens such as phenytoin that several concomitant mechanisms may contribute variably to particular fetal anomalies (Winn and Wells, 1995a). Given the relative absence of such molecular and biochemical studies in pregnant women, the human relevance of reactive intermediate-mediated teratogenicity and its associated determinants of predisposition remain speculative, as do potential therapeutic approaches for reducing xenobiotic-initiated embryonic and fetal damage. In light of the substantial nature of such damage in humans, and a long-standing awareness dating back to the discovery of human

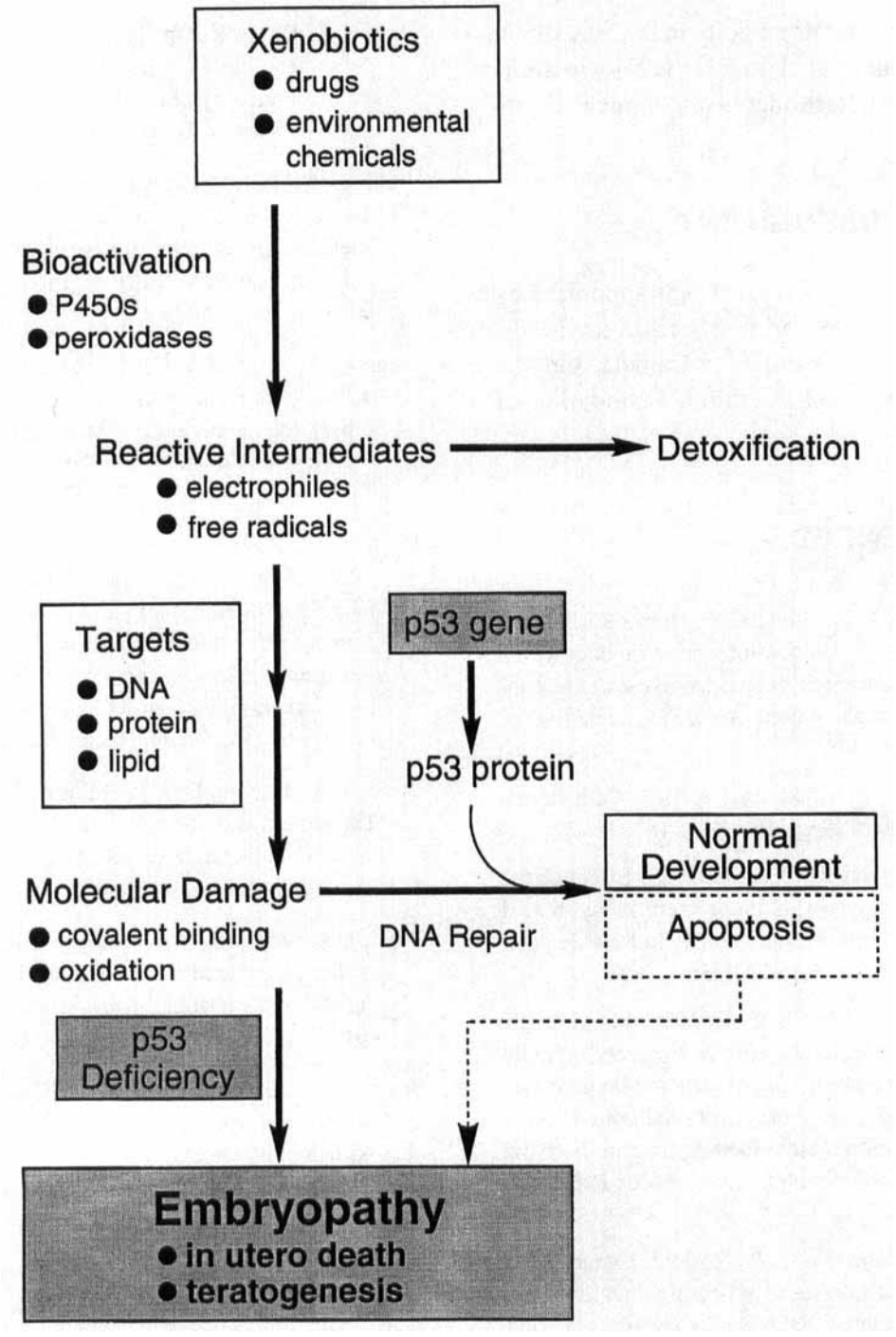

FIGURE 10. Potential embryoprotective role of DNA repair in chemical teratogenesis, exemplified by the p53 tumor suppressor gene. p53-facilitated DNA repair is generally embryoprotective for the DNA-damaging teratogens benzo[*a*]pyrene and phenytoin, with teratogenicity enhanced in p53-deficient dams. However, in some cases, particular fetal anomalies, and/or anomalies initiated by different teratogens or mechanisms, can be increased in p53-normal dams (dashed line), possibly due to the initiation of p53-dependent apoptosis (see text). (Modified from Nicol, C. J., Harrison, M. L., Laposa, R. R., Gimelshtein, I. L., and Wells, P. G. 1995. *Nature Genet.* 10: 181–187. With permission.)

thalidomide teratogenicity in the late 1950s, it is surprising that so little is known about the human determinants of chemical teratogenesis.

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