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Chemical-Biological Reactions Common to Teratogenesis and Mutagenesis

by Raymond D. Harbison*

Cytotoxic chemicals have in common the ability to act specifically on cells in cycle. Bacteria are more sensitive in the exponential growth phase than when growing slowly in media. Similar observations have been made on a variety of systems ranging from bacteria, yeast, higher plants and invertebrates to vertebrates including primates. The embryo and fetus are highly susceptible to cytotoxic agents because they have continuous groups of cells in the growth phase. Acutely toxic doses may cause cellular death and result in developmental defects or fetal death. Cytotoxic agents can be grouped as alkylating agents, electrophilic reactants, antimetabolites, intercalating agents, amino acid antagonists, spindle poisons, and an additional group of chemicals which covalently bind to DNA. These cytotoxic groups of chemicals may also be mutagenic by interacting with DNA to produce changes in sequences of nucleotides resulting in heritable defects either in a somatic cell line or in a germinal cell line. The mechanisms of chemical-induced teratogenicity and mutagenicity are similar. This commonality is further discussed in the text.

The biological functions that make up the processes of reproduction and organogenesis are varied in nature and in their susceptibility to adverse influences in the environment. A graphic display of the developmental sequences of embryonic and fetal growth is shown in Figure 1. The critical periods during human development can be divided into an embryonic period and a fetal development period, with most organogenesis complete during the first trimester. A relative comparison development chart is shown in Table 1. This table sequences several developmental stages of man, mouse, rat, hamster, and rabbit.

The most prominent example of specific chemical-induced alteration during a specific time of gestational development is thalidomide. Thalidomide was teratogenic when taken during gestational days 34 through 50 (*2*). Generally, ear anomalies were associated with intake between days 34 and 38, arm anomalies were seen between days 40 and 44, and aplasia of the femur or tibia between days 44 and 48. A variety of malformations of internal organs, such as intestinal atresia, imperforate anus, and aplasia of the gallbladder and appendix also occurred, either associated with skeletal defects or occasionally alone. A teratogenic response is determined by both the time of exposure as well as the chemical reactivity of the material.

Although it is widely known that under certain conditions some aspects of reproduction are vulnerable to physical and chemical factors in the environment, for example, both the germ cells and the early embryo may be damaged by relatively small doses of ionizing radiation or reactive chemicals, it is generally assumed that a placental barrier protects the embryo and or fetus against most levels of chemical exposure. The placenta which performs admirably in maintaining the growing embryo, does not selectively protect the intrauterine organism from harmful agents administered during pregnancy. The placental barrier has turned out to be a sieve. Except for compounds of large molecular weight and those with strong electronegative or electropositive charges (heparin and most neuromuscular blocking agents), almost all pharmacologic substances can and do pass from the maternal to fetal bloodstream. Generally, substances with a molecular weight of less than 600 pass the placental barrier.

Variances in placental transport of drugs and chemicals can be demonstrated between species and during gestation. A difference in placental transfer of diphenylhydantoin (DPH) occurred at different developmental periods (see Table 1). A significantly greater DPH concentration was found

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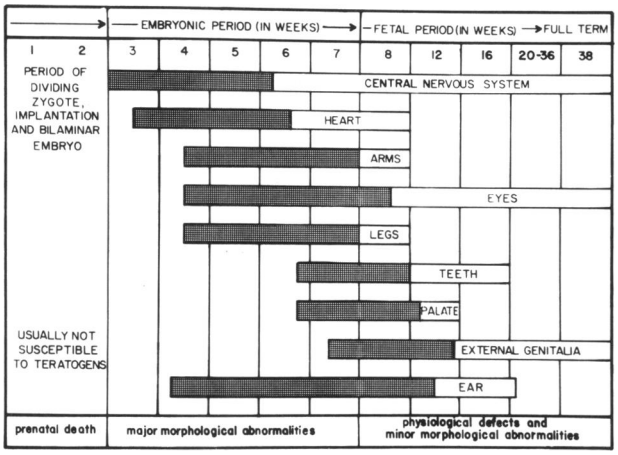


FIGURE 1. Schematic representation of human development and sensitive periods for production of maldevelopment. Shaded areas represent highly sensitive periods; clear areas represent stages that are less sensitive to teratogens.

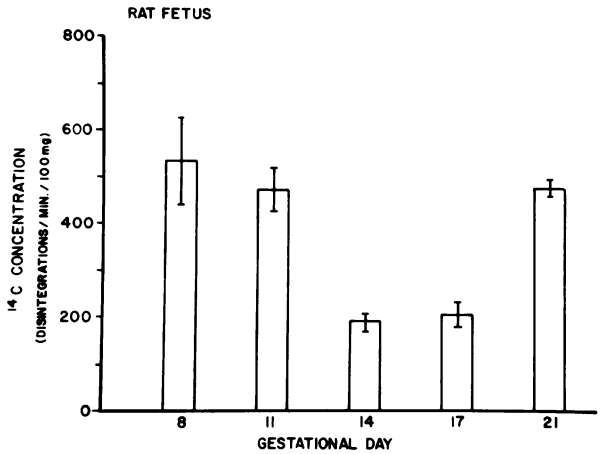


FIGURE 2. Concentration of DPH plus metabolites in rat fetal tissue. Each bar represents the mean ± SE of samples from 10-13 animals killed 4 hr after IV administration of 50 mg/kg [^{14}C] DPH (2.5 μCi/kg).

Table 1. Comparative gestational development.[a]

	Man	Rat	Mouse	Rabbit	Hamster
Implantation period	6½ days	8 days	5 days	9 days	7 days
13 to 20 somites	27 days	11 days	9 days	10 days	9 days
End of embryonic period	12-14 wk.	14 days	13 days	11 days	10 days
End of metamorphosis	20 wk.	17 days	17 days	15 days	14 days
Fetal development	20-34 wk.	18-22 days	18-20 days	16-32 days	15-16 days
Parturition	36-40 wk.	21 days	19 days	32 days	15 days

[a] Developmental data derived from Witshi (*1*) and Rúgh (*2*).

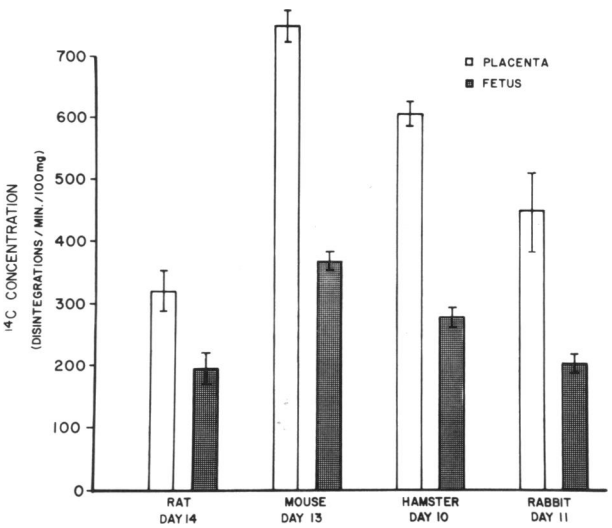


FIGURE 3. Concentration of DPH plus metabolites in placenta and fetal tissue of rat, mouse, hamster, and rabbit compared at the end of the embryonic period (see Table 1). Each bar represents the mean ± SE of samples from 5-10 animals killed 4 hr after IV administration of 50 mg/kg [^{14}C] DPH (2.5 μCi/kg).

in rat fetal tissue on gestation day 8, the period of implantation, and day 11, the 13-20 somite stage, than on days 14 and 17, the period of fetal maturation (Fig. 2). Finally, on day 21 of gestation, the fetal DPH concentration was elevated 2.5-fold, being at the level seen in early pregnancy. Similar gestation dependent transport was observed in mouse, rabbit and hamster for diphenylhydantoin. There were no significant maternal plasma drug level changes that would produce the observed fetal drug level differences.

Differences of placental transfer were observed between species (Fig. 3). The maternal rat, mouse, hamster and rabbit were treated on gestation days corresponding to the end of the embryonic period in each species, as previously described. Mouse fetal and placental tissues contained significantly higher concentrations of DPH than those of any of the other species, whereas hamster fetal and placental levels were higher than in the rabbits and rats. Although the concentration in the rabbit tissues was higher than the rat, the difference is not significant. These differences become even more important when compared with the maternal plasma levels. Mice which had the highest fetal level, and rats which had the lowest, had essentially identical plasma levels of DPH. Rabbits and hamsters had plasma levels twice that of the mice, yet both had fetal levels significantly less than that of the mice.

Similar gestational-dependent transfer was observed in human tissue (Fig. 4). Intracellular uptake for this substrate varied with gestation. Thus placental transfer can be expected to vary during gestation and between species making generalizations and anthropomorphizing animal data difficult.

Extraplacental transfer of drugs must also be a consideration when drugs are administered intraperitoneally in an experimental situation. The contribution of extraplacental transfer is shown in Figure 5. Uterine arteries were ligated, and no drug was detected in placental or fetal tissue or amniotic fluid 30 min following IV injection, indicating complete isolation of the uterus from the maternal blood supply. Increasing amounts of drug were measured in fetal and placental tissues and amniotic fluid following IP administration of drug at both 30 min and 4 hr. Transfer of significant amounts of drug directly to the fetoplacental unit can occur extraplacentally following intraperitoneal drug treatment.

Toxic chemicals that cross the placenta may produce morbidity if administered after the stage of organogenesis (second and third trimesters) or malformations if administered during the first trimester.

Mutagenic changes are distinguished from teratogenic responses, in that the former is transmissable to future generations, whereas the later is confined to a single generation. However certain chemical-biological interactions may be common to both biological alterations, and it is on this chemical-biological interaction, that I would like to focus.

The term, "cytotoxic chemical," was initially used to describe a number of chemically unrelated agents which had in common the ability to inhibit the growth of tumors. They act specifically on cells in cycle. The most sensitive vegetable cells were growing root tips and shoots of plants and germinating seedlings. Bacteria are more sensitive in the exponential growth phase than when growing slowly in minimal media. Similar observations have been made on a variety of systems ranging from bacteria, yeast, higher plants and invertebrates to vertebrates including primates. Acutely toxic doses cause death by damaging proliferating tissues such as bone marrow and intestinal mucosa. Survivors may show symptoms of damage to epithelial surfaces to ovarian and testicular tissues and to any system which contains cells in cycle and with a short cycle time. Chronic administration of low doses may produce cellular abnormalities and nuclear gigantism. Cytotoxic agents, because of their broad spectrum of action, have been used not only in treatment of cancer as immunosuppressants and as insect chemosterilants, but also for studying mechanisms of carcinogenesis, mutagenesis and teratogenesis.

The mechanism by which chemicals cause cell death is not clear, but effects have been ascribed to some form of unbalanced growth, RNA and protein synthesis being out of phase with DNA synthesis.

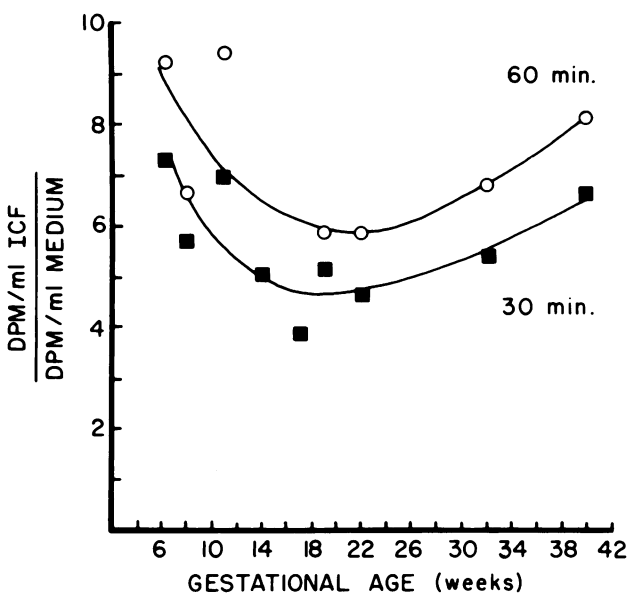


FIGURE 4. Effect of gestational age on diphenylhydantoin (DPH) uptake in the human placenta. Each point represents the mean of two to nine determinations of uptake of ^{14}C-DPH, 5 × $10^{-7}M$, measured after 30 or 60 min of incubation. ICF denotes intracellular fluid.

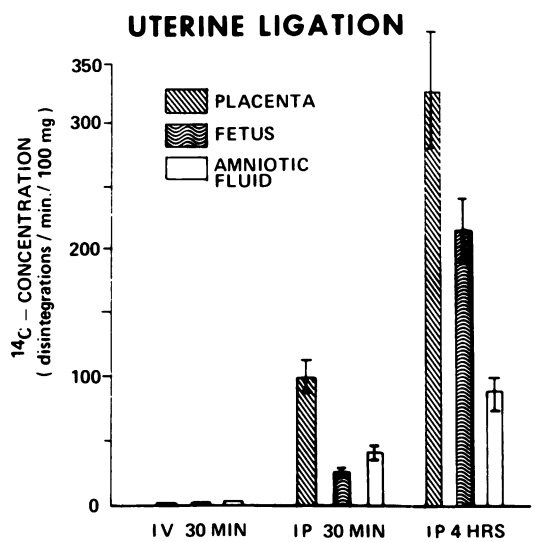
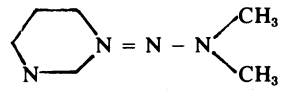
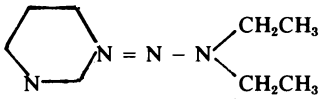


FIGURE 5. Concentrations of DPH plus metabolites in rat placenta, fetus, and amnionic fluid after uterine ligation. Each bar represents the mean ± SE of samples from 5-8 animals treated IV with 50 mg/kg [^{14}C] DPH (2.5 μCi/kg) after ligation of the uterus.

Table 2. Comparative cytotoxicity of two related dialkyltriazenes.

	Dosage producing 50% fetal mortality, mg/kg		Teratogenicity		Carcinogenicity	
	Day 10	Day 14	Day 10	Day 14	Day 10	Day 14
 1-(3-pyridyl)3,3-dimethyltriazene	10	100	0	+++	0	+
 1-(3-pyridyl)3,3-diethyltriazene	60	170	+++	0	++	++

The phenomenon of unbalanced growth has been described in detail for a number of cytotoxic agents.

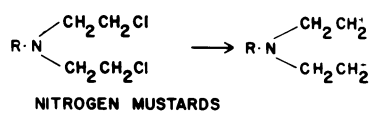
The biological reactivity of cytotoxic agents is similar but the teratogenic effects produced are dependent on the gestational time of treatment. Dimethyltriazene, for example, when injected into pregnant rats on gestational day 10, has little teratogenic action, but it is extremely effective if administered on day 14 (Table 2). Conversely, a closely related diethyltriazene is teratogenic at day 10 but not at day 14 (Table 2). There is also no correlation between median lethal dosage, teratogenicity and carcinogenicity. Thus, although a compound may be classed as a cytotoxic agent, its biological effects will vary from system to system.

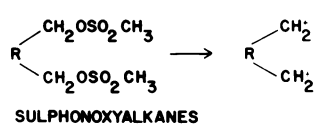
Differences in toxicity or biological properties between cytotoxic agents do not imply any major differences in their mechanisms of action, but reflect differences in their physical-chemical properties. For example, there may be differences in chemical or biological half-life, in tissue distribution, in metabolism and excretion of the chemical and in the ability of target cells to repair damage.

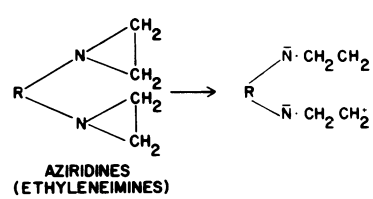
There are several clear mechanisms of teratogenic and mutagenic actions of cytotoxic agents in biological organisms: alkylating agents, electrophilic reactants, antimetabolites, intercalating agents, amino acid antagonists, spindle poisons, and miscellaneous agents.

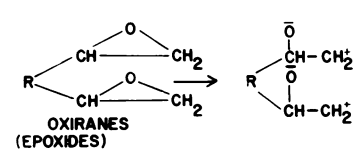
Cytotoxic agents destroy or alter dividing cells and exert their characteristic biological properties by several different mechanisms and vary as to site of action in the cell.

Biological properties of alkylating agents have been known for many years. Alkylating agents in polar solvents are very reactive towards molecules which have negative charges (nucleophiles) such as ionized carboxylic and phosphoric acids and thiols, or which have negative areas due to presence of amine groups. These agents react with many biological constituents including nucleic acids, proteins, nucleotides, and amino acids. Some alkylating agents have cytotoxic properties as parent compounds. These include nitrogen mustards, sulfonoxyalkanes, aziridines (ethylenamines), and oxiranes (epoxides). Their chemical reactivity depends on the positively charged form which reacts with negatively charged nucleophiles. The teratogenicity and mutagenicity of these chemicals depend on their electrophilic or positively charged character. Thus chemical reactivity is dependent on electrophilic character.





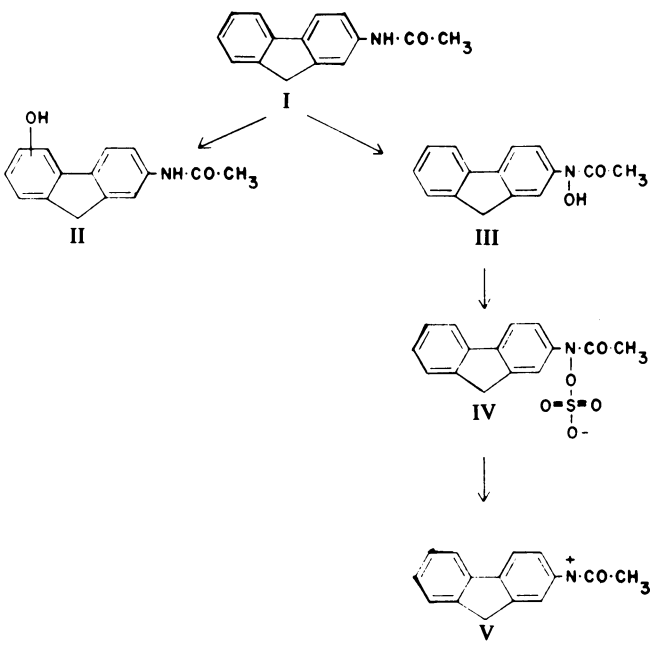




Many chemicals are inherently electrophilic or are converted *in vivo* to electrophilic reactants. Already, a number of organic chemicals have been identified as inherently electrophilic or converted *in vivo* to electrophilic reactants that are carcinogenic. Characteristically, these compounds are acutely toxic if given as a single dose and carcinogenic on chronic administration. For example, ingestion of large amounts of aflatoxin contaminated foodstuffs is known to cause acute hepatotoxicity, while injection of small amounts can cause hepatocellular carcinoma. Any carcinogen which acts by an intermediate electrophilic reactant will have the general properties of cytotoxic agents and under the appropriate conditions will probably be a carcinogen. This same hypothesis can be extended to include electrophilic reactants as mutagens and teratogens.

Many variables are involved in determining whether a chemical can be transformed *in vivo* to an electrophilic reactant, and one of the most comprehensive studies has been carried out on 2-acetylaminofluorene (*4*). This compound is a potent liver carcinogen in a variety of species, although the guinea pig is resistant. There is a correlation between the binding of 2-acetylaminofluorene (AAF) to nucleic acids and proteins and the degree of carcinogenicity. However, since AAF did not bind to these macromolecules *in vitro*, it was clear that some form of metabolic activation was taking place *in vivo*. The scheme for activation of AAF is shown in Eq. (1). AAF is metabolized in the liver to ring-hydroxylated products, but a small amount of *N*-hydroxylation also takes place. The *N*-hydroxylated metabolite is a more potent carcinogen, but the ultimate carcinogen is the sulfate ester of AAF which is rapidly rearranged to an electrophilic reactant which covalently binds to macromolecules. The guinea pig is not efficient in *N*-oxidation nor does it possess significant sulphotransferase activity, thus explaining its resistance to AAF-induced cancer.

The electrophilic AAF reactant attacks any cellular molecule in its vicinity which has a negatively charged region, a nucleophile. If the appropriate level of reaction takes place with the essential macromolecule, then a cellular transformation will occur. The degree and nature of the cellular lesion will depend on a number of variables, the most important of which are: stage of embryo or fetal development when chemical administered; dosage of chemical, route of administration, and exposure schedule; ability of chemical to pass placenta and penetrate embryonic tissues; ability of maternal tissues (especially liver) to detoxify or bioactivate chemical; the biological and chemical half-life of the material or its metabolite; capacity of embryonic



(1)

tissues to detoxify or bioactivate chemical; stage of the cell cycle during the period when the drug is present at a cytotoxic concentration; ability of the damaged cells to repair or recover from the cytotoxic lesion.

These variables explain why an agent may be found to be highly teratogenic or mutagenic under one set of experimental conditions and inactive in another.

AAF is predominantly a liver carcinogen, presumably because the sulfate metabolite is such a reactive chemical that it cannot travel far from its site of formation in the liver. AAF is a teratogen. A generalization can be made that a compound known to be an electrophilic reactant must be considered to be a potential teratogen. An important feature is the chemical half-life which determines whether the electrophilic reactant can travel from the site of bioactivation to sensitive embryonic or fetal tissue. If the intermediate metabolite is very reactive such as the $CCl_3 \cdot$ radical formed from CCl_4 it will be unstable and react at the site of formation. Hence CCl_4 is very toxic to the adult liver and only a weak teratogen, because the reactive chemical intermediate is not transported to embryonic or fetal tissue. Nitrosamines are similarly activated in the liver to highly reactive carbonium ions which act at the site of formation, the liver, and are not transported to embryo or fetus to produce alterations. These compounds are, however, mutagens when tested in

the Ames assay using liver microsomes as an enzyme source to bioactivate these materials to reactive electrophiles.

Glutathione is an abundant and ubiquitous sulfhydryl compound in cells and appears to serve as an electrophilic sink because of its nucleophilic properties. Thus it serves as a protective substance to inhibit binding to essential macromolecules. Alterations in cellular glutathione levels clearly alter the toxicity of reactive electrophiles.

Bifunctional alkylating agents which are teratogenic and mutagenic include busulphan (*5, 6*), chlorambucil (*7–10*), cyclophosphamide (*7, 11, 12*), METEPA (*13*), nitrogen mustard (*5, 7, 14–16*), thio-TEPA (*5, 17, 18*), triethylenemelamine (*7, 14, 19, 20*), and uracil mustard (*7*). The proposed mechanism of teratogenic activity of the bifunctional alkylating agents is inter- or intrastrand crosslinking of DNA as well as alkylation of other essential macromolecules.

Electrophilic reactants which are teratogenic and mutagenic are acetylaminofluorene (*21*), aflatoxin (*22*), alkyltriazenes (*23*), aminoazobenzene (*24*), benzanthracenes (*25*), benzpyrenes (*26*), carbon tetrachloride (*27, 28*), cycasin (*29*), 1,2-diethylhydrazine (*30*), ethionine (*31, 32*), methylcholanthrene (*33, 34*), nitrosourea (*35–37*), pyrollizidine alkaloids (*38*). These cytotoxic agents require metabolism or bioactivation. Liver microsomes most frequently produce a variety of electrophilic reactants which, like the alkylating agents, covalently bind to macromolecules. Metabolism or bioactivation may also be extramicrosomal or by gut bacteria. Some cytotoxic agents may also break down spontaneously to chemically reactive intermediates.

Antimetabolites which are teratogenic and mutagenic include aminopterin (*38–42*), azaguanine (*43*), azathioprine (*44*), azauracils (*7, 28, 45–48*), cytosine arabinoside (*7, 49–51*), diaminopurine (*7, 19*), halogenated pyrimidines (*7, 40, 52, 53*), mercaptopurine (*7, 28, 54, 55*), methotrexate (*29, 56, 57*), primethamine (*58–60*), and thioguanine (*7, 17*). These antimetabolites inhibit pathways of purine or pyrimidine biosynthesis and formation of thymidylic acid. The mechanism for inhibition of nucleic acid replication is by incorporation into nucleic acids as base analogs and subsequent inhibition of replication.

Intercalating agents which are teratogenic and mutagenic include acriflavin (*61*), actinomycin D (*62–66*), chloroquine (*67–69*), daunomycin (*7*), quinacrine (*70*), and mithramycin (*7*). Intercalating agents insert between base pairs of DNA and interfere with transcription and replication.

Amino acid antagonists which are teratogenic and mutagenic include asparaginase (*71*), azaserine (*72–74*), DON (*7, 75*), *p*-fluorophenylalanine (*43*), hadacidin (*75, 77*), and mimosine (*78*). Amino acid antagonists inhibit protein and nucleic acid synthesis by interfering with specific amino acids required for protein or nucleic acid synthesis. Specific interference with asparagine, aspartic acid, glutamine, methionine, phenylalanine, and tyrosine utilization has been reported.

Spindle poisons which are teratogenic and mutagenic include: colchicine (*28*), griseofulvin (*79*), podophyllotoxin (*7, 80, 81*), vinblastine (*31, 82–84*), and vincristine (*31, 82–84*). Spindle poisons condense with microtubular protein and interfere with formation of cellular organelles and the spindle apparatus.

Miscellaneous cytotoxins which are teratogenic and mutagenic include caffeine (*85*), formaldehyde (*86*), hydroxyurea (*10, 87–89*), procarbazine (*7*), and urethane (*90*). These agents covalently bind with DNA and inhibit DNA synthesis and repair.

Alkylating agents are effective for suppressing various types of tumor growth. To be effective anticancer agents they must have an optimal level of chemical reactivity (*91*). Alkylating agents in polar solvents are reactive towards molecules which have negative charges (nucleophiles) such as ionized carboxylic and phosphoric acids and thiols, or which have negative areas due to presence of amine groups. Alkylating agents react with many biological constituents including nucleic acids, proteins, nucleotides and amino acids. Nitrogen mustards, ethyleneimines (aziridines), sulfonoxy-alkanes, and epoxides (oxiranes) are alkylating agents possessing cytotoxic properties. The chemical reactivity of the alkylating agents results from formation of a positively charged species which subsequently reacts with negatively charged molecules. This reaction inactivates essential macromolecules necessary for cellular multiplication, proliferation and growth. This interaction is responsible for anticancer activity. Teratogenicity also depends on the chemical reactivity of the alkylating agent. Alkylating agents can kill dividing cells at any stage of the cell cycle. Defects found and their severity are dependent on the day of gestation on which the compounds are administered (*11*). Some alkylating agents must be biotransformed to chemically reactive species to be teratogenic. Factors which influence metabolism, e.g., stimulation or inhibition alter the teratogenic properties of the compound. Cyclophosphamide, for example, requires metabolic activation (*92*), and any factor which influences this activation will affect the teratogenicity of this compound. Similarly, mitomycin C probably requires activation by initial reduction of its guinone. The ability of embryonic

tissues to reduce mitomycin may be the main factor which determines its teratogenicity, since the active metabolite probably has a very short half-life and would not reach the embryo if it were generated only in maternal liver.

Alkylating agents react nonspecifically with a variety of cellular macromolecules. They all share a common mechanism of cytotoxicity. Their chemical reactivity is a result of inherent electrophilicity acquired by dissolution in polar solvents or metabolic activation. This physical characteristic imposes a chemical reactivity that stimulates an attraction for nucleophiles. A nucleophile is able to share or contribute an electron with subsequent covalent binding of the two reactants. This covalent bonding effectively biologically inactivates target macromolecules. At a molecular level this binding can be to cell membranes, nucleoproteins, glycolytic enzymes, DNA, RNA, cyclic AMP, etc. This nonspecific binding results in impairment of cellular proliferation and growth. During embryonic development impairment of cellular function is especially critical to future development of organ components and systems. Covalent binding of the alkylating agents to essential macromolecules disrupts cellular proliferation and growth and depending on the stage of development disrupts normal development resulting in malformed or abnormal offspring. Alkylating agents may also damage DNA producing functional changes which lead to malignant change or some other form of mutation.

Many synthetic and natural carcinogens are converted *in vivo* to alkylating agents or some analogous form of electrophilic reactant. These compounds are acutely toxic at high doses and carcinogenic on chronic administration. Ingestion of large amounts of aflatoxin contaminated foodstuffs is known to cause acute hepatotoxicity, while injection of small amounts can cause hepatocellular carcinoma in a wide range of animal species. A carcinogen acting by electrophilic intermediates will have the general properties of cytotoxic agents. A number of carcinogenic chemicals have been shown to be potent teratogens.

One of the most studied biotransformations of a chemical to an electrophilic reactant is that of 2-acetylaminofluorene (AAF) (*4*) [Eq. (1)]. There is a correlation between binding of AAF metabolites to nucleic acids and proteins and carcinogenicity. AAF (I) is metabolized in the liver mainly to ring hydroxylated products (II), but a small amount of *N*-hydroxylation (III) also takes place. The *N*-hydroxy intermediate is a more potent carcinogen than AAF. However, neither the AAF or *N*-hydroxy metabolite bind with cellular macromolecules. Sulfation of the *N*-hydroxy metabolite IV produces the proximate carcinogen. The sulfate group forms an excellent leaving group for spontaneous chemical rearrangement to form the reactive electrophilic amidonium ion (V). Synthetic esters of III do covalently bind to cellular macromolecules. The electrophilic reactant formed attacks any cellular molecule in its vicinity which has a negatively charged region (nucleophile). A critical level of electrophilic reactant is necessary for cytotoxicity. The level of reactant formed will depend on the balance between bioactivation and detoxification.

AAF is a liver carcinogen because the active sulfate is such a reactive chemical that it cannot travel far from its site of formation in the liver. AAF also induces tumors in mammary glands, epithelium of small intestine, and in sebaceous ear duct glands, which implies these tissues also activate AAF or that another unidentified metabolite is formed in the liver which is stable enough to diffuse from the liver and reach distant tissues. AAF produces skeletal defects when administered to mice between gestational days 8 and 15 (*21*). Fetal liver does not metabolize AAF since no hepatotoxicity was observed, but some form of cytotoxic agent is being produced by maternal tissues which reach the embryo in active form.

Electrophilic reactants are highly carcinogenic as well as teratogenic. Without exception, it has been found that the active metabolite is an electrophilic reactant (Table 3) (*93*). A compound known to be an electrophilic reactant (for example, an alkylating agent) must be considered a potential teratogen. The chemical half-life of the reactant determines whether it can travel from the site of injection to sensitive embryonic tissues. Compounds with no inherent reactivity to nucleophiles but biotransformed to chemically reactive intermediates must be considered potential teratogens. Very reactive intermediates, such as the $CCl_3 \cdot$ radical, formed from carbon tetrachloride will be unstable and react at the site of formation. Carbon tetrachloride is very toxic to adult liver where it is generated but not very toxic in newborn rats whose metabolic enzymes are not fully developed (*94*). This is in agreement with the weak teratogenic action of carbon tetrachloride which caused no congenital defects in offspring of rats (*27*). Aflatoxins are metabolized, by the liver to an epoxide which has a very short half-life and acts mainly on the liver (*95*). However, despite the short half-life of the metabolite, the aflatoxins are teratogenic (*22*).

Nitrosamines are similarly activated in the liver to reactive electrophilic carbonium ions which are hepatotoxic. Nitrosamines are not powerful inducers of transplacental cancer because the active metabolite is too unstable to reach the fetus. The

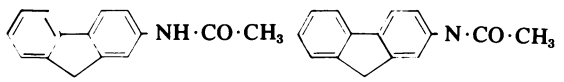
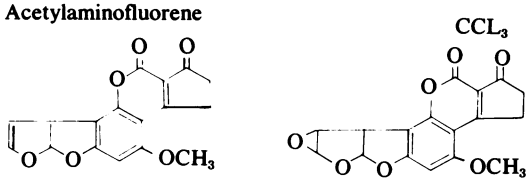
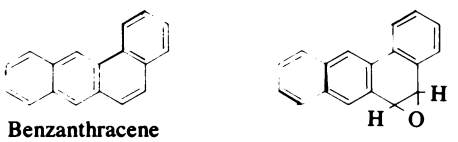
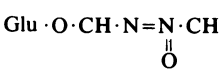
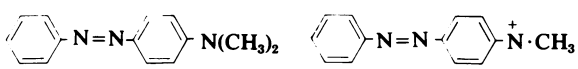
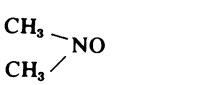
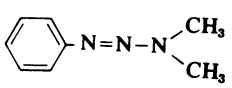
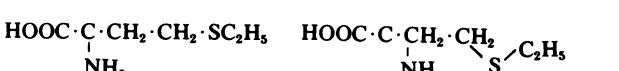
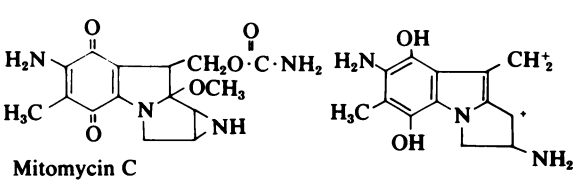
fetus is unable to activate the nitrosamines until shortly before birth (*93*). Chemically related nitrosoureas are teratogenic because they are stable enough to cross the placenta and be demonstrable in the embryo (*96*). Monoalkyltriazenes which are formed from dialkyltriazenes by metabolic demethylation are chemically stable having chemical half-lives of the order of ten minutes. The chemical stability of the monoalkyltriazenes allows time for diffusion from maternal liver and penetration of embryonic tissue resulting in teratogenicity (*23*). Removal of the sugar residue from cycasin *in vivo* produces an electrophilic reactant that is stable enough to be carcinogenic and teratogenic. Metabolic activation of the cycasin is dependent on species, age and condition of the test species, thus teratogenicity of the compound is widely variable. Ethionine is bioactivated in the liver to *S*-adenosyl-L-ethionine which can ethylate macromolecules causing cell abnormalities. Adenosylethionine is chemically stable and can penetrate to embryonic tissues and cause teratogenic effects. Aminoazobenzenes are teratogenic (*24*). These chemicals are bioactivated to amidonium ions by *N*-hydroxylation and subsequent esterification in a manner analogous to that previously described for acetylaminofluorene. Polycyclic hydrocarbons are metabolically converted to epoxides which are very reactive electrophiles. This class of chemicals is highly teratogenic and mutagenic. Not all the hydrocarbons are alike in their teratogenic properties. Benzpyrene is not an effective teratogen (*97*). However, 7,12-dimethylbenzanthracene produces a high incidence of defects (*25*). Pyrolizidine alkaloids have a similar structure as mitomycin C and both are probably active only after conversion to a pyrrole analog.

Glutathione is the most abundant and ubiquitous sulfydryl compound in cells and appears to serve as an electrophilic sink by nature of its nucleophilic properties. Thus glutathione serves as an electrophile scavenger to inhibit binding of bioactivated chemicals to essential macromolecules. Alterations in cellular glutathione levels clearly alters the toxicity of reactive electrophiles. Decreasing cellular glutathione levels enhances the toxicity of bioactivated materials.

Diphenylhydantoin (DPH) is bioactivated to a teratogen. DPH binds covalently to both maternal and fetal tissues; 132 pg of DPH bound to maternal liver and 225 pg of DPH bound to fetal tissue 4 hr following a dosage of 50 mg/kg DPH administered IP. Covalent binding was measured as previously described (*98*).

Several metabolites of DPH have been identified *in vivo*. Conclusive evidence for the presence of

Table 3. Chemical structure of teratogen and active metabolite.

Teratogen	Active Metabolite
 Acetylaminofluorene	
 Aflatoxin B_1	
 Benzanthracene	
CCl_4 Carbon Tetrachloride	
 Cycasin	CH_3^+
 Diethylaminoazobenzene	
 Dimethylnitrosamine	CH_3^+
 3,3-Dimethyl-i-phenyltriazene	CH_3^+
 Ethionine	Adenosine
 Mitomycin C	
 Pyrrolizidine Alkaloids	

diphenylhydantoic acid and α-aminodiphenylacetic acid *in vivo* following the administration of DPH, by using melting point, elemental analysis, and chromatography, was provided by Maynert (*99*) in identifying these products in the urine of dogs, rats, and people. In addition to these two degradative products Butler (*100*) demonstrated that one of the phenyl groups in DPH is hydroxylated in the para position. He also found that unchanged DPH is excreted in very small amounts if at all in urine and concluded that DPH is biotransformed by dogs and people to 5-*p*-(hydroxyphenyl)-5-phenylhydantoin (HPPH), conjugated, and excreted. Maynert (*99*) also found the major urinary product to be HPPH. Chang et al. (*101*) identified 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin as a metabolite of DPH in rat and monkey urine. Atkinson et al. (*102*) identified 5-*m*-hydroxyphenyl-5-phenylhydantoin in conjugate form in the urine of dogs and patients treated with DPH. HPPH is probably the major hydroxylated metabolite of DPH. A proposed schematic for the metabolism of DPH is shown in Figure 6. DPH is converted to a dihydrodiol in mouse, rat, dog, and man (*103*). The existence of a dihydrodiol is evidence for an intermediate epoxide. The DPH epoxide may be in equilibrium with the chemically stable oxepin. DPH-dihydrodiol may be further converted to catechols and conjugates.

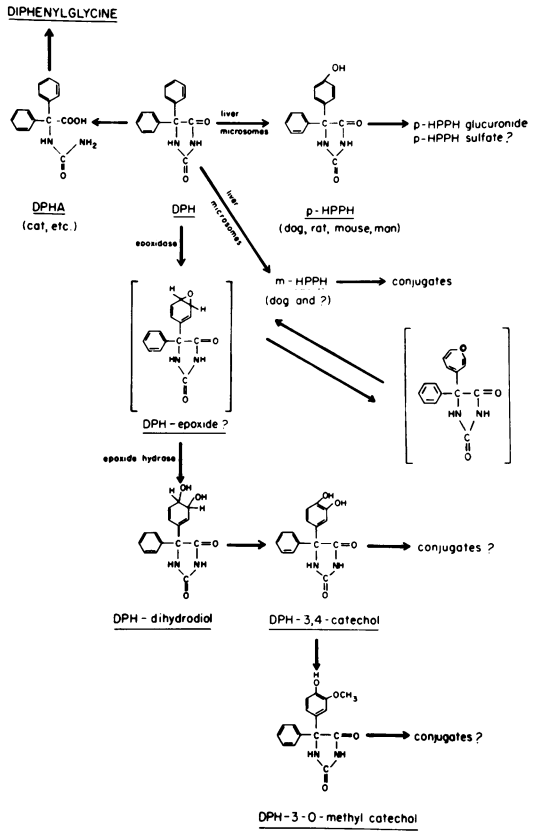


FIGURE 6. Proposed biotransformation of diphenylhydantoin *in vivo* to an electrophilic reactant.

The metabolism of polycyclic aromatic hydrocarbons by the hepatic mixed function oxidase system has been extensively studied. It has been demonstrated that the arene oxide and dihydrodiol derived from benzypyrene are very reactive electrophiles which bind *in vitro* and *in vivo* to biological macromolecules. Covalent binding of these macromolecules is responsible for the tissue damage observed on exposure to these metabolites or the parent hydrocarbon. Comparatively little work has been done to identify the chemical species responsible for tissue damage on treatment with monocyclic aromatic derivatives such as DPH. The dihydrodiol and other hydroxylated metabolites of DPH have been isolated suggesting an intermediate arene oxide.

Pretreatment of pregnant mice with diethyl maleate DEM (a depletor of glutathione) enhanced the teratogenicity of DPH. DPH at a dosage of 50 mg/kg administered orally produced 2% incidence of cleft palate (Table 4). Pretreatment of pregnant mice with DEM increased the DPH-induced incidence of cleft palate to 24% (Table 4). Similarly, DEM and trichloropropane (TCP), an epoxide hydrase inhibitor, increase DPH-induced fetal resorptions (Fig. 7). DPH, 50 mg/kg administered orally, induced about 10% incidence of fetal resorptions. DEM increased by 4-fold the incidence of DPH-induced fetal resorptions. TCP also significantly increased the incidence of fetal resorptions. SKF (β-diethyl-aminoethyldiphenyl propylacetate), an inhibitor of microsomal metabolism also enhanced DPH-induced fetal resorptions. DEM also increased the covalent binding of DPH to fetal tissues (Table 5). DEM pretreatment doubled the binding of DPH to fetal tissue. DPH binding was increased from 250 pg to 570 pg/mg of fetal tissue by DEM pretreatment. A reactive metabolite of DPH, an epoxide, may be responsible for its teratogenicity.

Alkylating agents induce mutations, chromosomal aberrations, and cancer (*104*). Rubratoxin B, a metabolite of Penicillium rubrum, is a mycotoxin that produces a midzonal hepatic necrosis and nephrosis (*105*). A substituted analog of byssochlamic acid, in which the ethyl group is replaced by a 6-α,β-unsaturated lactone, rubratoxin B is a potential alkylating agent. Chronic treatment with rubratoxin B produced both hepatic and renal histopathological changes but no tumorigenic activity was noted (*105, 106*). However, Umeda et al. (*107*) reported that rubratoxin B increased the mitotic rate of HeLa

Table 4. Effect of diethyl maleate pretreatment on diphenylhydantoin (DPH)-induced cleft palates.[a]

Treatment	Incidence of cleft palate, %
DPH	2 ± 0.9
DPH + DEM	24 ± 3

[a] Diethyl maleate (DEM) pretreatment, 150 mg/kg, IP, 30 min prior to DPH, 50 mg/kg, PO. Values are the mean percent incidence ± SE. Pregnant animals were treated on gestational days 12, 13, and 14.

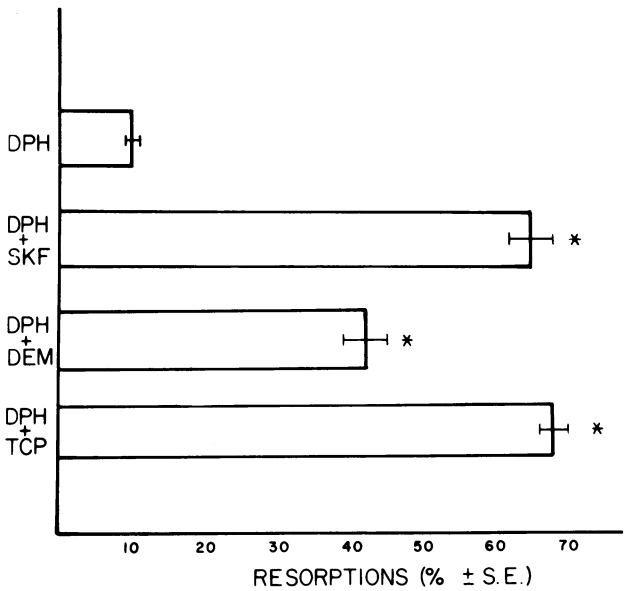


FIGURE 7. Effect of SKF-525A (SKF), diethyl maleate (DEM), and 1,2-epoxy-3,3,3-trichloropropane (TCP) pretreatment on diphenylhydantoin (DPH)-induced fetal resorption rates. DPH, 50 mg/kg PO, was administered on gestational day 12, 13, and 14; DEM, 150 mg/kg IP, was administered 30 min prior to each DPH treatment; SKF, 40 mg/kg IP, was administered 1 hr prior to each DPH treatment; TCP, 150 mg/kg SC, was administered 1 hr prior to each DPH treatment.

cells and produced chromosomal damage.

Aflatoxin, a toxic metabolite of Actinomyces flavus is a known carcinogen and mutagen and also contains an unsaturated lactone ring. The mechanism of aflatoxin toxicity is probably related to formation of an epoxide of the dihydrofuran ring which binds to nucleophilic groups of nucleic acids (*108*).

To assess evidence for dominant lethal mutations, male mice were treated with rubratoxin for 5 days (*109*).

During the first 4 weeks of mating, rubratoxin B induced a significant dose-related increase in the number of pregnant females having early fetal deaths. Rubratoxin B, 1.5 mg/kg, produced early fetal deaths in 100% of the females during the first 3

Table 5. Effect of diethyl maleate (DEM) pretreatment on diphenyl hydantoin (DPH) binding to fetal tissue.[a]

Treatment	Fetal DPH binding pg DPH/mg tissue
DPH	250 ± 90
DPH + DEM	570 ± 50

[a] Diethyl maleate pretreatment, 150 mg/kg, IP, 30 min prior to DPH, 50 mg/kg, PO. Values are the mean ± SE. Pregnant animals were treated with DPH (20 μCi/kg) on gestational day 14 and sacrificed 4 hr following treatment.

weeks of mating. Frequency of females with early fetal deaths returned to control levels during the fifth week of mating. A dose-response relationship was observed.

Rubratoxin B induced a significant increase in the number of early fetal deaths per pregnancy during the first, second, third, and fourth weeks of mating. Again, a dose-response relationship was observed. Rubratoxin B, 1.5 mg/kg, increased the number of early fetal deaths 300 to 400% above controls at these intervals.

Hydrogenation of rubratoxin saturates the α,β-unsaturated lactone ring and inhibits the bioactivation of this ring to a reactive electrophilic intermediate.

Hydrogenated rubratoxin did not increase the frequency of females with early fetal deaths nor did the hydrogenated rubratoxin increase the number of early fetal deaths per pregnancy.

Embryotoxicity was observed in pregnant mice administered rubratoxin B. Control resorption rate was about 2%. Rubratoxin B administered on gestational days 8, 9, and 10 or 12, 13, and 14 produced a significantly higher resorption rate. Rubratoxin B, 0.5 mg/kg, killed 90% of the fetuses when injected on gestational days 8, 9, and 10, but only 22% of the fetuses were killed when rubratoxin administration was on gestational days 12, 13, and 14.

Hydrogenated rubratoxin (10 mg/kg) did not increase resorption rate when administered on gestational days 8, 9, and 10 or on gestational days 12, 13, and 14 when compared to control. Similarly, it did not alter the body weight of surviving fetuses when administered at dosages greater than 20 times the dosage of rubratoxin B.

Various anomalies were noted after single and multiple-day treatment with rubratoxin B. Head, eye, ear, and kidney anomalies were dominant following treatment on day 8 of gestation. Orofacial, brain, lung, liver, and kidney anomalies were dominant following treatment on day 12, 13, or 14 of gestation. No treatment-associated defects were observed following administration of hydrogenated

rubratoxin on gestational days 8, 9, and 10 or days 12, 13, and 14 at a dosage of either 1.0 or 10 mg/kg.

Compounds containing a lactone structure possess a wide range of pharmacological and cytotoxic properties (*110–112*). Since many lactones are electrophilic reacting, the biological activity of these compounds has been attributed to their action as alkylating agents (*113*), and alkylation of nucleic acids, particularly the N-7 position of guanine, has been suggested as the site of action for many lactones in susceptible cells (*114*). Previous studies have demonstrated that rubratoxin B is a potent toxin, but, unlike aflatoxin and other lactone-containing compounds, rubratoxin B did not produce observable chromosomal damage. However, our studies demonstrated that rubratoxin B, possessing an α,β-unsaturated lactone ring, is a potent mutagen and teratogen.

Rubratoxin B is biologically more reactive than its hydrogenated analog. Hydrogenation of the lactone ring decreases the electrophilic properties of this moiety and also the possible oxidation or bioactivation of this moiety to a reactive intermediate with strong electrophilic characteristics. The mechanism of rubratoxin-induced toxicity to the reproductive process is probably electrophilic attack by the lactone moiety to bind to essential macromolecules irreversibly and inhibit cellular proliferation.

Antimetabolites are antitumor agents and are divided into purine and pyrimidine antagonists and antifolates. Amino acid antagonists may also be considered antimetabolites and alter cellular growth and development.

The 6-substituted purines such as azathioprine, mercaptopurine, thioguanine, and chloropurine and heterocyclic ring analogs such as 8-azaguanine are teratogenic and mutagenic. These agents prevent the formation of the nucleic acid bases, adenylic and guanylic acids from inosinic acid. The base analogs operate at the pathways at the nucleotide di- and triphosphate levels. Thus, the base analogs are bioactivated to the appropriate ribotide or deoxyribotide before they act as antagonists. The level of the activating enzyme in a cell will be one factor determining its sensitivity to the antimetabolite. The teratogenic and mutagenic antimetabolites are reversible antagonists acting specifically at one stage of the cell cycle, usually the DNA-synthetic phase. The antimetabolite effects are dose schedule-dependent, unlike the electrophilic reactants which react irreversibly at all stages of the cell cycle. A single high dose of antimetabolite may not be effective, while a smaller dose administered over a period of time may be much more effective. To be effective, the antimetabolite must be present for the time it takes all target cells to enter and pass through the sensitive phase of the cell cycle. Thus, a single dose of an antimetabolite may not be effective because only a portion of the cells are exposed to the drug when they are in a sensitive phase. However, a small minimum effective dose of the compound administered for at least one cell cycle may be effective. Fetal tissues contain cells growing almost synchronously and of a very short cycle time. Therefore, a single dose of an antimetabolite given at the critical time of development can be teratogenic (*7, 17, 43, 44, 55*). Antipyrimidines must also be converted to nucleotides before having teratogenic or mutagenic activity. They act on specific phases of the cell cycle and are dose schedule dependent. The antimetabolites inhibit various stages of pyrimidine biosynthesis, particularly the formation of thymidylic acid from deoxyuridylic acid. Antifolates inhibit the production of tetrahydrofolate, which eventually results in inhibition of nucleic acid and protein synthesis. Three antifolates which have been shown to be teratogenic are aminopterin, methotrexate, and pyrimethamine.

Intercalating agents form very stable complexes with DNA. Unlike alkylating agent–DNA complexes, they do not form covalent linkages, since these compounds may be dissociated in unaltered form by modification of the ionic environment. The effect of complexing is to modify the configuration of the DNA double helix and to interfere with its template activity. Planar molecules containing a number of conjugated aromatic rings are most efficient in binding to DNA. Intercalating agents react with the double helix DNA to cause unwinding and insertion of single molecules between stacked base pairs. Intercalating agents are teratogenic and the effects produced are dependent on gestational day of administration (*64*). These agents are also mutagenic by a similar mechanism.

Amino acid antagonists inhibit protein synthesis. A few amino acid antagonists are teratogenic (*43, 76–78, 115*). Azaserine and DON are two glutamine antagonists which are teratogenic (*7, 72–75*). Asparaginase enzymes hydrolyze circulating asparagine to aspartic acid. Rapidly growing embryonic tissues are not able to synthesize sufficient asparagine from aspartic acid and asparaginase at low doses is teratogenic (*71*). Amino acid antagonists are similarly teratogenic.

Some materials are able to inhibit growth by arresting cells at mitosis. They inhibit cells in metaphase by interfering with formation of the mitotic spindle apparatus. Spindle poisons are teratogenic and mutagenic (*31, 83*). They condense microtubular protein and prevent formation of essential cell

structures.

Other cytotoxic agents which are teratogenic are hydroxyurea and urethane which inhibit nucleic acid synthesis by preventing reduction of ribonucleotides and reacting directly with nucleic acid bases. Procarbazine breaks down to a number of products which covalently bind with DNA. Formaldehyde is mutagenic and teratogenic and acts by reacting with amino groups of the purines of nucleic acids.

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