

REGULATORY RESEARCH PERSPECTIVES

Impact on Public Health

On the Chemical Causation of Methyl Deficiency and its Attendant Pathologies

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Abstract: It is now fairly well established that physiological methyl group deficiency and abnormal DNA methylation are involved in the etiology of several pathological processes. The proceedings of a recent meeting on this subject held at the NIH campus in Bethesda, MD, the "Trans-HHS Workshop: Diet, DNA Methylation Processes and Health", have recently been described [*Journal of Nutrition*, 132 (8S):2329S-2484S, 2002]. The pathologies considered at the workshop included: cancer, atherosclerosis, birth defects, aging, diabetes and pancreatic toxicity, and hepatotoxicity. Two major causes of the diseases related to methyl group insufficiency were considered at this conference: dietary deficiency and genetic polymorphism. A third prospective cause, chemicals, was not treated in depth. The purpose of this article is to explore the evidence that exogenous chemicals, like dietary imbalances and genetic defects, may produce the abnormal methylation processes linked to the etiology of disease. The pathologies considered are cancer, pancreatic toxicity and diabetes, atherosclerosis, birth defects, and neurotoxicity. Four categories of toxic chemicals are considered: the antimetabolites of the methyl donors, the anticonvulsants, polyhalogenated compounds, and metals. The results indicate that a number of such agents appear to exert their pathological effects, at least in part, through abnormal methylation processes.

Introduction

In August 2001, a meeting entitled, "Trans-HHS Workshop: Diet, DNA Methylation Processes and Health" was held at the NIH, which explored the relation between abnormal methylation and several disease processes (1). It considered in some depth the role of dietary factors in DNA methylation processes and attempted to delineate the causes and mechanisms of abnor-

mal DNA methylation in various physiological conditions involved in growth, normal development and differentiation, and disease prevention. The underlying premise of the meeting was that abnormal methylation was a common feature of many diseases and that derangement in methylation reactions could foster the development of several diseases by similar or common mechanisms. These pathologies included cancer (2-5), pancreatic

toxicity and diabetes (6,7), atherosclerosis (8), aging (9,10), birth defects (11-13), and neurological disturbances (14,15). Many of these pathologies could be induced by dietary deficiencies (16,17), genetic alterations (18-20) and chemicals (3,21,22). The Workshop was principally focused on the nutritional causation of disease but did consider, to a lesser extent, genetic al-

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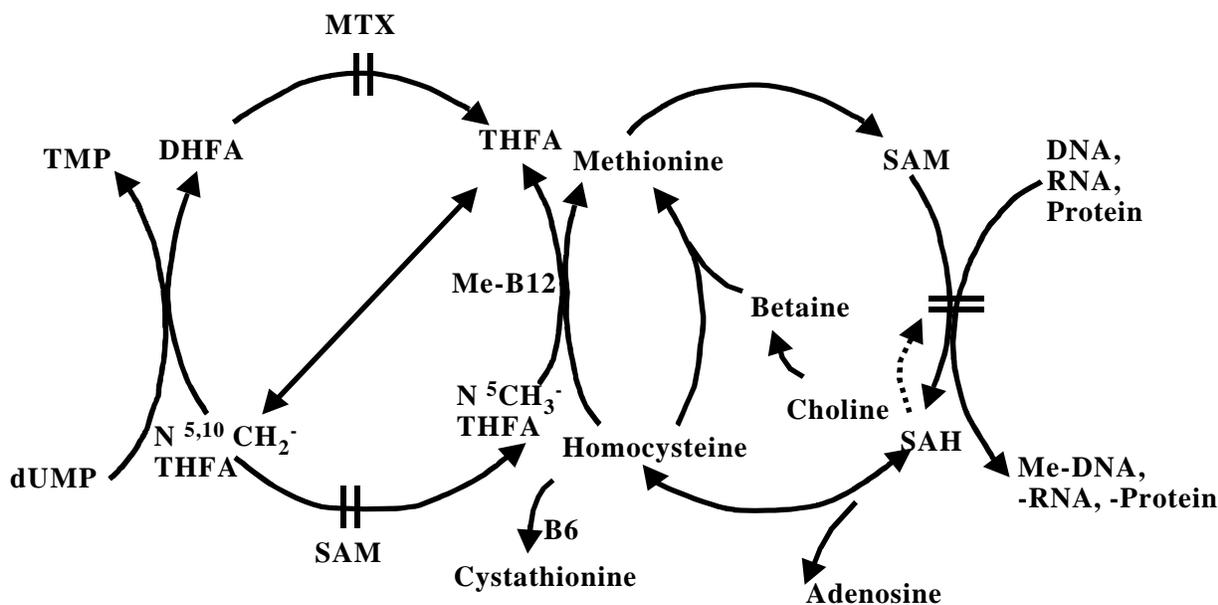
terations (18-20) especially in conjunction with dietary imbalances (18). Because of the Workshop's focus on diet, the chemical causation of methyl deficiency and of the diseases associated with abnormal methylation processes did not receive much emphasis (1). However, three excellent reviews (2,23,24) have recently described the possible role of abnormal DNA methylation as a major factor in chemical carcinogenesis by epigenetic agents. The present review attempts to summarize evidence that specific categories of epigenetic carcinogens exert a pattern of pathological effects caused, at least in part, by abnormal methyl group metabolism and abnormal DNA methylation.

At the core of any consideration

of abnormal methyl metabolism in the etiology of disease are the complex metabolic interrelations between the various sources and uses of the methyl intermediates (Figure 1). While all such schema are of necessity incomplete, the main dietary and metabolic features relative to disease etiology can be illustrated. There are four major dietary sources of methyl groups (Figure 1). Two, methionine and choline, provide preformed methyl groups; two, the vitamins B₁₂ and folic acid, are used in the *de novo* synthesis of methyl groups *in vivo* (Figure 1). Deficiencies of one or more of these four essential nutrients have been shown to exert pathologic effects in humans and/or experimental animals (1). Such pathologies include: cancer (25,26), atherosclerosis (27,28), pancreatic toxicity and dia-

betes (29,30), birth defects (31,32), neurological disturbances (33,34) and abnormal neurological development (33,34), and hepatotoxicity (35,36). While each of these nutrients exerts important roles other than those listed in Figure 1, they do share one metabolic feature in common: the biosynthesis of S-adenosylmethionine (SAM). SAM is the body's chief physiological methyl group donor. It is used to methylate a host of substrates, including proteins, phospholipids, RNA, and DNA (37-39). DNA is the macromolecule on which most attention is focused in studies on abnormal methylation processes and disease. The demethylated metabolic product of SAM, S-adenosylhomocysteine (SAH) is produced in all SAM-dependent methylations and is a

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Abbreviations:

SAM S-adenosylmethionine
 SAH S-adenosylhomocysteine
 THFA Tetrahydrofolic acid
 DHFA Dihydrofolic acid
 dUMP Deoxyuridine monophosphate
 TMP Thymidine monophosphate

B₆ Vitamin B-6
 B₁₂ Vitamin B-12
 Me- Methyl-
 MTX Methotrexate
 CH₃- Methyl group

Figure 1. The metabolism of methionine and related compounds.

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strong competitive inhibitor of all methylases studied (38,40). Two other compounds deserve special mention because of their close association with SAM and with methylation reactions: homocysteine (HCys) and vitamin B₆. In humans, hyperhomocysteinemia has long been associated with the development of atherosclerosis (41). In recent studies, homocysteinemia has been associated with the development both of birth defects (42,43) and of cancer (44). The accumulation of HCys in blood can result from nutritional deficiencies of folate and vitamin B₁₂, as well as of vitamin B₆ (45-49). The effect of B₆ on HCys is thought to be due to its role as a coenzyme in the enzyme cystathionine β synthetase (CBS) (46,50). Hence a deficiency of vitamin B₆ is becoming increasingly associated with high risks of atherosclerosis (49,51,52), cancer (53,54), and birth defects (55,56). While

several mechanisms have been brought forth to explain the pathological effects of HCys (57,58), it is its role as a source of SAH that has received most of the recent attention (30,59,60). By reversing the normal direction of the enzyme SAH hydrolase, high levels of HCys may produce high intracellular levels of SAH and, consequently, a hypomethylating environment (60-62).

Alterations in the levels or activities of enzymes associated with the formation and utilization of SAM have also exerted pathologic effects resembling those produced by methyl group insufficiency caused by methyl-deficient diets (Table I). For example, alterations in the polymorphism or expression of the enzyme methylenetetrahydrofolate reductase (MTHFR), the enzyme actually responsible for the formation of labile methyl groups *in vivo* (Figure 1) have been associated with increased risk of cancer (depending upon tissue and dietary

factors) (4,18,63) and birth defects (64,65). Abnormal MTHFR activity in diabetics has been associated with the more advanced stages of the disease rather than with its inception (30,66,67). The enzyme catechol-*O*-methyltransferase (COMT) uses SAM to methylate catechols, including the estrogen metabolites 2- and 4-hydroxyestradiol. In most studies, the low activity polymorphic form of COMT (met/met) is associated with increased risk of breast cancer formation compared to that observed with the high activity polymorphic form of this enzyme (val/val) (68-70). However, in some populations, particularly post-menopausal women with a low body mass index (BMI), the low activity polymorph is associated with decreased risk of developing breast cancer (71,72). One hypothesis suggested for these observations is that in the sensitive women the less active form of the enzyme allows more of the tumorigenic estrogens to remain in circulation, while in the protected women, the less active form of COMT places less of a drain on the available SAM. Finally, diminished activity of methionine adenosyltransferase, the enzyme that actually synthesizes SAM, is associated both with demyelination of nervous tissue in man (73) and with hepatotoxicity in rodents (74).

Because of time constraints, the recent meeting "Trans-HHS Workshop: Diet, DNA Methylation Processes and Health" was unable to adequately consider chemicals as prospective causes of the methyl deficiency-related diseases. This was a bit of a paradox, for early studies utilizing the chronic administration of toxic chemicals, particularly carcinogens, were among the first to link an insufficiency of "labile" methyl groups with multiple pathologies (75,76). Particularly noteworthy in this regard were the synergistic hepatotoxic and hepatocarcinogenic effects by aflatoxin and choline deficiency (75) and the hepatotoxicity, hepatocarcinogenicity and

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Table I		
Associations between disease and methyl insufficiency caused by diet or enzyme defects		
PARAMETER	AGENTS	
	DIET	ENZYME DEFECT
Disease		
Cancer	+ (2-5,16,25,53,54)	+ (4,18,63,68-70)
Pancreatic toxicity	+ (6,29)	+ (66,67)
Atherosclerosis	+ (8,26-28)	+ (40,41,79)
Birth defects	+ (11,12,31,32,55,56)	+ (42,64,65)
Neurotoxicity	+ (14,15,33,34)	+ (73)
Biochemical alterations		
SAM/SAH	+ (3,18,19,33,34)	+ (19,20,30,73,74)
Homocysteine	+ (13,18,19,44-46,48,51,52,80,81)	+ (19,79)
DNA methylation	+ (3,11,16,17,19)	+ (19,20)
MTase	+ (82)	

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pancreatotoxicity in rats of the methionine antagonist ethionine (76). Interestingly, in 1953 J. A. and E. C. Miller suggested, "The Methyl Deficiency Hypothesis" as a possible explanation for the carcinogenic activity of the aminoazo dye hepatocarcinogen *N,N*-dimethyl-4-aminoazobenzene (77). Twenty years later, direct biochemical evidence that physiological methyl insufficiency resulted from the chronic feeding of a hepatocarcinogen was published (78).

The current evidence, from the Bethesda Workshop as well as from other sources, shows that five biochemical parameters related to methylation: SAM, SAH, HCys, DNA, and gene methylation may be reasonably used to assess overall methylation status *in vivo* and in particular organs (1). The major diseases linked to abnormal methylation reactions and treated at the Bethesda Workshop were: cancer (2-5,25), atherosclerosis (8), pancreatic toxicity and diabetes (6,7), birth defects (11-13), neurological disturbances and abnormal neurological development (14,15), hepatotoxicity (21,22), and aging (9,10). The effect of diet and genetic alterations on methylation status and on five related pathologies is summarized in Table I. The aim of this manuscript is to provide evidence that specific categories of chemicals cause the same pathologies that result from an insufficient supply of physiological methyl groups produced by dietary deficiencies or by enzymatic changes resulting from genetic polymorphisms or gene expression. Since the body of literature in this area is both broad and diffuse, some practical limits had to be set on the agents and the parameters to be evaluated. The major categories of chemicals to be considered are 1) the antimetabolites of the methylation reactions, 2) the anti-convulsants, 3) the polyhalogenated organics, and 4) the metals. As was described above with the dietary and genetic causes of abnormal methyl group metabolism, the bio-

logical endpoints to be focused upon will be cancer, pancreatic toxicity and diabetes, atherosclerosis, birth defects, and neurotoxicity. Hepatotoxicity has been excluded as a separate pathologic category since the hepatotoxic chemicals whose effects on methyl metabolism have been investigated are almost invariably hepatocarcinogens as well. Aging has also been excluded as a separate category because, apart from the five pathology endpoints that will be discussed, there are few studies linking the chemical prolongation of life with improvements in methylation status. The biochemical parameters altered by the chemicals will include SAM and SAH, HCys, DNA methylation, and DNA methyltransferase (MTase). Except in the cases of cancer and aging, there are relatively few studies in which alterations in methyl metabolism and DNA methylation have been examined in the target tissue. Hence, the general, rather than the tissue-specific, effects of toxic agents on methylation reactions will often be considered.

Section II: The Antimetabolites

Ever since the early studies on vitamins, antimetabolites have demonstrated their usefulness in determining the essentiality and mechanism of action of various nutrients (83). Later (84,85), antimetabolites of specific nutrients and their metabolites found great applicability as chemotherapeutic agents against cancer. The pathological and biochemical effects of four antimetabolites, ethionine, azacytidine, methotrexate (MTX), and fluorodeoxyuridine (FUDR), which inhibit the formation and/or use of physiological methyl groups, often resemble those associated with methyl group deficiencies produced by dietary deficiency or enzyme polymorphisms (Table II). Of these, it is the methionine antagonist ethionine whose reported effects appear to resemble most closely those produced by a dietary methyl group deficiency

(Table II). Ethionine is a good hepatocarcinogen in both rats and mice (76,86-88), and its carcinogenic activity is inhibited by methionine (76) and enhanced by dietary methyl group deficiency (86). Ethionine is also toxic to the pancreas of rats and other species (76,89) and is a part of a regimen that results in the hepatization of pancreatic acinar cells in hamsters (90). Ethionine pancreatotoxicity in rats is marked by destruction of the exocrine pancreas, is enhanced by choline deficiency, and can be prevented by methionine (89). Ethionine treatment has also caused birth anomalies in rats and chicks (76,91). Ethionine is an effective competitive inhibitor of methionine in the synthesis of SAM (76,92,93), especially in the liver. It produces a hypomethylating environment in the liver, both by reducing the hepatic contents of SAM, as well as by the accumulation of the ethyl analogue of SAM, S-adenosylethionine (SAE) (76,92,93). SAE is an effective inhibitor of several SAM-dependent methylases, including MTase (94). As expected, both the acute (94) and the chronic (95,96) treatment of rats with ethionine results in global DNA hypomethylation. SAE, like ethionine, transforms rat liver epithelial cells in culture (97). The administration of ethionine has also resulted in increased hepatic levels of HCys (93).

Both *in vivo* and in cell culture, azacytidine is an excellent DNA hypomethylating agent (Table II) (98-101). Azacytidine is incorporated into DNA as an analogue of cytidine and, through its subsequent chemical interaction with MTase, prevents the normal methylation of DNA (99,101). Azacytidine has exhibited tumorigenic activity in several animal studies (Table II) (102-105). It has also shown cell transforming activity in cell culture (105-108). Azacytidine is teratogenic (Table II) and has produced neural tube defects in developing rat embryos *in vitro* (109,110). While we could find no evidence of any direct effect of

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TABLE II
THE ANTIMETABOLITES

PARAMETER	AGENTS			
	ETHIONINE	AZACYTIDINE	MTX	FUDR
Disease				
Cancer	+ (76,86-88)	+ (102-105)	+/- (111-114)	+/- (141-145)
Pancreatic toxicity	+ (76,89,90)		+/- (115-117)	
Atherosclerosis			+/- (118,119)	
Birth defects	+ (76,91)	+ (105,109,110)	+ (120-122)	+ (147-149)
Neurotoxicity			+/- (124-131)	+ (152-154)
Biochemical alterations				
SAM/SAH	+ (76,92,93)		+ (47,127,128,133)	
Homocysteine	+ (93)		+ (134-136,138)	
DNA methylation	+ (94-96)	+ (98-100,105)	+/- (137-140)	- (140)
MTase	+ (94)	+ (99,102)		

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azacytidine on SAM, SAH or HCys, it did reverse the chemopreventive effects of SAM during the early stages of liver tumor promotion by phenobarbital in rats (100).

Unlike ethionine and azacytidine, the one-carbon antimetabolites MTX and FUDR have not been shown to inhibit SAM-related enzymes. Their pattern of biological effects might thus be expected to differ from those of the former two antimetabolites. This is somewhat the case with MTX. MTX is a very well established chemotherapeutic drug, which exerts its activity principally by the inhibition of the enzyme dihydrofolate reductase leading to a deficiency of reduced folate intermediates (84). MTX has not shown marked carcinogenicity either in humans or in experimental animals (Table II). IARC considers the evidence for carcinogenicity both towards humans and towards experimental animals to be *inadequate* (111,112). In experimental studies, MTX appeared to exert co-

carcinogenic activity towards the buccal pouches of hamsters treated with benzpyrene (113) and towards the mammary glands of male rats receiving procarbazine (114). In one study, MTX has caused, significant but moderate, pancreatic toxicity in mice, as evidenced by fatty metamorphosis and diminished levels of amylase, lipase and other enzymes; but other studies showed no clear evidence of such toxicity either in humans or in rats (115-117). Similarly, in its effects on heart disease, methotrexate treatment caused varying effects: In cholesterol-fed rabbits, MTX treatment protected against the formation of aortic atherosclerotic plaques, but in patients with rheumatoid arthritis, it increased cardiovascular comorbidity associated with atherosclerosis (118,119) (Table I). MTX is a well-known teratogen in both humans and experimental animals (120-122) (Table II), with cleft palates as a major presenting lesion. In addition, MTX enhanced the formation of neural tube defects (NTDs) in the

embryos of pregnant mice treated with valproic acid (123). MTX therapy exerts significant effects on the nervous system (Table II), but these vary with the disease treated and the administration protocols used (124-131). When used to treat acute lymphocytic leukemia or non-Hodgkin's lymphoma, MTX caused neurologic problems, neuropsychological deficits, white matter changes in the brain, leukoencephalopathy, and demyelination (125-129). However, MTX has been used with some benefit to treat two other disorders associated with demyelination: multiple sclerosis (MS) (130) and chronic inflammatory demyelinating polyneuropathy (131).

In one study (127), the leukoencephalopathy caused by MTX treatment of ALL patients could be associated with decreased SAM levels, increased SAH and decreased SAM/SAH ratios in the cerebrospinal fluid (CSF). In another study (128), the demyelination produced by MTX was accompanied by low

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levels of SAM, methionine and 5-methyltetrahydrofolic acid in the CSF. Consistent with the effects of MTX on SAM in the CSF and demyelination is the observation that treatment with the methyl donors methionine, betaine and SAM induced remyelination in patients with inborn errors of folate and one-carbon metabolism (132). In addition to the clinical studies described, MTX has also altered the SAM/SAH ratios in the livers of rats and mice (47,133). Increases in plasma and CSF levels of HCys have been described for patients treated with high doses of MTX (134-136). As are its effects on some other parameters, the effects of MTX on DNA methylation are mixed (Table II). MTX treatment decreased the extent of DNA methylation in the brains and livers of rats (137,138). On the other hand, MTX reversed the hypomethylation seen in the peripheral blood mononuclear cells of patients with arthritis and, like other chemothera-

peutic inhibitors of DNA synthesis, induced DNA *hypermethylation* in cells (139,140).

FUDR is an analogue of deoxyuridine that exerts its chemotherapeutic activity through the inhibition by its metabolite FdUMP of the enzyme thymidylate synthetase (85). Cell transformation by FUDR has been described with both hamster and mouse fibroblast cells in culture (141,142), but no solid studies appear to have been published demonstrating the carcinogenic activity of this compound *in vivo*. An early report with newborn Swiss mice appeared to show the production by FUDR of lung adenomas in this strain (143), but a larger study using lung adenoma formation in Strain A mice failed to demonstrate significant tumorigenicity by this compound (144). FUDR enhanced skin tumor formation in mice treated with 3-methylcholanthrene (145), but this experiment did not use control groups treated with FUDR only. We could find no significant effect of

FUDR on pancreatic changes or on atherosclerosis. It does, however, exhibit significant teratogenic activity in rats, with cleft palates being among the major anomalies described (Table II) (146-149). The effects of FUDR on SAM, SAH and HCys levels either in animals or in cell culture do not appear to have been widely investigated. However, like MTX and several other cancer chemotherapy agents, FUDR, at cytotoxic levels, caused *hypermethylation* of DNA in human cancer cells in culture (140).

Very recent studies have described another antimetabolite of methyl metabolism with carcinogenic activity (150,151). The compound is diethanolamine (DEA). DEA acts as an antagonist of choline, and its chronic administration causes an increased incidence of liver cancer in B6C3F1 mice (150,151). The administration of DEA to mice for four weeks led to markedly diminished hepatic levels

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TABLE III
THE ANTICONVULSANTS

PARAMETER	AGENTS			
	PHENOBARBITOL	PHENYTOIN	DIAZEPAM	OTHER
Disease				
Cancer	+ (157-160)	+ (161,162)	+ (163,164)	+ (163,165)
Pancreatic toxicity	+ (177,214,215)	+ (167)		+ (168-174)
Atherosclerosis	-/+ (178,179,181,182,184)	-/+ (182,184)	- (180)	-/+ (181,182,184)
Birth defects	+ (185-188)	+ (185-187,189-191)	+ (186,187,192)	+ (161,185,186,188-190,193,194)
Neurotoxicity		+ (196-198)		+ (199)
Biochemical alterations				
SAM/SAH	+ (200-202)	+ (202,203)	+ (204)	+ (193,205,206)
Homocysteine	+ (155,209)	+ (155,208,209)		+ (208-210)
DNA methylation	+ (201,211-213)			+ (205)
MTase				

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of the methyl intermediates choline, phosphocholine, and SAM, as well as to elevated levels of SAH (150). DEA may thus provide another tool in detailing the respective contribution of various methylation processes in the development of disease.

Section III The Anticonvulsants

Introduction and Cancer: After the antimetabolites of the methyl group donors, it is probably the anticonvulsants that constitute the category of agent whose correlation with methyl metabolism has been most widely investigated. The major reason for this is the widely recognized antagonism between the anticonvulsants and folic acid (155,156), which has often resulted in fetal abnormalities in epileptic mothers treated with anticonvulsants. In addition, the anticonvulsants, as a chemical class, have frequently been tested as potential carcinogens or tumor promoting agents (Table III) (157-166). In its assessment of the sporadic tumors arising in patients treated with one or more of the anticonvulsants, IARC had generally categorized the information supporting the human carcinogenicity of these compounds as "inadequate" or "limited" (161,164). However, in standard animal carcinogenicity studies, a number of anticonvulsants of varying structures have demonstrated carcinogenic or liver tumor promoting activity in mice and rats (157-166). In fact, it was the use of phenobarbital as a tumor promoter that provided the first direct experimental evidence of the applicability of the two-stage model of carcinogenesis in the rodent liver (166). Several studies since then have amply confirmed the tumorigenicity of phenobarbital and of other anticonvulsants in rodents.

Pancreatic toxicity and diabetes: Pancreatotoxic effects of the anticonvulsants have been described in both humans and in experimental animals (Table III). Pancreatitis in

epileptic patients has occasionally been observed following treatment with phenytoin (167), carbamazepine (168-171) and valproic acid (172-174). Phenobarbital treatment of rats has been reported to diminish replication of the beta-islet cells (175) and to increase the levels of amylase and lipase in blood, signs of pancreatic injury (176). The combination of KBr and phenobarbital to treat epileptic dogs has led to the formation of pancreatitis in this species (177).

Atherosclerosis: With regard to the formation of heart disease and atherosclerosis, the anticonvulsants present a mixed picture (Table III). In most studies, both in humans and in experimental animals, administration of anticonvulsants has resulted in an improved distribution of the lipids associated with atherosclerosis. Thus, phenobarbital treatment increased the ratio of HDL cholesterol to total serum cholesterol in human subjects and decreased the serum cholesterol levels and the formation of atheromata in rabbits fed an atherogenic, cholesterol-containing diet (178,179). Diazepam also protected against the formation of atherosclerotic lesions in roosters fed an atherogenic diet (180), while treatment of epileptic children with valproic acid lowered the total cholesterol and the ratio of total cholesterol to HDL cholesterol in blood (181). A mini-review on the subject (182) described the improved ratios of HDL cholesterol to total cholesterol and noted the diminished mortality from coronary deaths in epileptic patients receiving long-term therapy with such anticonvulsants as phenobarbital, diphenylhydantoin and carbamazepine. On the other hand, anticonvulsant therapy has been shown to cause a rise in serum HCys levels (Table III) and to cause a rise in total cholesterol and/or LDL cholesterol in epileptic children (181). In addition, lipoprotein levels and carotid intimal thickness were reported to be elevated in epileptic patients receiving long-term medication with carbamazepine, pheny-

toin, phenobarbital or valproate (183,184). To quote Eiris *et al.* (181), "The literature on the effects of hepatic enzyme-inducing anti-inflammatory drugs [AEDs] on serum lipid profiles and, by extension, on risk of atherosclerosis, is thus contradictory."

Birth defects: The teratogenic activity of the anticonvulsants both in humans and in experimental animals is now well established (Table III) (155,185-195). The compounds exhibiting such activity include the three representative anticonvulsants listed in Table III: phenobarbital, phenytoin and diazepam, as well as carbamazepine and valproic acid. The principal developmental defects seen both in humans and in experimental animals were NTDs (185,189,192,193), although congenital heart malformations and facial clefts were also associated with the ingestion of barbiturates and phenytoin during pregnancy (189).

Neurotoxicity: In three small, separate studies (196-198) neuropathologic changes were seen in the brains of patients treated with phenytoin; in each case the changes were accompanied by reversible demyelination of the tissue. In another clinical study, the progression of a demyelinating disease was exacerbated by treatment with valproic acid (199).

SAM/SAH: Several studies have demonstrated the direct effects of anticonvulsants on SAM and SAH in the target tissues of experimental animals (Table III) (193,200-206). The chronic administration of phenobarbital under liver tumor-promoting conditions, as well as the similar chronic administration of phenytoin both gave rise to decreased levels of SAM and/or SAM/SAH ratios in the livers of rats (200,201,203). In addition, phenytoin, phenobarbital and diazepam appeared to prevent both the seizures and the rise in SAM/SAH ratios in the brains of rodents treated with the convulsant methionine sulfoximine (202,204). The effects of valproic acid on the hepatic con-

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TABLE IV
POLYHALOGENATED COMPOUNDS

PARAMETER	AGENTS			
	CCl ₄	DDT	DIOXIN	OTHER
Disease				
Cancer	+ (216,217)	+ (158,231-236)	+ (250-254)	+ (278,296)
Pancreatic toxicity	+ (218,219)	+ (241-243)	+ (252,255,256,258)	+ (279-281)
Atherosclerosis		+ (244)	+ (262-264)	+ (244,282,283)
Birth defects	+ (221,222)	+ (246,247)	+ (251,272-274)	+ (284-287)
Neurotoxicity	+ (223-226)	+ (232,248,249)	+ (224,225,274-277)	+ (285,288)
Biochemical alterations				
SAM/SAH	+ (227)	+ (200)		
Homocysteine	+ (227)			
DNA methylation	+ (227)			+ (289-292)
MTase				+ (293)

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tents of SAM and SAH were varied (193,205,206). In one mouse study, an acute dose of valproate caused a sharp rise in the hepatic contents of SAM and SAH, along with a decrease in the SAM/SAH ratio (193). In a chronic study with pregnant rats, valproic acid administration led to altered hepatic contents of SAM and SAH, but no changes in the SAM/SAH ratios (205), while in another chronic study, valproic acid treatment led to elevated hepatic contents of SAM (206).

There is much indirect evidence, as well, linking methylation status with the biological effects of the anticonvulsants. The antagonism between folate and the anticonvulsants in humans has already been noted (155,156). Methionine and SAM have both been shown to inhibit liver tumor promotion by phenobarbital in rodents (158-160). Phenytoin treatment of pregnant mice under a regime known to cause birth defects caused a significant drop in the MTHFR activity in

maternal liver (191,207). Treatment with folinic acid and a combination of vitamins B₁₂ and B₆ effectively inhibited the fetal malformation induced by valproic acid in mice (195). Folate, but not exogenous SAM, protected against the teratogenic effects of valproic acid in rats (194). Diminished levels of folate were seen in the plasma of mice treated with phenytoin (207) and in the livers of rats receiving valproate (206). Finally, the co-administration of the folate antimetabolite MTX enhanced the formation of neural tube defects produced in mice by valproic acid (123).

Homocysteine: HCys is one metabolite whose blood levels are frequently elevated with anticonvulsant therapy (Table III) (155,208-210). In epileptic patients treated with phenobarbital, phenytoin, primidone or carbamazepine, both the fasting and the post-methionine load levels of HCys in serum were elevated compared to those of control subjects; correspondingly, their blood folate levels were depressed

(155,209,210). Valproic acid did not exert any significant effect in these studies, either on plasma HCys or on blood folate levels. Finally, epileptic patients who were homozygous for the 677T mutation in the enzyme MTHFR exhibited high HCys levels if they received the anticonvulsants phenytoin and carbamazepine, but not if they were treated with valproic acid (208).

DNA methylation: Both global and gene-specific DNA hypomethylation has been observed in the livers of rats and mice during carcinogenesis or tumor promotion with phenobarbital (Table III) (201,211-213). Phenobarbital treatment induced hypomethylation and increased expression of the hepatic oncogenes *c-Ha-ras* and *c-raf* in mice and of *c-myc*, *c-Ha-ras* and *c-K-ras* in rats (201,211-213). Similarly, the administration of a teratogenic dose of valproic acid to pregnant Wistar rats resulted in DNA hypomethylation in the fetal livers (205).

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Section IV The Polyhalogenated Compounds

Carbon tetrachloride: The polyhalogenated compounds encompass a large number of chemicals that exert carcinogenic and other toxic effects. Many produce one or more of the pathologic and biochemical changes described in this review; three are described in Table IV: CCl₄, DDT, and dioxin. Of these, the one exhibiting the broadest range of effects is CCl₄ (Table IV). CCl₄ has exhibited hepatocarcinogenic and liver tumor promoting activity in rodents and has been associated with increased risk of rectal cancer in humans (216,217). In rats it also causes pancreatic toxicity (Table IV) (218,219). While we did not find direct evidence of a causal association between CCl₄ ingestion and atherosclerosis, the treatment of arteriosclerotic and nonarteriosclerotic rats with sufficient CCl₄ to induce cirrhosis led to myocardial necrosis, calcification and a high incidence of atrial thrombi (220). The ingestion of CCl₄ has been associated with the formation of birth defects in humans as well as in rats (Table IV). The contamination of public drinking water with CCl₄ has been associated with several birth defects including: CNS defects, NTDs and oral cleft defects (221). CCl₄ was shown to be teratogenic in rats, significantly decreasing the crown-rump length in the embryos of CCl₄-treated dams (222). CCl₄ is a neurointoxicant (223-226), and its activity has been suggested to occur through abnormal signal transduction (226). The studies by the group of J. M. Mato (227) have provided convincing evidence that many of the hepatotoxic effects of CCl₄ occur through a deficiency of SAM (Table IV). Hepatotoxic doses of CCl₄ have produced decreased hepatic levels of SAM and of SAM/SAH ratios while increasing the blood levels of HCys, as well as the extent of DNA hypomethylation in the livers of rats (Table IV) (227). In

addition, the exogenous provision of the physiological methyl donors SAM and betaine has inhibited the toxicity of CCl₄ toward hepatocytes (228-230).

DDT: Dichlorodiphenyltrichloroethane (DDT), a halogenated hydrocarbon, was introduced as an insecticide in the 1940s. Its use was banned in the 1970s by most developed countries due to its persistence in the environment, bioaccumulation in alimentary chains, negative impact on wildlife, and presumed adverse effects on human health. Rodent studies showed a significant association between DDT administration and lymphoma, respiratory cancer, liver cancer, and estrogenic effects on mammary tissue (158,231). The tumorigenic potential of DDT, however, was negligible after dosing for 15-22 years in nonhuman primates; few animals administered DDT developed tumors, although hepatic and central nervous system toxicity was commonly observed (232). In humans, epidemiological studies have shown controversial results. In fact, the International Agency for Research on Cancer qualified early studies as providing inadequate evidence for carcinogenicity in humans since several of them were deficient in satisfactory sample sizes and were not exempt from such confounding factors as multiple chemical exposure, lifestyle factors, genetic, and other environmental influences (231,233-237). Recent investigations, in which those confounding factors were more controlled, have reported some risk of lymphoma, leukemia, pancreatic cancer, and breast cancer in humans exposed to DDT (237-241). Adipose levels of DDE, one of the main DDT metabolites that persist for long periods in human tissues, have also been associated with high mortality rates for liver cancer, but not for pancreatic cancer, multiple myeloma, non-Hodgkin lymphoma, breast cancer, and uterine cancer (240). High exposure to or internal levels of DDT or its metabolites, as well as other chlorinated compounds, have been

associated with such other diseases as diabetes, atherosclerosis, some neurological afflictions, and reproductive alterations (Table IV) (232,242-249). For example, significant amounts of DDT were found in the serum of patients suffering from slight to severe atherosclerotic lesions, compared to the corresponding levels seen in normal subjects (244). Acute DDT intoxication causes tremor and hyperexcitability that may be related to its effects on the sodium channel (248). Low-dose exposure to DDT during the 'brain growth spurt', a period occurring in the course of perinatal development of the brain that can be critical for its normal maturation, can lead to irreversible changes in adult brain function in the mouse. Furthermore, neonatal exposure to a low dose of a neurotoxic agent can lead to an increased susceptibility in adults to an agent having a similar neurotoxic action, resulting in additional behavioral disturbances and learning disabilities (249).

Recent reports have raised new concerns DDT may disrupt normal reproduction and development through inhibition of androgen receptor function (245). Normal development of male genitalia in mammals depends on androgen action. DDE was found to inhibit binding of androgen to its receptor and to block androgen action in rodents, yet studies in humans are still inconclusive (246). Although there are several indications that chronic exposure to chlorinated compounds, such as polychlorinated biphenyls and dioxins, could induce diabetes mellitus, there is as yet no solid evidence linking DDT exposure with this disease (242). Two studies have linked abnormal methyl group metabolism with liver tumor formation by DDT (158,200). The subchronic feeding of the liver tumor DDT led to a 50% drop in the hepatic levels of SAM (200), and this effect could be partially prevented by supplemental dietary methionine. In a long-term study, the same supplementation by methionine signifi-

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cantly diminished the formation of metastatic hepatocellular carcinomas by DDT in diethylnitrosamine-initiated rats (158). We were unable to find any publications describing the effects of chronic DDT administration on the serum levels of HCys, on MTase activity or on DNA methylation.

Dioxin: Dioxin appears to be a carcinogen both in humans and in experimental animals (Table IV) (250-254). In humans, this increased tumor incidence was most notable with cancers of the rectum, the lung and the lymphohemopoietic system (251,252,254). In rodent species, liver cancer is the major tumor studied (250-252). In addition to its cancer-causing potential, dioxin induces many non-neoplastic pathologies (Table IV). Several studies have associated high human exposures to dioxin with the eventual formation of diabetes (252,254-257). Others observed either little or no correlation between diabetes and dioxin exposure (258-260). However, this apparent disparity may be attributable to differences in exposure levels in the populations concerned (252). As another manifestation of its pancreatotoxicity, dioxin induced hepatic transdifferentiation in the pancreas of hamsters (261). In addition, dioxin treatment in experimental animals produced hypoinsulinemia (255,259). The evidence in favor of a causal role of dioxin in atherosclerosis is indirect (262-266) and consists of: 1. the hyperlipidemia, resembling that of atherosclerosis, seen in dioxin-treated animals (262,265-268), 2. the elevated incidence of ischemic heart disease found in the populations exposed to high levels of dioxin (263,269), and 3. the shared risk factors previously described for cancer and atherosclerosis (264,270,271). Dioxin has produced a variety of birth defects, including spina bifida in humans and cleft palates in mice (251,272-274). Similarly, exposure to dioxin has resulted in peripheral neuropathy in humans (275,276) and polyneu-

ropathy in rats (277) (Table IV). In humans at least, the neuropathy has been proposed to result from an incipient diabetes (275,276), also associated with exposure to dioxin. The effects of dioxin on the methylation parameters considered in this review do not appear to have been described in the scientific literature to a significant degree.

Others: A number of additional polyhalogenated compounds have exhibited carcinogenic activity (274,278). Many have demonstrated the pattern of pathologic effects and the alterations in methylation processes seen with the previously considered carcinogens. For example, pancreatitis has resulted from methylene chloride poisoning in humans and chloroform injection into dogs (279,280). The serum levels of polychlorinated biphenyls in pregnant women with diabetes were found to be significantly higher than in the corresponding pregnant control subjects (281). Similarly, the blood levels of total organochlorine residues were highest in severely atherosclerotic patients compared to the corresponding levels in control subjects or moderately atherosclerotic patients (244). In different studies, polychlorinated biphenyls diminished blood levels of HDL in one animal model for atherosclerosis (282) and caused vascular endothelial cell dysfunction in another (283). The developmental toxicity of the organohalides has been amply demonstrated in experimental animals. PBB treatment of pregnant animals led to the formation of cleft palate in developing mouse embryos and to decreased crown-rump lengths in male rat embryos with eventual behavioral toxicity in the resulting rat pups (284,285). Chloroform inhalation by pregnant dams produced fetal anomalies in both rats and mice (286,287); these included decreased fetal crown-rump lengths in both rats and mice, delayed ossification and missing ribs in rats and cleft palates in mice. Polybrominated biphenyls have exhibited neurotoxic effects both in developing rats and in humans

(285,288).

In a series of recent studies (289-292), the group of Pereira has examined the effects on DNA methylation by the carcinogen tetrachloroethylene (TCE), its metabolites dichloroacetic acid (DCA) and trichloroacetic acid (TCA), and several related compounds in the livers of mice. The subchronic administration of chloroform and CHBrCl_2 caused global DNA hypomethylation in livers of B6C3F1 mice; the similar administration of chloroform, CHBrCl_2 , CHBr_2Cl and CHBr_3 led to the hypomethylation of the promoter region of the *c-myc* oncogene (292). In the model system employed, TCE, DCA and TCA all promoted liver tumor formation in initiated B6C3F1 mice and produced both global and gene-specific hypomethylation of hepatic DNA (290,291,293). The genes shown to be hypomethylated following treatment with DCE, DCA, and TCA are the protooncogenes *c-jun* and the *c-myc*, with hypomethylation of both genes shown to occur in their promoter regions (289,291,293). The hypomethylation of both oncogenes was accompanied by increased gene expression (290). Liver tumor promotion by DCA and TCA led to increased MTase activity in the resulting liver adenomas and to decreased MTase activity in the adjacent non-tumorous tissue (293). The possible involvement of SAM in the hypomethylation produced by subchronic doses of DCA, TCA and DCE was shown by the observations that the simultaneous administration of methionine reversed both the decrease in *c-myc* and *c-jun* methylation and the accompanying increase in the expression of these genes produced by DCA and TCA (291). Additional links between abnormal methyl metabolism and the toxicity of the polyhalogenated carcinogens are seen in the formation of methylmercapto metabolites of PCBs (294) and of the inhibition by both carbon tetrachloride and chloroform of methionine biosynthesis in a B_{12} -dependent auxotroph of *E.*

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coli (295).

Section V The Metals

Arsenic: Exposure to inorganic arsenic in drinking water affects millions of people, primarily in Asia and South America where the concentration of arsenic considerably exceeds the standard of 50 µg/l, recommended by the U.S. Environmental Protection Agency. Chronic exposure to inorganic arsenic compounds is responsible for the prevalence of various tumors, as well as of other diseases in human beings such as diabetes, cardiovascular and peripheral vascular disease, developmental anomalies, neurological and neurobehavioral disorders, portal fibrosis of the liver, and lung fibrosis (242,297-312) (Table V). Several mechanisms of action have been proposed to explain the observed associations between chronic arsenic exposure and human diseases. For instance, some data suggest that inorganic arsenic increases oncogene expression, cellular proliferation and apoptosis (312-318). In addition, *in vitro* and *in vivo* studies, as well as examination of people exposed to high concentrations of inorganic arsenic in drinking water indicate that this metalloid may intensify toxic effects of other physical and chemical agents, especially by DNA repair inhibition. Inorganic arsenic compounds also cause DNA damage, changes in the cell redox potential, and alter DNA methylation and phosphorylation of cell cycle control proteins (312,318-326).

As mentioned above, perturbation of DNA methylation has been expected as a potential mechanism whereby arsenic could induce human diseases such as cancer. Inorganic arsenic exhibits a complex metabolism. Arsenate is first reduced in the blood and enzymatically in the liver to arsenite, which is then methylated to monomethylarsonic acid and dimethylarsinous acid (DMA) (327). It has been pro-

posed that inorganic arsenicals were the species that accounted for the toxic and carcinogenic effects of this metalloid, and that methylation was a mechanism for detoxification of arsenic. Methylation of inorganic arsenic consumes SAM in the process. Since DNA methyltransferases require this same methyl donor, it is possible that arsenic-induced initiation results from DNA hypomethylation caused by continuous methyl depletion, and that over-methylation of specific CpG sequences may exist over the entire genome in response to arsenite exposure (323). Experiments with rat liver cells showed that whereas transcription of MTase was elevated, its enzymatic activity was reduced following arsenic transformation (326). Of possible relevance is the observation of a chemical reaction between the arsenic metabolite DMA and bacterial MTase (328).

Recent experimental and epidemiological evidence has shown that arsenic biotransformation including methylation capacity may have a role in the development of arsenic-induced DNA damage and diseases (311,329,330). Although it has not been possible to isolate an arsenic methyltransferase in humans, the putative enzyme has been isolated from the liver cytosol of rats; its mRNA could be detected by reverse PCR in human cell lines that methylate arsenite but not in a cell line that does not methylate arsenite (331). In non-human primates, only 4 out of 17 investigated species had arsenite methyltransferase activity *in vitro* suggesting that methylation of inorganic arsenic is not a detoxification mechanism for many non-human primates (332). A recent study suggested that the uptake of dimethylated arsenic by red blood cells is a contributing factor to the animal species differences in the metabolism of arsenic, in addition to the reduction and methylation capacity in the liver (333). Methylated and dimethylated arsenicals that contain arsenic in the trivalent oxidation state are more cytotoxic, genotoxic and potent inhibitors of

the activities of some enzymes than are inorganic arsenicals that contain arsenic in the trivalent oxidation state (329,334). The administration of a methyl-deficient diet to mice, prior to treatment with sodium arsenite, resulted in a hepatic methyl donor deficiency, altered arsenic metabolism and modulated the target organ specificity of arsenic-induced DNA damage with an apparent shift from liver and bladder to skin (335). In methylation defects such as MTHFR deficiency, the concentration of methyl donors is severely reduced, and it has been suggested that this defect may increase the neurotoxicity of arsenic (336). Other methyl-metabolism defects such as impaired folate transport have been suspected to play an important role for arsenic teratogenicity (337). Even more, the eventual involvement of arsenic methylation on the generation of acute and chronic toxicity promoted research on the use of compounds that interfere with arsenic methylation as potential preventive measures (338,339).

Nickel: Nickel is a well-established carcinogen both in humans and in experimental animals (Table V) (340,341). It is also toxic towards the pancreas (Table V). Acute injection of Ni in rats produces hyperglycemia and hypoinsulinemia (340,342), which may be caused by the induction of nitric oxide synthase. Ni also played a role in the development of heart disease (Table V). In rats, arteriosclerotic lesions could be observed 7-9 weeks after an intrarenal injection of Ni₃S₂; hypertension and hyperlipidemia were excluded as contributing causes to the Ni-induced cardiovascular lesions (343). In humans, the serum levels of Ni were found to be elevated in patients with unstable angina or with acute myocardial infarction, but not in patients with atherosclerosis (344). Ni is also teratogenic (Table V). The children born of Ni-exposed mothers exhibited an elevated incidence of malformations, especially cardiovascular de-

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**TABLE V
THE METALS**

PARAMETER	AGENTS			
	As	Ni	Cd	Zn Deficiency
Disease				
Cancer	+ (311-313,316,319,452,453)	+ (340,341)	+(353)	+ (385-392,454)
Pancreatic toxicity	+ (242,301-303,309,312)	+ (341,342,455)	+ (354-360,456)	+ (394-397,401,403,404)
Atherosclerosis	+ (264,298,299,307,308,312,322,453,457)	+ (343,344)	+ (361-368)	+ (398,399,406-411)
Birth defects	+ (337,453,458-460)	+ (345-347)	+(353,373,374,421,461)	+ (412-419,421)
Neurotoxicity	+ (312,336,453)		+ (375-380)	+/- (422-427)
Biochemical alterations				
SAM/SAH	+ (326,327,335,462,463)	- (349)		+ (428,429)
Homocysteine		- (352)		+/- (429,430)
DNA methylation	+ (313,315,316,323,326)	+ (350,351)		+ (429)
MTase	+ (328)	+ (350,351)	+ (384)	+ (384)

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fects (345). The progeny of mice and hamsters treated with Ni manifested several defects including exencephaly and cleft palate (346,347). While we could find no direct evidence of the neurotoxic effects of Ni, there was an association between high soil content of Ni, as well as Zn and Pb, in a high cluster population of multiple sclerosis cases (348).

Compared to other carcinogens, Ni's effects on methylation processes appear somewhat paradoxical (349-352). In rats, Ni treatment reversed both the elevated urinary levels of FIGLU and the decreased hepatic contents of SAM produced by the chronic administration of a folate-deficient diet (349). In vitamin B₁₂-deficient pigs, Ni decreased the elevated levels of serum HCys and

increased the serum and hepatic contents of vitamin B₁₂ (352). In cell studies, Ni induced time-dependent changes in global and gene-specific DNA methylation; the Ni-induced DNA hypermethylation was observed even as the level of MTase was decreased (350,351).

Cadmium: Cd is another metal that is deemed by IARC to be carcinogenic both to man and to animals (353). Cd exhibits multiple toxicities towards the pancreas (Table V). In rats and hamsters it causes hepatization of pancreatic acinar cells (354,355). In rats, subchronic doses of Cd were diabetogenic, causing hyperglycemia and decreased serum insulin levels in otherwise normal rats (356,357) and exacerbating the hyperglycemia and diabetic glomerulopathy in rats with pre-existing diabetes (358,359). In

mice, acute, nontoxic exposure to Cd inhibited pancreatic protease activities (360). The evidence linking Cd exposure with the development of atherosclerosis is quite strong (Table V). Chronic Cd administration led to the formation of atherosclerotic plaques and to hypertension in pigeons (361,362) and to thrombi, altered lipid profiles and atherosclerotic changes in rabbits (363,364). In another study with rabbits, Cd inhibited the atherogenic effects of a high cholesterol diet (365). In a series of human studies (366-368), the tissue levels of Cd correlated well with the degree of atherosclerosis in autopsied subjects, and a significantly elevated incidence of atherosclerosis was detected in subjects living in a specific region of the Netherlands con-

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taminated with Cd and Zn. Consistent with the animal and human data correlating Cd with atherogenesis *in vivo* is the finding that Cd stimulates the proliferation of vascular smooth muscle cells in culture (369,370). Cd has been shown to be teratogenic in a number of animal species, including mice, rats, hamsters, and frogs (Table V). Among the anomalies found were decreased crown-rump length and cleft palate (371,372). Cd's teratogenicity was inhibited by Zn (373). One report indicates that the formation of neural tube defects in human embryos could be correlated with maternal exposure to Cd (374). The neurotoxicity of Cd has been well studied, especially in occupationally exposed populations (Table V). The chief symptoms are peripheral polyneuropathy and neurobehavioral and neuropsychological deficits (375-378). Rats receiving a long-term exposure to Cd in the drinking water developed a peripheral polyneuropathy that was accompanied by myelin degeneration (379). Consistent with these findings was a conclusion from an epidemiologic study showing that an outbreak of 30-40 cases of multiple sclerosis, and other demyelinating syndromes in Galion, Ohio, could be correlated with an excess concentration of heavy metals, particularly of Cd and Cr in the local sewage and river water (380). The inhibition by Cd of superoxide dismutase has been proposed as the mechanism responsible for Cd's neurotoxic effects (381).

We could find no studies showing direct effects of Cd on the levels of SAM, SAH, or HCys or on DNA methylation *in vivo* or *in vitro*. However, synergistic interactions between Cd and hypomethylating environments have been observed. The induction of metallothionein by Cd was markedly enhanced in the livers of rats pretreated with azacytidine and in hepatocytes in culture treated with azadeoxycytidine (382,383). Recent studies have shown that Cd is an effective inhibi-

tor of mammalian MTase and that the kinetics of such inhibition is quite different with DNA samples from the livers of methyl-deficient rats than with DNA samples from control livers (384).

Zinc Deficiency: Zn deficiency appears to play a significant role both in experimental and in human carcinogenesis (Table V). In rats, Zn deficiency has acted as a co-carcinogen, or as a tumor promoter, in the esophagus as well as in other organs (385-389). Zn deficiency has also enhanced the formation of Cd-induced injection site sarcomas and testicular carcinomas in rats (390). Zn deficiency also enhanced the development of tumors induced by the Moloney sarcoma virus (391). Further, Zn deficiency alone has been shown to cause esophageal cancer in rats (388). Two clinical studies have shown that patients with esophageal cancer have decreased levels of Zn in their sera and in other tissues (385,392), suggesting that Zn deficiency may play a similar role in humans as it does in rats. A number of associations have been made between diabetes, pancreatic toxicity and Zn deficiency (Table V). Patients with chronic pancreatitis and diabetes frequently display low serum (393-397) and tissue (398-400) levels of Zn, while low groundwater Zn levels were associated with the eventual development of type 1 diabetes (401). Experimental studies with a diabetes-prone strain of rat showed that Zn supplementation in the diet delayed or prevented the onset of the disease (402). Zn deficiency also led to decreased zymogen granules and increased lipid droplets in rat pancreatic acinar cells both *in vivo* and *in vitro* (403,404).

Zinc deficiency has frequently been linked to atherosclerosis and other forms of heart disease (Table V) (367,398,399,405-411). Most of the evidence supporting a role for Zn deficiency in the development of atherosclerosis is indirect and consists of biochemical markers of oxidative stress *in vivo* or of inflammatory response and increased apop-

toxis in vascular endothelial cells *in vitro* (406,408,410,411). Similarly, the weight of evidence indicates that zinc deficiency can produce birth defects both in animals and in humans (Table V). In pregnant rats, Zn deficiency led to the formation of skeletal malformations, altered pancreatic function and neural tube defects in their embryos (412-414). In humans, zinc deficiency and low maternal zinc levels have been associated with the birth of children with neural tube defects (415,416). In this regard, the impact of the low Zn levels, described above for diabetic patients, has been examined in studies with rats. Pregnant rats with experimentally induced diabetes produced progeny with an elevated incidence of birth defects as well as with diminished fetal liver contents of Zn (417,418), and feeding a Zn-deficient diet to the diabetic dams markedly enhanced the formation of such defects (417). Other studies (419,420) have failed to correlate fetal abnormalities in children with the maternal Zn or folate levels or with Zn supplementation. Finally, Zn itself has shown teratogenic activity toward frog embryos (421). The ties between Zn and neurological disturbances appear to be mixed (Table V). Many studies have examined the correlations between Zn and the demyelinating disease multiple sclerosis (MS). A high exposure to Zn was associated with an increased incidence of MS in an industry-based cluster (422), but the blood levels of Zn in the patients were not found to be significantly different than those of the non-MS workers in the same plant. MS patients typically exhibit higher blood levels of Zn than do control subjects (423). The associations between high Zn levels and neurotoxicity are consistent with the observations that Zn is toxic to neuronal cells *in vitro* and led to the proposal that Zn itself may cause demyelination (424,425). On the other hand, women with MS were found to consume less Zn than did control subjects (426). Zn deficiency has also

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been shown to alter neurotransmitter activity in rats (427).

Zn deficiency alters methyl group metabolism in experimental animals (Table V). In liver perfusion studies with rats fed Zn-adequate and Zn-deficient diets, it was found that the livers of Zn-deficient rats had lower SAM/SAH ratios, produced more HCys and were less capable of enzymatic methylation, including DNA methylation, than were the corresponding Zn-adequate animals (428,429). Other studies showed increased hepatic methionine synthetase activity and reduced plasma HCys and folate in Zn-deficient rats (430,431). The importance of Zn for the normal interaction of the Zn-binding domain of mammalian MTase with its catalytic site has recently been described (432).

Other Metals: Two other metals exhibit many of the same pathologic and biochemical effects described above: lead and cobalt. Both are recognized carcinogens (433,434). A number of cardiovascular abnormalities, including atherosclerosis, have been associated with Pb exposure in humans (367,435). In addition, Pb, similar to HCys *in vivo*, stimulates the proliferation of vascular smooth muscle cells *in vitro* (370). Co appears to exert mixed effects on the cardiovascular system of mammals. In humans, elevated Co exposure resulted in elevated mortality from ischemic heart disease (433). In sheep, it partially prevented the cardiovascular lesions caused by Co and/or vitamin B₁₂ deficiency (436); this finding is probably due to the fact that Co is an essential component of vitamin B₁₂. Co is teratogenic to mice, rats and frogs (437,438). Pb appears to produce neural tube defects in humans (439,440). In a matched case-control study, English districts with high levels of Pb exhibited increased rates of NTDs (439). Another study, comparing the amniotic fluid levels of B₁₂, Ca, methionine, and Pb in NTD and non-NTD pregnancies (440), found: 1) the NTD pregnancies were marked by lower

levels of Ca, methionine, and B₁₂ and higher levels of Pb compared to the non-NTD pregnancies, and 2) a negative correlation existed between Pb and both B₁₂ and methionine. Pb is also a neurotoxicant that can cause hypo- and demyelination (441).

There are a number of interrelations between the toxic metals Pb and Co and the processes of methyl metabolism and methylation. Treatment with SAM protected against Pb toxicity in mice and rats (442,443) and lowered blood Pb levels and raised GSH levels in Pb-intoxicated patients (444). Similarly, dietary methionine protected against the toxic and growth suppressing effects of Pb in chicks (445,446). The effects of Co on methyl metabolism appear to be due less to heavy metal toxicity than to the essentiality of Co in vitamin B₁₂. Thus, in sheep, low Co intake resulted in decreases in the hepatic contents of SAM, of methionine synthetase activity, and of phospholipid methylation (447). Low Co intake in sheep also resulted in hepatotoxicity, low blood levels of B₁₂ and high serum levels of methylmalonic acid and HCys (448); these changes were prevented by vitamin B₁₂ injections. In cattle Co intake was inversely related to serum levels of HCys (449), while in pigs vitamin B₁₂ deficiency and hyperhomocysteinemia could be partially prevented by Co supplementation (352). Biochemical studies showed that Pb inhibited mammalian MTase, but that divalent Co stimulated the activity of MTase from rat kidney, brain and spleen (450). In an *in vitro* enzymatic study, methylcobalamine, the Co-containing cofactor for methionine synthetase, acted as a substrate in DNA methylation by Mtase and was an inhibitor of SAM in that reaction (451).

Section VI Summary

The present review summarized evidence supporting the generalization that physiological methyl group

deficiency and abnormal methylation processes caused by specific categories of chemicals, like those caused by dietary deficiencies and enzymatic defects, produce a pattern of pathological alterations. Such pathologies include cancer, pancreatic toxicity, atherosclerosis, birth defects, and neurotoxicity. The categories of agents that appear to act via abnormal methylation are the antimetabolites of essential methyl intermediates, the anticonvulsants, polyhalogenated compounds and certain metals. It is interesting to note that these agents are thought to act via epigenetic, and not by classical genotoxic, mechanisms. The evidence compiled here presents a reasonable case that the pathologies induced by these chemical categories of agents share common causal mechanisms centered on abnormal one-carbon metabolism.

The evidence presented above showed that representative compounds from each of the categories significantly altered methyl group metabolism, particularly DNA methylation. In addition, the SAM precursors methionine, choline, betaine, and folic acid inhibited many of the toxic and biochemical effects of the representative compounds discussed above. The toxic compounds whose activity was inhibited by the methyl donors included: the antimetabolites ethionine and MTX; the anticonvulsants phenobarbital, diphenylhydantoin, and valproic acid; the polyhalogenated compounds DDT and CCl₄, and the metal Pb (*vide supra*). Also, as noted above, azacytidine inhibited the chemopreventive effects of SAM.

Three, more specific, toxic endpoints of the representative agents included in this study provided additional links with abnormal methyl metabolism: NTDs, demyelination of nervous tissue, and hepatic transdifferentiation of the pancreas. NTDs, demyelination, and pancreatic hepatization have all been described in animals or humans that

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were deficient in methyl donors due to diet, defective enzymes or disease (*vide supra*, (464,465)). The same biological effects have been noted in animals or humans exposed to the chemicals in the various categories of this report. NTDs have been observed following exposure to the anticonvulsants in general, to the polyhalogenated compounds CCl₄ and dioxin, and finally to the metals Pb and Cd, as well as to Zn deficiency (*vide supra*). In addition, the antimetabolite MTX enhanced the formation of NTDs by valproic acid, while azacytidine itself produced NTDs *in vitro* (*vide supra*). Similarly, demyelination, particularly of peripheral nerves, has been observed following treatment with, or exposure to: methotrexate, the anticonvulsants, and the metals Pb, Cd and As ((466) *vide supra*). Hepatization of the pancreas has been induced in rats and hamsters by Cd, by dioxin, and by a regimen that included ethionine ((465) *vide supra*).

The results compiled in the present review may provide useful lessons regarding the mechanism of action of the diverse agents examined. For example, ethionine and azacytidine clearly exhibit tumorigenic activity, while FUDR does not. Ethionine and azacytidine also inhibit SAM-dependent DNA methylation, while FUDR does not appear to do so. These observations indicate that the SAM-dependent methylation may be more directly involved in the carcinogenic process than is the inhibition of thymidylate synthetase and of DNA synthesis. In this regard, the fact that MTX acts to block both DNA synthesis and DNA methylation may explain why it has so seldom been found to exhibit tumorigenic activity. Further evidence on the role of SAM-dependent DNA methylation in carcinogenesis is seen in comparing the tissue specificities of ethionine and azacytidine. Azacytidine exerts its tumorigenicity and DNA hypomethylating activity in several organs; to date, the carcinogenic and

DNA hypomethylating activity of ethionine has been confined to the liver. It is in the liver that ethionine is most readily converted to its hypomethylating metabolite SAE.

Altered methyl metabolism in the liver appears to play a particularly important role in the toxicology of the anticonvulsants and the polyhalogenated compounds. In rodents, both categories of chemicals are hepatotoxic and generally exert their major carcinogenic effects in this organ (*vide supra*). Specific anticonvulsants and polyhalogenated compounds have been effective in decreasing SAM/SAH ratios and increasing the extent of DNA hypomethylation in this organ. Two different mechanisms may be proposed for the diminished availability of SAM in the livers of animals treated with hepatotoxic agents: diminished synthesis and increased utilization. Defects in the expression of the liver-specific methionine adenosyltransferase MAT1A gene, and thus the activity of the corresponding enzyme, have been shown to be a major cause of SAM insufficiency caused by hepatotoxic agents (*vide supra*) (467,468). A second possible mechanism is the drain on hepatic SAM caused by many anticonvulsants and polyhalogenated compounds tested in rodents (*vide supra*, (469)); SAM is used for the synthesis of the phosphatidylcholine required in the induction of the hepatic cytochrome P450s caused by these agents. Such biosynthesis also results in the elevated formation of SAH.

The demonstration of the validity of either mechanism is not without implications for the regulatory agencies. As has frequently been noted (470), liver tumor formation in rats and mice is responsible for a high proportion of the carcinogenic compounds detected by the NTP. Many of these compounds also appear to act as secondary agents or epigenetic carcinogens. Demonstration that the tumorigenic activity of such compounds towards the liver is dependent upon an altered methylation status *in vivo* would strengthen

the hypothesis that these compounds do in fact have a threshold of activity.

The hypomethylating environment produced in the liver by the anticonvulsants and the polyhalogenated compounds may affect other organs as well. A high degree of correspondence was seen between the decreased SAM/SAH ratios in liver and the rise in serum HCys by the same compounds (Tables III and IV). The elevated serum levels of HCys may thus contribute both to the formation of a hypomethylating environment and to the development of extrahepatic pathologies caused by the chronic administration of the anticonvulsants and the polyhalogenated compounds.

Time did not permit a consideration of other categories of chemical agents, which also alter methyl group metabolism and cause pathological changes. These include some electrophilic carcinogens, nitrous oxide, sulfa drugs, and alcohol. The same constraints did not permit a consideration of alternate mechanisms of toxicity not centered on abnormal methylation processes. The authors are aware of the fact that in some biological systems, particularly in rodent liver, methyl deficiency leads to oxidative damage and, correspondingly, oxidative damage can lead to methyl insufficiency and abnormal DNA methylation ((468,471), *vide supra*). In this case the alternate potential mechanisms are not mutually exclusive.

In conclusion, the results indicate that abnormal methylation processes caused by chemical agents, like those caused by dietary deficiencies or by metabolic defects, can be associated with a general pattern of specific pathological effects.

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Glossary

Antimetabolite: A structural analogue of a nutrient or metabolite which prevents the proper utilization of the nutrient or metabolite in biological systems.

DNA hypomethylation: The process by which nuclear DNA, either global or gene-specific, loses a portion of its normal complement of 5-methyldeoxycytidine as a fraction of total cytidine residues. Its opposite is DNA hypermethylation, which

describes an *increase*, compared to normal, in the fraction of 5-methyldeoxycytidine in DNA.

Epigenetic: The characteristic describing an agent which produces a stable, heritable change in the ge-

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netic and/or phenotypic expression in cells without a direct, chemical reaction of the agent or its metabolites on cellular DNA. Also used to describe the alteration thus produced.

FIGLU: Abbreviation for the his-

tidine catabolite formiminoglutamic acid, the elevated presence of which in urine indicates a folate deficiency.

Methyltransferase: An enzyme which transfers a methyl (CH₃-) group from S-adenosylmethionine (SAM) to one of any number of low and high molecular weight sub-

strates; e.g., DNA methyltransferase (MTase).

Polymorphism: That characteristic of a specific gene which, because of relatively minor mutations, exists in multiple forms within a single species or population. Also applied to the enzyme product of such a gene.

The Authors



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Lionel A. Poirier is a research chemist within the Division of Molecular Epidemiology (DME) at the National Center for Toxicological Research (NCTR). He received his Ph. D. at the McArdle Laboratory of the University of Wisconsin for his work in identifying the activation pathway for the aromatic aminoazo dye carcinogens. He began his studies on abnormal methyl metabolism in carcinogenesis during a four-year stage at l'Institut du Cancer de Montréal, in Canada. He then moved to the National Cancer Institute (NCI) in Bethesda, Maryland, where he set up NCI's mutagenicity prescreens for chemical carcinogens. At NCI he also showed that dietary methyl group deficiency alone caused cancer, that such cancer formation was preceded by abnormal DNA and gene methylation and that both resulted from the hypomethylating environment caused by the deficient diet. In 1987, he joined the NCTR to establish the Division of Nutritional Toxicology and to ex-

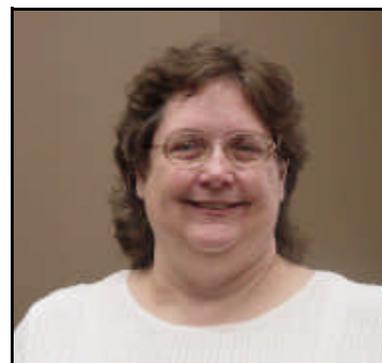
pand his studies on abnormal methylation processes and cancer. He joined DME in 1996 and began clinical studies on the role of methyl group deficiency development of diabetes and atherosclerosis. In 1997, he received the DeWitt S. Goodman Award from the American Association for Cancer Research for his contributions to the fields of nutrition and cancer and cancer prevention.



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Luis A. Herrera is a researcher at the Unidad de Investigación Biomédica en Cáncer, Instituto de Investigaciones Biomédicas-Instituto Nacional de Cancerología, México. He received his Ph.D. in biomedical research at the Universidad Nacional Autónoma de México in 2000 under the supervision of Dr. Patricia Ostrosky. Since 1986, he has been actively engaged in the area of genetic toxicology. Dr. Herrera has also been a visiting scientist at the Institut für Humangenetik der Universität Er-

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Carolyn K. Wise

Carolyn K. Wise is an FDA Research Chemist in the Division of Molecular Epidemiology at the National Center for Toxicological Research (NCTR), Jefferson, Arkansas. She graduated with a B.A. in Chemistry from Southwestern College in Winfield, Kansas, in 1968, and with a M.S. in Chemistry from Ft. Hays State University in Hays, Kansas, in 1974. After teaching chemistry for several years in the

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Two years later, she joined the group of Dr. Lionel Poirier to investigate the role of abnormal methyl metabolism and DNA methylation in cancer. Since then she has expanded her studies to include heart

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To conduct peer-reviewed scientific research that provides the basis for FDA to make sound science-based regulatory decisions and to promote the health of the American people through enforcement and compliance. This involves fundamental and applied research specifically designed to define biological mechanisms of action underlying the toxicity of products regulated by the FDA. This research is aimed at understanding critical biological events in the expression of toxicity and at developing methods to improve assessment of human exposure, susceptibility and risk and apply these scientific findings to FDA's pre-market application review and product safety assurance effort.

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