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BIOACTIVATION IN CHEMICAL TERATOGENESIS

M. R. Juchau

Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98195

INTRODUCTION: DEFINITIONS AND SCOPE

Teratology is a science that has undergone relatively recent maturation and expansion. It deals primarily with persistent (permanent or semi-permanent) developmental defects that are manifest subsequent to parturition or hatching, but that originate prior to parturition or hatching. Most of the earlier observations and investigations dealt with grossly observable morphological abnormalities, sometimes referred to as "terata", associating the science primarily with the discipline of anatomy. Classical teratology is still regarded by many as a science that addresses principally issues of anatomical anomalies. Research performed during the past two decades, however, has served to emphasize that prenatally originating, persistent developmental defects are not restricted to morphologic abnormalities. It is now clearly evident that persistent defects involving behavior, mentation, reproductive capacity, metabolic function, motor coordination, neoplasia, immune function, and a host of other malfunctions associated with specific organs or organ systems (heart, kidney, liver, etc.) can occur in the absence of readily detectable morphologic changes. It is also becoming increasingly appreciated that such defects are often not recognizable at birth. Indeed, a significant fraction of the individual's lifespan may elapse prior to detection of prenatally initiated neoplasms, deficiencies in reproductive capacity, etc. Seemingly largely unappreciated at present is the concept that exposures of embryos to chemicals may produce permanent/semi-permanent functional or metabolic defects at lower exposure levels than those required to produce classical morphologic "terata."

It has become abundantly clear that exposures of the human conceptus to drugs and other chemicals can result in not only transient, reversible toxicity,

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but also in those far more serious permanent or semipermanent detrimental defects that can handicap and/or disfigure for an entire lifetime. This realization has been relatively recent, but was markedly punctuated by the thalidomide tragedy and has since been reemphasized by experiences with diethylstilbestrol, ethyl alcohol, retinoids, various anticonvulsants, androgens, cancer chemotherapeutic agents, organomercurials, and coumarin-type anticoagulants. Chemical substances currently regarded as established human teratogens are listed in Table 1. It is of importance that a large proportion of those chemicals have been added to the list relatively recently, which suggests that many more chemicals will be added in future years. The rapidly increasing number of chemicals listed as teratogenic in experimental animals (1) tends to reinforce this suggestion.

The purpose of this review is to provide an update on recent research that has shed light on the role of bioactivation and reactive intermediates in the chemical causation of dysmorphic manifestations. The generation and toxicity of reactive intermediary metabolites of organic chemicals of low molecular weight has been a research area of considerable interest to investigators concerned with mechanisms of chemical carcinogenesis (2–4), mutagenesis (4, 5), cytotoxicity, and tissue damage (6, 7), atherogenesis (8), ageing

Table 1 Chemicals commonly regarded as having high teratogenic potential in humans

Chemicals	Abnormalities
Thalidomide	Phocomelia, etc
Androgens	Masculinization
Folate antagonists	Multiple
Diethylstilbestrol	Vaginal adenosis, etc
Methyl mercury	CNS lesions
Inorganic iodides	Congenital goiter
Ethanol	Fetal alcohol syndrome
Phenytoin	Fetal hydantoin syndrome
Trimethadione	Fetal tridione syndrome
Alkylating agents	Multiple
Thiourea compounds	Congenital goiter
Warfarin	Saddle nose, etc
Tetracyclines	Tooth enamel staining
Chlorinated biphenyls	Multiple (cola babies)
Cigarette smoke	IUGR, etc
13-*cis*-retinoic acid	Hydrocephalus, etc
Valproic acid	Neural tube defects
Penicillamine	Loose skin, etc
Aminoglycosides	8th cranial nerve damage
Cytarabine	Limb, ear defects

processes (9), allergenic manifestations (10, 11), and possibly a host of other disease processes and pathological conditions (12, 13). Interest in the idea that reactive intermediates may be important determinants of the capacity of chemicals to elicit persistent deleterious effects on developmental parameters prenatally, however, has been relatively recent and the topic has been reviewed only rarely (14, 15). The high complexity of developmental processes, the frequent necessity of performing research with minute quantities of biological material, the potentially confounding influences of a host of maternal factors, the multitude of possible mechanisms whereby chemicals may elicit developmental defects, and a general lack of past appreciation for the enormous impact of birth defects on society and the capacity of chemicals to act as causative agents appear to have been the major impediments to progress in this research area.

INVESTIGATIONS WITH MODEL PROTERATOGENS

Cyclophosphamide

Some of the earliest recorded attempts to ascertain whether a reactive metabolite(s) could account for the dysmorphogenic effects of an administered chemical were reported by Gibson & Becker (16). At that time it was well known that cyclophosphamide required P450-dependent bioactivation in order to exert its cytotoxic and antitumor effects and it seemed reasonable to speculate that its teratogenic effects would likewise require bioactivation. Thus it was somewhat unexpected that administration of a P450 inducer, phenobarbital, reduced the toxic manifestations and administration of a nonspecific P450 inhibitor, SKF-525A, resulted in increased embryotoxicity. The results appeared to suggest that a reactive alkylating intermediate was not responsible for the teratogenic effects manifest. This idea was later reinforced in studies by the same investigators with a series of structural cognates with varying alkylating activities (17). Analyses of the alkylating activities vs. patterns of teratogenesis by these congeners indicated a lack of correlation. Consistent with those results, Welsch (18) later reported that P450 induction with polychlorinated biphenyls would also reduce cyclophosphamide's teratogenicity. In contrast, however, Hales reported (19) that induction in vivo with phenobarbital led to increased teratogenicity. The discrepancy may be explained by species differences since the studies of Gibson & Becker and Welsch were performed with mice, whereas those reported by Hales were with rats.

Regardless, it is extremely difficult to draw satisfactory conclusions from these studies in vivo because of complicating toxicokinetic factors (e.g., administration of phenobarbital results in marked increases in hepatic blood

flow, in bile flow, phase II metabolic reactions, etc.) that could influence the capacity of reactive metabolites generated in the maternal liver (or even elsewhere) to gain access to critical embryonic targets in an in vivo setting. In view of these uncertainties, it is of great interest that a very large number of recent investigations from several independent laboratories have provided substantial evidence to indicate that a reactive intermediate(s) of cyclophosphamide can and will elicit considerable dysmorphogenesis in vitro. The topic has been covered in two extensive recent reviews by Mirkes (20, 21). Cyclophosphamide probably provides the best current example of chemical teratogenesis via bioactivation, even though numerous questions still remain. Currently, the belief is widespread that reactive metabolites are also responsible for the observed permanent/semipermanent teratogenic effects producible via the usual routes of administration of cyclophosphamide to pregnant experimental animals. It does appear likely that phosphoramide mustard and acrolein represent the most important proximate intermediary metabolites responsible for cyclophosphamide-induced abnormal morphogenesis in rat embryos in vitro. In vivo, the P450-dependent conversion to the 4-hydroxycyclophosphamide precursor likely depends largely on isozymes present in the maternal liver. The 4-hydroxy metabolite is commonly regarded as a "transport" metabolite and appears to be active only after subsequent conversion to phosphoramide mustard, acrolein, and perhaps other active species such as carboxyphosphamide (Figure 1). Slott & Hales have provided

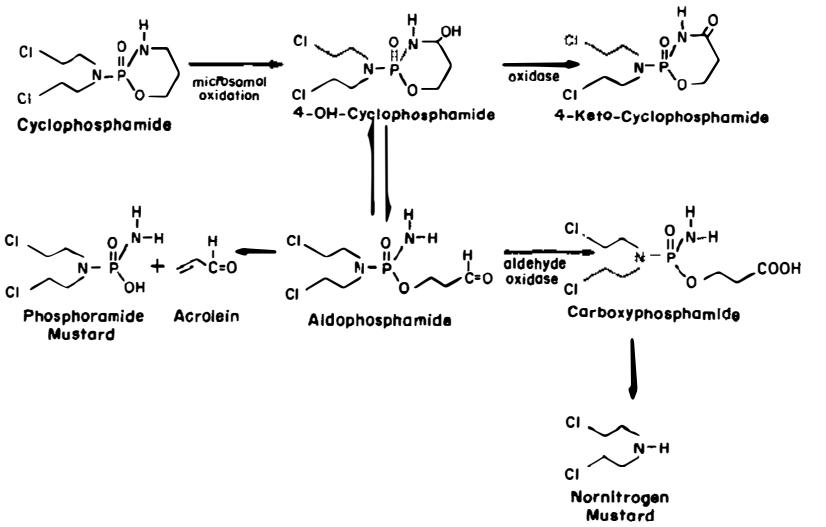

Figure 1 Conversion of cyclophosphamide to various metabolites. From Colvin & Hilton, *Cancer Treat. Rep.* 65:89, 1981, with permission.

recent evidence to suggest that the 4-hydroxy intermediate may also subserve the role of a "transport" metabolite in cyclophosphamide teratogenesis (22). It is noteworthy that both acrolein and phosphoramide mustard elicited significant embryotoxicity after intra-amnionic injections in vivo as well as after additions to whole embryo cultures (23–25). Of course, other metabolites could conceivably participate, and questions as to which metabolite or combination of metabolites act as ultimate dysmorphogens for specific defects remain open. Additionally, the participation in the bioactivation process of target cells/tissues within the conceptus is an important area of future research. An attractive feature of research with cyclophosphamide is that defects elicited in whole embryos in vitro are remarkably similar to those observed after administration of the drug to pregnant rats in vivo (20, 21). This greatly enhances the promise for future elucidation of mechanisms.

2-Acetylaminofluorene

A series of investigations was recently reported (26–30) on studies of the bioconversion of 2-acetylaminofluorene (AAF) to intermediates dysmorphogenic to rat embryos in vitro. Use of this chemical as a model for dysmorphogenic bioactivation is appealing because of extensive background information about relationships between AAF biotransformation (Figure 2) and overt manifestations of its toxicity, including carcinogenicity, mutagenicity, genotoxicity, clastogenicity, and cytotoxicity. In addition, AAF is teratogenic in vivo in rats, mice, and chicks (31–34).

Investigations of the capacity to produce malformations in cultured whole embryos indicated that P450-dependent bioactivation was required (26) and

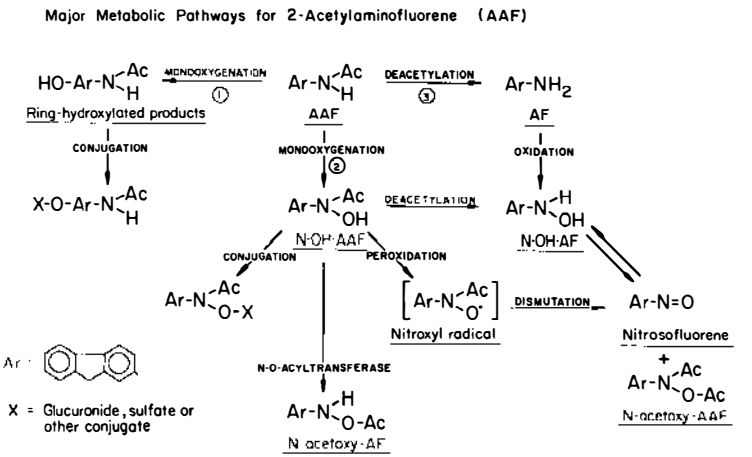

Figure 2 Conversion of 2-acetylaminofluorene to various metabolic products.

that several intermediary metabolites of AAF would produce dysmorphic effects when coincubated with the cultured embryos (27–29). Very remarkably, however, none of the originally suspected metabolites produced the same spectrum of malformations as did AAF when the latter was added together with an hepatic bioactivating system. Specifically, the large increase in incidence of neural tube defects produced by AAF plus the added bioactivation system were not elicited by N-hydroxy-AAF, N-acetoxy-AAF, N-hydroxyaminofluorene, N-O-sulfonoxy-2-acetylaminofluorene or 2-nitrosofluorene. The two former metabolites each produced characteristic prosencephalic hypoplasia with little observable effect on the neural tube per se. The latter three metabolites each elicited large increases in incidence of axial rotation abnormalities, with little detectable effects on the incidence of either prosencephalic abnormalities or neural tube defects. Surprisingly, later experiments (29, 35, 36) revealed that ring-hydroxylated metabolites, particularly purified 7-hydroxy-AAF, when added directly to the culture medium containing no exogenous bioactivating system, would cause the same type of abnormal neurulation as bioactivated AAF. The results were unexpected because ring-hydroxylated metabolites of AAF are commonly regarded as totally inactive products of metabolism. Similarities between the structures of 7-hydroxy-AAF and acetaminophen, however, prompted us to postulate that acetaminophen would produce the same neural tube abnormality as 7-hydroxy-AAF or S-9-bioactivated AAF. Experiments designed to test the hypothesis proved it to be valid (35). It may seem logical to suggest that, in analogy to the metabolite commonly believed to be responsible for the hepatotoxicity of acetaminophen, a quinoneimine may be involved in causing abnormal neurulation by 7-hydroxy-AAF. In addition, the similar neural tube defects elicited by bioactivated AAF, 7-hydroxy-AAF and acetaminophen differed morphologically from those producible by valproic acid or cytochalasin D under the same conditions (36); this further indicates the specificity of the effect. Of future interest will be the degree to which specific types of malformations can be predicted from chemical structure within a series of aromatic amides. Of course, the identity of the reactive intermediate(s) ultimately accountable for evocation of the neural tube defect will be of paramount interest. Several possibilities for interconversions that result in the generation of electrophiles and free radical intermediates are conceivable and some of these are depicted in Figure 3.

Nitroheterocycles

The discovery that niridazole, a potent, antischistosomal, nitrothiazolic antibiotic, would produce a striking embryotoxicity apparently affecting only the right half of cultured embryos (37) led to a series of investigations that have provided insights into mechanisms whereby nitroheterocycles and other

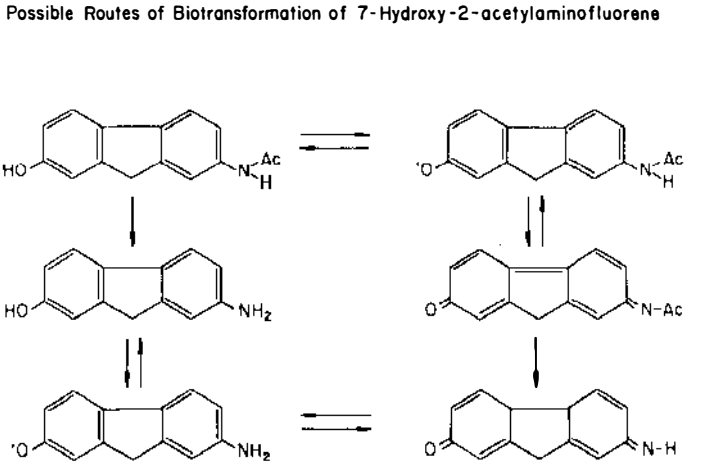

Figure 3 Conversion of 7-hydroxy-2-acetylaminofluorene to potential proximate embryotoxins.

redox-cycling chemicals may be capable of generating dysmorphic phenomena (37–40). The subject has been reviewed recently (41) and thus is dealt with here only briefly. The only chemicals thus far shown to be capable of eliciting the unusual asymmetric defect have been compounds with five-membered heterocyclic rings and bearing nitro groups with redox potentials higher than −0.480 mv. Thus far, these include ronidazole, 2-nitroimidazole, niridazole, nitrofurazone, furazolidone, nifuroxime (39), and misonidazole (unpublished). Except for a puzzling result obtained with nitrofurantoin, a good correlation between single electron redox potentials of the nitro groups and capacity to elicit the defect was also observed. The incidence and severity of the unusual dysmorphic effect was increased under culture conditions in which the oxygen tension was lowered (38). A close relative of niridazole, 4'-methylniridazole, which lacks antischistosomal activity, also failed to produce asymmetric malformations at any of the tested concentrations. It has been reported that the schistosome is unable to reduce the 4'-methyl derivative, probably owing to reduction of the single electron redox potential and/or steric hindrance that results from the presence of a methyl group adjacent to the nitro group. It is well known that the mutagenic activity of nitroheterocycles correlates loosely with the single electron redox potentials of their nitro groups. Similar correlations have been established for the radiosensitization of hypoxic cells by such agents, as well as for their cytotoxicity toward hypoxic microorganisms and other cells.

In vivo (and presumably in vivo), aromatic nitro groups will accept electrons singly from reduced flavoenzymes such as NADPH-cytochrome P450 reductase, the reduced forms of other enzymes such as xanthine oxidase,

aldehyde oxidase, quinone oxidoreductase, and other cellular reducing agents with appropriate redox potentials, including free flavins such as riboflavin (42, 43). Acceptance of the single electron results in the formation of the nitro anion radical. Autooxidation of the radical by molecular oxygen results in the generation of superoxide anions (Figure 4) with potential subsequent formation of highly reactive (and presumably very toxic) hydroxyl radicals (44, 45). However, the fact that the dysmorphogenic effect is ameliorated by increases in oxygen tension suggests strongly that reactive oxygen species are not causally involved in the genesis of the observed axial asymmetry. For the same reasons, a radical-initiated lipid peroxidation mechanism seems somewhat unlikely, although the possibility has not been excluded. Direct effects on critical macromolecules by the nitro anion radical, local loss of reducing equivalents such as NADPH, NADH, or GSH via futile redox cycling and/or local hypoxia that result from the redox cycling-induced depletion of molecular oxygen in critical cells or tissues (Figure 4) represent other possibilities. Recently we have obtained preliminary evidence in support of the last-mentioned possibility (46, 47). Future investigations of the dysmorphogenic effects of other bioreducible chemicals promise not only to reveal mechanisms of chemical teratogenesis but also of the control of normal developmental processes.

Thalidomide

Although the evidence is far from conclusive, a growing body of published data has accumulated to suggest that some or all of the teratogenic effects producible by thalidomide may be mediated via reactive intermediary metabolites generated by P450-dependent catalysis. Braun and his coinvestigators

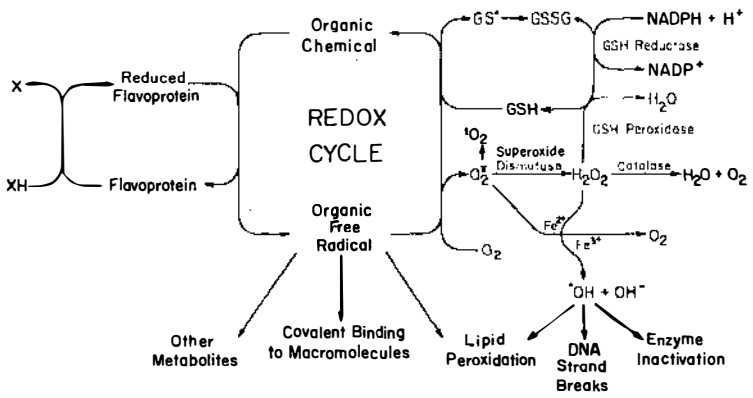

Figure 4 Generation of reactive intermediates via redox cycling. From reference #41, with permission.

(48–54) have published a series of articles in which indirect evidence for a causative role of oxidative thalidomide metabolism was presented. Initial investigations (48, 49, 51) demonstrated that a large number of agents known to have teratogenic potential would also inhibit the attachment of ascitic mouse ovarian tumor cells to concanavalin A-coated polyethylene surfaces at noncytotoxic concentrations. A good correlation between inhibition and teratogenicity was reported, but thalidomide yielded negative results in the attachment assay. However, they reported that treatment of thalidomide with liver microsomes yielded a metabolite(s) that inhibited attachment. In the presence of the appropriate cofactors for P450-dependent monooxygenation, hepatic microsomes from uninduced mice, rats, or dogs effected the bioactivation reaction, but microsomes from rats induced with polychlorinated biphenyls, 3-methylcholanthrene, or phenobarbital, or from mice pretreated with thalidomide were less effective than microsomes from untreated controls (52–54). Nevertheless, the bioconversion was inhibitable by classical inhibitors of P450-catalyzed reactions such as carbon monoxide, SKF525-A, metyrapone, and N-octylamine. In addition, thalidomide elicited a type I binding spectrum in canine hepatic microsomes and rates of conversion were dependent upon oxygen tension. The results were consistent with the concept that thalidomide bioactivation in the cell attachment system is dependent upon an as yet uncharacterized, minor form of a constitutive P450. Interestingly, attachment-inhibiting material (presumably thalidomide metabolites) was extractable from incubation vessels with successive hexane and chloroform extractions; this suggests that the reactive metabolite(s) was lipid-soluble and relatively stable. Of great interest would be to determine whether this putative metabolite(s) would exhibit characteristic thalidomide teratogenicity. In view of the reports of other investigators (59), it is of importance that bioactivation in the cell-attachment system was unaffected by either epoxide hydrolase or 1,2-epoxy-3,3,3-trichloropropane, a classical epoxide hydrolase inhibitor. (A thalidomide epoxide also would probably be quite unstable.) It is also of note that several thalidomide analogs, including EM8, EM12, EM16, EM87, EM136, EM255, E350, phthalimide, phthalimido-phthalimide, indan, 1-indanone, and 1,3-indandione, were all converted to inhibitors of cell attachment in the presence of an hepatic bioactivating system. Glutarimide, glutamic acid, phthalic acid, a number of spontaneous decay products of thalidomide, EM12, or EM87 (54) were not bioactivated by liver microsomes in the cell attachment system.

Other investigators have also reported results of studies suggesting that the biotransformation of thalidomide to active metabolites is important for its teratogenicity. Klein et al (55) noted aberrant development of whole rat embryos when cultured in serum from monkeys treated with thalidomide, whereas direct introduction of the drug into the culture medium produced no

detectable effect. Newall & Tesh (56) reported that an S9 bioactivating system was required for dysmorphogenesis in cultured whole rat embryos. Studies with cultured limb buds (57, 58) suggested that thalidomide metabolites were responsible for observed maldevelopment. Gordon et al (59) reported that the liver microsomes of pregnant rabbits, as well as the fetal hepatic microsomes of rabbits, monkeys, and humans (but not rats) each were effective in catalyzing the conversion of thalidomide to metabolites toxic (assessed by trypan blue dye exclusion) to isolated human lymphocytes and that the toxicity could be enhanced by addition of epoxide hydrolase inhibitors. The toxicity was also abolished by addition of purified epoxide hydrolase. They interpreted these results to indicate that microsomes of sensitive (but not insensitive) species would catalyze the bioconversion of thalidomide to teratogenic arene oxide intermediates. In addition, two teratogenic analogs of thalidomide, phthalimidophthalimide and phthalimidoglutarimide, were converted to toxic metabolites in the lymphocyte dye exclusion system, whereas two nonteratogenic metabolites, phthalimide and hexahydrothalidomide, were nontoxic, even in the presence of epoxide hydrolase inhibitors. It seems unfortunate that no follow-up on this research has been reported. Of particular interest are the apparent inconsistencies in the results of Braun's research group vs. those of Gordon et al (59). Most recently, Hales & Jain (60) reported that thalidomide had no direct effect on cultured embryos, but when an hepatic S9 bioactivating system from uninduced pregnant mice, pregnant rats, or adult male monkeys (but not pregnant rabbits or hamsters) was added, teratogenicity was markedly increased. Clearly, much remains to be learned about the oxidative conversion of thalidomide to embryotoxic metabolites.

In addition to the oxidized metabolites discussed above, other metabolites of thalidomide have been implicated as active teratogens. Possibly the most commonly implicated are hydrolytic products, of which there are a relatively large number. Unfortunately, many of such products were tested in mice with Tween 20 as vehicle—this detergent is now known to exert toxic effects on pregnant as well as non-pregnant mice. At least one hydrolytic metabolite, N-phthaloyl-L-glutamic acid, does appear to exhibit teratogenicity independent from that of the injected vehicle (61, 62), although much further work will be required to establish the precise roles of hydrolytic reactions and hydrolases in thalidomide teratogenicity. Some recent, definitive, studies of the teratogenicity of thalidomide and various derivatives in marmosets (63, 64) provide hope for the eventual resolution of these kinds of questions.

Miscellaneous proteratogens

Investigations indicating that several other chemicals undergo bioconversion to more proximate dysmorphogenic intermediates have been reported in the

literature. Most of these agents have been discussed in relatively recent reviews (14, 15, 65), and thus are not discussed further here; the reader is referred to those reviews for further details and references. Included are phenytoin, procarbazine, ethanol, rifampicin, diethylstilbestrol, certain benzhydrylpiperazine antihistamines, adriamycin, testosterone (via reduction to dihydrotestosterone), benzo(a)pyrene, methoxyethanol, caffeine, aflatoxin (66), and paraquat. The evidence for and mechanisms of dysmorphogenic bioactivation of these agents are generally less extensive and/or convincing than for the four chemicals discussed in previous sections. Of more recent interest is the demonstration that an *endogenous* chemical, estradiol-17β, will undergo P450-dependent bioactivation to a dysmorphogenic intermediate in vitro (67, 68). The implications of this finding also remain to be investigated.

It should be emphasized that it is very difficult to demonstrate definitively that a specific metabolite of any chemical is responsible for in vivo teratogenicity of the parent compound. A number of criteria may, however, be regarded as evidence for the involvement of such a metabolic intermediate:

1. The parent chemical should be convertible to the postulated intermediate within the test system used.

2. The intermediate should be generated within, or be accessible to, the target tissues/cells at the appropriate time.

3. Concentrations of the metabolite within target tissues/cells (at the appropriate time) should correlate with the magnitude of the toxic effect.

4. Inhibition of conversion to the intermediate should attenuate the toxic response to the parent chemical (unless the inhibitor elicits counteracting exacerbating effects).

5. Acceleration of conversion to the intermediate (e.g. via induction of the pertinent bioactivating enzyme) should exacerbate the toxic response to the parent chemical (unless the agent responsible for acceleration elicits counteracting attenuating effects).

6. Modulation of conversion via inhibition or acceleration should affect the incidence and/or severity but normally not the qualitative nature of the toxic effect.

7. Compromise of a biochemical defense system that would ordinarily protect embryos from the toxic effects of reactive intermediates (discussed below) would normally be expected to increase the incidence and/or severity of the observed embryotoxicity.

EMBRYONIC BIOACTIVATION

In pregnant animals, the major site of bioactivation of drugs and chemicals is generally presumed to be the maternal liver. However, many if not most reactive intermediates generated at this site would not be expected to reach

embryonic targets because of their high reactivity and short half lives. Export from the hepatocytes, transport in the maternal blood stream, passage across chorioallantoic and/or yolk sac membranes, and transport in the circulation of the conceptus all are required prior to contact with embryonic cells. Thus, only the most stable and least reactive/toxic metabolites would be expected to traverse this route completely. Consequently, interest in the capacity of tissues of the conceptus per se to catalyze bioactivating reactions has increased in recent years. Even though most textbook references still suggest that tissues of the early conceptus have minimally few or no enzymes capable of catalyzing the bioactivation of foreign organic chemicals, recent publications indicate that bioactivation in the conceptus (during organogenesis) can be surprisingly consequential. The subject has been discussed in a number of recent reviews (69–72).

P450-Dependent Bioactivation

A number of studies have shown that P450-dependent oxidation of benzo(a)pyrene (73–75) or diethylstilbestrol (76) will occur in the embryos of experimental animals at extremely early stages of development, i.e. during the preimplantation period. Studies by Nebert and coworkers (77, 78) and by Manson and coworkers (79, 80) suggested that genetic variability in the capacity of embryos to respond to inducers of P450IA1, a monooxygenase for which benzo(a)pyrene is a good substrate, was a determinant of the susceptibility of the embryos to benzo(a)pyrene-elicited embryotoxicity. These investigations pointed to the possibility that P450-dependent embryonic bioactivation could play a significant role in chemical teratogenesis. A series of more recent investigations with 2-acetylaminofluorene and cultured whole embryos has greatly reinforced this concept (30, 81, 82). It was discovered that day 10 rat embryos contained enzymes that would catalyze the N- and ring-hydroxylation of 2-acetylaminofluorene, a compound previously demonstrated to act as a proembryotoxin in culture in the presence of an exogenous, P450-dependent bioactivating system (discussed above). These later investigations showed definitively that embryos preinduced in utero and subsequently explanted in the culture system contained more than adequate cytochrome P450 to catalyze the bioconversion of 2-acetylaminofluorene to reactive intermediates in quantities amply sufficient to produce grossly observable malformations in the selfsame embryos. The implications of these results are enormous because they demonstrate that only extremely low levels of observed monooxygenase activity in the target cells of the conceptus are sufficient to elicit major malformations and that the *ratio* of P450-dependent bioactivation to inactivation and/or repair within embryonic cells may be a crucial determinant for many chemical teratogens.

In more recent investigations with rat embryos, fairly conclusive evidence

has been obtained (83) for the presence and functionality of P450IA1, or a very closely related isozyme(s), during an early stage of organogenesis. (The recently standardized P450 nomenclature recommended by Nebert et al (84) is used in this review). Using phenoxazone ethers as probe substrates, inhibitory anti-P450IA1 (also commonly referred to as P450c) antibodies and 7,8-benzoflavone as specific inhibitors, and 3-methylcholanthrene as an inducing agent, it could be shown that the yolk sac as well as the embryo per se appeared to contain functional P450IA1, or a very closely related isozyme(s) following induction. Interestingly, higher P450 levels were observed in the yolk sac than in the embryos per se. It was considered unlikely that the induced isozyme was P450IA2 because the latter isozyme exhibits specificity for catalysis of the O-demethylation of methoxyphenoxazone, and embryonic demethylation was undetectable, even after induction. This is of further interest because recent attempts to detect messenger RNA for either P450IA1 or P450IA2 during early development in rats were unsuccessful (85). Neither message could be detected until six to eight days subsequent to parturition. In view of the results reported by Nebert et al for mouse embryos (86), these results were somewhat surprising, but may indicate that, in spite of close resemblances in catalytic activity and immunologic characteristics, the message for the embryonic, inducible isozyme in rats does not cross-hybridize extensively with the message probe for the adult, inducible P450IA1 and that the observed embryonic P450 in rats may be a developmentally specific isozyme. Alternatively, extremely low levels of the transcript (undetectable in routine assays) may be capable of highly efficient, rapid rates of mRNA translation. Further studies are indicated and should be highly interesting.

In addition to the inducible isoform(s), the studies with a series of phenoxazone ethers (83) also provided evidence for the presence of at least three functional, *constitutive,* xenobiotic biotransforming P450 isozymes in tissues of the rat conceptus at day 11 of gestation. One or more of these isozymes catalyzed the carbon monoxide-inhibited depentylation of pentoxyphenoxazone, a reaction frequently regarded as specific for phenobarbital-inducible P450s such as P450IIB1 or P450IIB2. Nevertheless, the embryonic depentylase did not appear to be induced by phenobarbital and its enzymatic activity could not be inhibited with metyrapone or anti-P450IIB1/2 antibody. Thus, the identity of this embryonic P450(s) remains an enigma. Preliminary data reported by Flint & Brown (87) have provided additional evidence for the presence of P450 isozymes in rat embryos during organogenesis. Further studies of embryonic P450 isozymes will be both interesting and challenging.

P450-Independent Bioactivation

It is now recognized that virtually *all* enzymes involved in xenobiotic biotransformation, including phase II conjugations, will catalyze bioactiva-

tion reactions (88, 89). Cytochrome P450-dependent oxidations have been accorded the greatest attention in the past, but much greater interest is now focusing on other reaction types. In terms of the role of P450-independent bioactivation in embryotoxicity and teratogenesis, reduction reactions appear to have attracted the greatest attention to date (41). However, the only well-documented example of reductive embryonic bioactivation leading to embryotoxicity of which I am aware is the reduction of niridazole by cultured rat embryos (40). A good correlation between rates of reduction by intact embryos and incidence of a unique, asymmetric defect was observed. In contrast to embryo and yolk sac homogenates, intact embryos were able to generate reduced metabolites under conditions of relatively high oxygen tension. Embryonic reduction of the nitro group of this nitrothiozole appeared to cause the asymmetric malformation, but participatory enzymic or other catalytic components within the tissues of the conceptus have not yet been identified. The exact mechanism by which niridazole reduction effects axial asymmetry also remains unclear, although, as discussed in a previous section, the available data suggest that localized hypoxia resulting from depletion of molecular oxygen via redox cycling (see Figure 4) of the nitro anion radical may be the most important factor. If so, asymmetrically distributed regions with inherently low tissue oxygen or with lower capacity to replete diminished supplies of oxygen might be envisioned. At present, no concrete evidence exists to support the idea that reductive embryonic bioactivation represents an important mechanism for teratogenicity in vito. It is to be anticipated, however, that future research will document its importance. It is of pertinent interest that earlier studies have provided evidence for the capacity of human embryonic tissues to effect aromatic nitro group reduction (90, 91). In view of these considerations and of the widespread exposure of human populations to chemicals bearing nitro groups, increasing interest in the developmental toxicology of such chemicals is to be expected.

EMBRYONIC DEFENSES AGAINST REACTIVE INTERMEDIATES

The consequences of reactive metabolite generation include the covalent binding of electrophilic intermediates to critical nucleophilic sites on functional macromolecules, free radical-initiated damage such as lipid peroxidation, DNA strand scission, etc., and redox cycling-effected loss of reducing equivalents and depletion of molecular oxygen. The qualitative and quantitative embryotoxicity caused by such reactive intermediates is dependent not only upon rates and quantities of their generation, but also upon the capacity of the organism to remove or inactivate them prior to initiation of damage and/or to repair or circumvent the effected damage (92). It seems possible that

either genetically acquired or chemically elicited deficiencies in these defense capacities could represent a major factor in causation of terata by chemicals. Unquestionably, the maternal organism possesses a number of systems important to the defense of the developing conceptus against reactive metabolites. Metabolic detoxication in maternal hepatic cells, elimination via maternal renal and biliary systems, and preferential distribution to maternal compartments undoubtedly play extremely important roles. Nevertheless, it is clear that maternally administered chemicals can escape these defenses and access tissues of the conceptus. As discussed above, it is also clear that reactive intermediates can be generated within embryonic target sites. Thus, the role of *embryonic* defenses assumes considerable importance. What embryonic defenses are available? What is their efficiency? How easily are they compromised? These are among the questions for which answers are only just now beginning to emerge.

Thiol-Dependent Defenses

Possibly the most important endogenous defenses against the toxic effects of reactive intermediates are the nonprotein thiols (92). Reduced glutathione (GSH) accounts for approximately 90% of the intracellular nonprotein thiol content, with intracellular concentrations ranging from 0.1 to 10 m*M* (93), and, aside from its very high tissue levels, is regarded as an ideal constituent for cellular protection systems for several additional reasons. Among these are: (*a*) it will act as a reducing agent for hydroperoxides and free radicals, (*b*) it is a soft nucleophile and will nonenzymatically react with and inactivate potentially harmful soft electrophiles, (*c*) it is the primary substrate for the highly important GSH-S-transferases—neither cysteine, N-acetylcysteine, pantotheine nor a range of simple exogenous thiols will substitute for it (89)—the transferases enable more efficient inactivation of soft electrophiles and can also catalyze the inactivation of harder electrophiles, (*d*) it is resistant to typical proteases because of its gamma-glutamyl linkage and its degradation is mainly an extracellular process catalyzed by gamma-glutamyltranspeptidase, (*e*) it serves as a source for inorganic sulfate, which is normally rate-limiting for detoxication via sulfation. Surprisingly, very little is known about GSH concentrations in specific tissues of the conceptus during organogenesis and even less about the regulation of tissue concentrations at this stage of gestation or of the role of GSH as a modulator of the embryotoxic effects of chemicals. Only in the last few years have publications appeared addressing these issues.

Possibly the first indication that thiols could modulate the teratogenic effects of bioactivatable chemicals was the report of Ashby et al (94) who reported that pretreatment of pregnant rats with either glutathione or cysteine would reduce the teratogenicity of cyclophosphamide. Later, Hales (95)

further examined the effects of modulating thiol levels on cyclophosphamide teratogenicity by pretreating pregnant rats with various thiols (glutathione or cysteine) or thiol-depleting agents (diethyl maleate) and concluded that thiols exerted a marked protective effect. Wells (96) also reported a protective effect of thiols on phenytoin-induced cleft palate in mice. At this point, however, there was no direct evidence to indicate that modulation of GSH levels in the conceptuses *per se* would affect the capacity of the chemical to elicit deleterious effects. The data did provide further evidence for the participation of a reactive intermediate(s) in the teratogenic response and also pointed to a metabolite(s) capable of reacting with thiol compounds as the responsible species. Of pertinent interest in this regard is the recent report by Robson et al (97) that resistance of cultured cells to the effects of nitrogen mustards appeared to be a consequence of an elevation in GSH-S-transferase activity and cellular GSH content.

A number of studies in vitro have indicated that addition of exogenous thiols to the culture medium can protect the conceptus against the embryotoxic effects of a number of chemicals, including mercuric chloride (98), acrolein (99), and certain reactive metabolites of 2-acetylaminofluorene (100). However, the demonstration by Harris et al (101) that levels of GSH in tissues of the cultured conceptus could be effectively and profoundly modulated without producing detectable embryotoxicity, made possible investigations on the importance of varying levels of GSH within *embryonic tissues* on chemical dysmorphogenesis. L-Buthionine-S,R-sulfoximine (L-BSO), a selective inhibitor of GSH synthesis, exhibited the capacity to deplete GSH in both the embryo proper and its associated visceral yolk sac after treatment of pregnant rats in vivo, as well as after direct addition of L-BSO (at day 10) to the culture medium. Following a 24-hr culture period, no embryotoxic effects of L-BSO could be detected, although GSH depletion (at day 10.5) followed by a 45 hr culture period reportedly resulted in measurable embryotoxicity (102).

In addition, inhibition in cultured embryos of the activity of gamma-glutamyltransferase (an important regulator of GSH turnover) with acivicin or antibodies raised against the enzyme, also depleted GSH levels and produced significant embryotoxicity (103). Very recent studies have shown that modulation of the glutathione status of the conceptus can also affect the capacity of chemicals to elicit open neural tube defects via mechanisms independent of interactions with reactive intermediates (104). Thus, experiments involving modulation of chemical dysmorphogenesis via alteration of embryonic GSH levels should be interpreted in light of these considerations and cannot be regarded as definitive evidence for or against the participation of reactive intermediates in the absence of other supporting data.

An additional useful tool for investigations of these phenomena is 2-oxothiazolidine-4-carboxylate (OTC), a compound that is enzymatically con-

verted to provide an additional precursor source of intracellular cysteine and increase synthesis of GSH when tissue levels are decreased (105). Under the appropriate conditions, this chemical also lacks embryotoxicity in vitro and has been used successfully to study the role of modulation of GSH levels on 2-nitrosofluorene-induced malformations in the whole embryo culture system (106). Addition of OTC (5mM) to the culture medium effectively eliminated both malformations and loss of viability caused by this reactive metabolite of 2-acetylaminofluorene. Use of L-BSO and OTC in combination thus appears to be a very useful approach in determining the role of intrinsic embryonic GSH as a determinant of embryotoxicity. At this juncture, it will be of further interest to investigate the complement of GSH-S-transferase isozymes present in embryos during the sensitive stages of organogenesis. Preliminary data (107) indicate that various of these transferases are present and functional in day 10 rat embryos. Embryonic transferase activities toward 1-chloro-2,4-dinitrobenzene, benzo(a)pyrene-4,5-oxide, p-nitrostyrene-7,8-oxide, and aflatoxin-8,9-oxide were reported as 3920 ± 618, 50 ± 5, 157 ± 9, and 15 ± 4 (mean $\pm$ SE) pmol/mg protein/min, respectively. Continued study of the embryonic GSH-S-transferase enzymes will be of considerable interest and importance.

Thiol-Independent Defenses

With respect to the capacity of the conceptus to defend itself against the toxic effects of reactive metabolites of drugs and other chemicals, virtually all of the attention thus far has focused on the role of thiols, with specific emphasis on GSH. However, from the voluminous number of published studies on the role of thiol-*independent* mechanisms in the protection of organisms against the carcinogenic and cytotoxic effects of chemicals (e.g. see reference 108), it should be obvious that such mechanisms could very likely play an important role as determinants of chemical teratogenesis. Probable nonthiol-dependent components in an embryonic defense arsenal would include naturally occurring antioxidants and radical scavenging substances such as the tocopherols, ascorbate, β-carotene and uric acid (92). β-Carotene is particularly effective against singlet oxygen and the tocopherols are probably the most important intracellular, endogenous radical scavengers. The tocopherols are especially effective in terminating lipid peroxidation reactions. Ascorbate readily scavenges superoxide anion radicals. Selenium is also regarded as important in cellular defenses, but primarily via its participation in selenium-dependent glutathione peroxidases. Also of probable importance in the conceptus are a number of enzymes known to constitute important cellular defenses in more mature organisms. These include superoxide dismutase, catalase, peroxidases, quinone oxidoreductases, epoxide hydrolases, and perhaps others. Some chemicals may be bioinactivated by xenobiotic biotransforming en-

zymes present in tissues of the conceptus. Transferrin and ferritin may prevent the generation of free radicals by binding iron. Damage to embryonic nucleic acids may be corrected by the appropriate repair systems (109). Although each of these defenses probably *exists* in the early conceptus, extremely little is known about their status (i.e. levels and/or activity at which they are present), the gestational-age dependency of their relative efficacy, or their biological compartmentation within the conceptus—factors regarded as extremely critical determinants of defense efficiency (92). Clearly, much remains to be learned about embryonic defenses against the toxic effects of reactive intermediates.

SUMMARY AND CONCLUSIONS

Within the past decade, interest has increased markedly in the elucidation of mechanisms whereby drugs and other chemicals can alter the normal developmental pattern of the developing conceptus. This has, in large measure, been attributable to the recent availability of methods for the successful long-term culture of whole embryos (110) as well as various embryonic tissues (e.g. limb buds). These preparations have enabled a more straightforward investigation of the direct effects of chemicals on the conceptus per se, without the complicating and frequently confounding participation of maternal factors. The demonstration that exogenous metabolic preparations could be incorporated into such culture systems (111, 112) has enabled investigators to pursue questions about the nature of proximate and ultimate chemical species responsible for producing abnormal morphogenesis. Demonstrations of the capacity of the early conceptus to effect profound dysmorphogenic bioactivation (81–83) provide additional relevance to such questions. Elucidation of the identity of the chemical species represents a first and necessary step in unraveling the pathogenic mechanism. Control of their rates of generation and inactivation or elimination are probable major determinants of incidence/severity of chemically induced embryotoxicity. Future investigations of these phenomena promise to yield key contributions to the discovery of mechanisms in chemical teratogenesis.

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