CURRENT AWARENESS DOCUMENT

NITROSAMINE CONGENER ALKYLAZOXYMETHANOL-DERIVED ALKYLATING AGENTS: CYCASIN AND RELATED COMPOUNDS

CARCINOGENICITY AND STRUCTURE ACTIVITY
RELATIONSHIPS. OTHER BIOLOGICAL PROPERTIES.
METABOLISM. ENVIRONMENTAL SIGNIFICANCE.

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5.3.2.2 Cycasin and Related Compounds

5.3.2.2.1 Introduction

Cycasin is a member of a group of naturally occurring azoxyglycosides produced by the palm-like cycad plants. Belonging to the ancient family Cycadaceae of the Gymnospermae, cycads are thought to represent an intermediate form in the phylogenetic evolution of plants from ferns to flowering plants. Currently, there are about 100 species of cycads, identiifed as members of 9 genera (Bowenia, Ceratozamia, Cycas, Dioon, Encephalartos, Macrozamia, Microcycas, Stangeria, and Zamia)*, growing in tropical and subtropical regions around the world. Various parts of these plants were and still are used as a source of food in some parts of the world, particularly during periods of natural disasters.

The historical development of early cycad research was eloquently described in a 1963 review by Whiting (1). The toxicity of cycads has long been known by the natives who have developed elaborate procedures to remove the poisonous substances. Explorers and early settlers were frequently the victims of toxic effects of cycads as they experimented with these unfamiliar native foods. In 1770, members of Captain Cook's crew were reported to have become violently ill after consuming cycad (Cycas media) nuts during their voyage to Australia. Outbreaks of cycad poisoning and heavy losses of livestocks occurred in Australia between the 1880's and the 1930's as the rapid growth of the sheep and cattle industry led to the expansion of grazing into

*Some taxonomists separate an additional genus (Lepidozamia) from Macrozamia and classify cycads into three families: Cycadaceae (1 genus, Cycas); Stangeriaceae (1 genus, Stangeria); and Zamiaceae (includes all the other genera).

the natural habitat of cycads. Scientific interest in cycad research began in the late 19th century, but it was not until 1941 when Cooper (2) succeeded in isolating a glycoside from Macrozamia spiralis. The compound, named macrozamin, was subsequently structurally identified as methylazoxymethanol-β-D-primeveroside (3, 4). Soon afterwards, Riggs (5, 6) and Nishida et al. (7) isolated cycasin from cycad plants found in Guam (Cycas circinalis L.) and Japan (C. revoluta Thunb) and identified its structure as methylazoxymethanol-β-D-glucoside.

During the mid-1950's, clinical observations of the natives of Guam (the Chamorros, a Malayo-Polynesian group) showed an unusually high incidence of a neurological disease diagnosed as amyotrophic lateral sclerosis (8). Based on evidence collected from reports of neurological disorders in cycad-poisoned cattles and sheeps (1), Whiting suggested a possible link between native consumption of cycad and the disease. An attempt to experimentally prove such a link using rats was unsuccessful. Instead, however, Laqueur et al. (9) found that rats fed a crude meal derived from cycad (C. circinalis L.) seeds from Guam developed a variety of tumors. Subsequent studies using purified cycasin and cycasin-free cycad meal (see Section 5.3.2.2.3.1) established cycasin as the active carcinogenic principle in the crude cycad meal. Methylazoxymethanol, the aglycone of cycasin, has been shown to be the proximate carcinogen of the azoxyglycoside (see Section 5.3.2.2.3.2). Products derived from a variety of other cycad species (including the Encephalartos spp. which produce macrozamin) also display carcinogenic properties. In addition, cycasin is teratogenic, when given to pregnant rats, and neurotoxic when given to various newborn animals (see Section 5.3.2.2.2.2). These findings have stimulated a wave of interest in cycad research culminating in a succession of six conferences between 1952 and 1972. The proceedings of three of these con-

ferences were published (10-12). Cycasin and related compounds have continued to attract considerable scientific interest which is reflected by the large number of recent reviews (13-19).

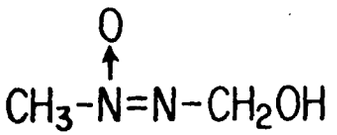
5.3.2.2.2 Physicochemical Properties and Biological Effects

5.3.2.2.2.1 PHYSICAL AND CHEMICAL PROPERTIES

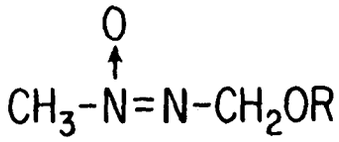
The physical and chemical properties of cycasin and related compounds were extensively studied by several groups of investigators in Australia and Japan (2-7, 20-25). The chemical structures and some physicochemical properties of these compounds are given in Tables XXXVII and XXXVIII, respectively. Cycasin is soluble in water or aqueous ethanol, sparingly soluble in pure ethanol, and insoluble in most organic solvents. The ultraviolet absorption spectrum of cycasin shows a pronounced maximum around 217 nm, and an inflexion at 275 nm. The infrared spectrum displays a strong absorption band around 1,540 cm^{-1}, characteristic of aliphatic azoxy compounds. Cycasin is readily hydrolyzed at 100°C with 0.1 N HCl, yielding methanol, formaldehyde, nitrogen and glucose. Mild alkali treatment also leads to decomposition, yielding formic acid, cyanide, nitrogen, ammonia and methylamine (7). Essentially identical physicochemical properties have been reported for other azoxyglycosides such as macrozamin (4, 7), and the neocycasins A, B, C, and E (20-23).

Methylazoxymethanol (MAM), the common aglycone of all the above azoxyglycosides, is a colorless liquid with an amine-like odor; it has a density of 1.208 and boils at 51°C under reduced pressure (0.56 mm Hg). It is totally miscible with water and aqueous ethanol, and is slightly soluble in chloroform and ether. Both pure and aqueous solutions of MAM are unstable at room temperature; approximately 12.5% of pure MAM and 21.3% of aqueous MAM decom-

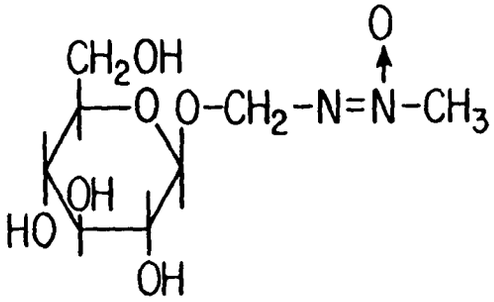
Structural Formulas of Cycasin and Related Compounds



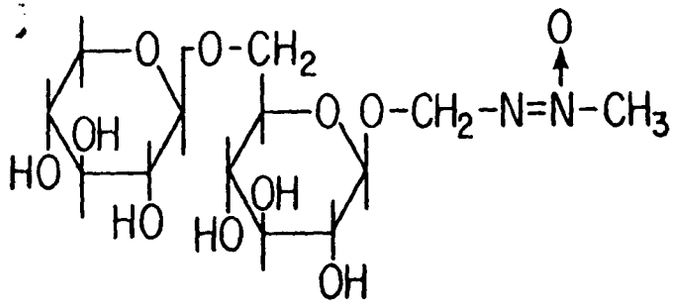
Methylazoxymethanol
(MAM)



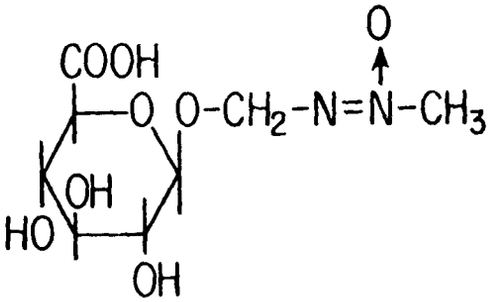
MAM Acetate (R=CH_3CO-)
MAM Benzoate (R=C_6H_5CO-)



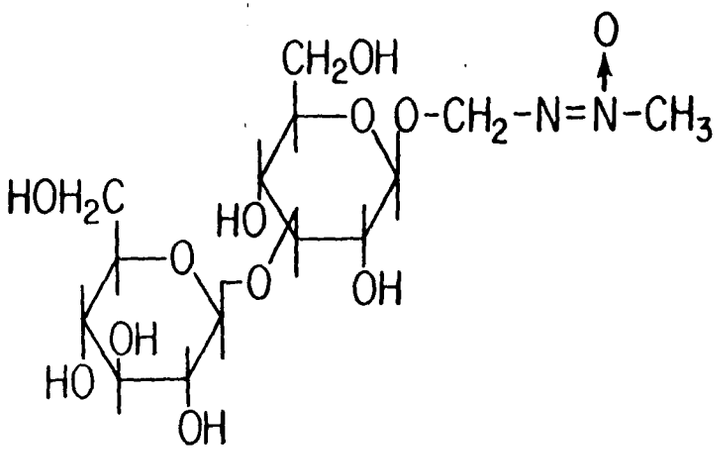
Cycasin



Macrozamin



MAM-β-D-glucosiduronic
acid



Neocycasin A

Table XXXVIII
Physicochemical Properties of Cycasin and Related Compounds

Compound	Synonyms and abbreviations	m.p.	b.p.	Optical rotation	UV absorption λ_{max}	UV absorption logϵ	IR- λ_{max}	References
Cycasin	MAM- β-D-glucoside; β-D-glucosyloxyazoxymethane	144-145°C	--	$[\alpha]_D^{28} = -41.3^\circ$	217 nm	3.8	1,540 cm^{-1}	(7)
Neocycasin A	3-O- β-D-glucosylcycasin; β-laminaribosyloxyazoxymethane; MAM- β-laminariboside	162-163°C	--	$[\alpha]_D^{29} = -35.1^\circ$	217 nm	3.9	1,537 cm^{-1}	(20)
Neocycasin B	6-O- β-D-glucosylcycasin; β-gentiobiosyloxyazoxymethane; MAM- β-gentiobioside	173-174°C[a]	--	$[\alpha]_D^{18} = -37.6^\circ$	215 nm	3.81		(21)
Neocycasin E	4-O- β-D-glucosylcycasin; β-cellobiosyloxyazoxymethane; MAM- β-cellobioside	156-158°C	--	$[\alpha]_D^{15} = -29.2^\circ$	215 nm	3.75		(23)
Macrozamin	6-O- β-D-xylosylcycasin; β-primeverosyloxyazoxymethane; MAM- β-primeveroside	199-100°C	--	$[\alpha]_D^{23} = -74^\circ$	217 nm	3.8	1,538 cm^{-1}	(4,7, 26)
Methylazoxymethanol (MAM)	Hydroxymethylazoxymethane	1-3°C	51°C (0.6 mm Hg)		215 nm	3.94	1,515 cm^{-1}	(25)
MAM acetate	MAM Ac; MAMA	--	191°C		215 nm	3.93	1,522 cm^{-1}	(25)
MAM benzoate	MAMB	66°C			230 nm	4.3	1,517 cm^{-1}	(25)
MAM- β-D-glucosiduronic acid	MAM glucuronide	109-110°C					1,540 cm^{-1}	(27)

[a] Acetate form.

pose after 5 and 5.6 hrs, respectively (25). Heat, acid and alkali greatly accelerate the rate of decomposition. Approximately 75% of MAM decomposes in aqueous solution by heating at 75°C for 30 min, and close to 100% when heated at 100°C for 10 min (25). Under physiological conditions (37°C; pH 7-8), the half-life of MAM in aqueous solution is about 11.5 hours (28). The half-life is considerably shorter in alkaline solutions, decreasing to 2.8 hours at pH 10. The ester of MAM with acetic acid has similar physical properties as MAM but is considerably more stable; MAM acetate can withstand decomposition in aqueous solution when heated at 75°C for 30 minutes (25). The benzoic acid and β-D-glucosiduronic acid esters of MAM are both solid at room temperature, with melting points of 66°C and 109-110°C, respectively (25, 27).

5.3.2.2.2.2 BIOLOGICAL EFFECTS OTHER THAN CARCINOGENICITY

Toxicity. The toxicology of cycasin and cycad products was the subject of several extensive reviews (1, 16, 29) and several international conferences (10-12). The acute LD_{50} data on cycasin and related compounds are summarized in Table XXXIX. Cycasin is acutely toxic to rodents when administered by the oral route, but nontoxic when injected intraperitoneally (9, 25, 31, 33), suggesting that enzymatic hydrolysis by the intestinal bacterial flora is required for the toxic action of cycasin. This is supported by the finding that both the aglycone of cycasin, methylazoxymethanol (MAM) and MAM acetate are toxic in rats by intraperitoneal injection producing essentially the same toxic effects as orally administered cycasin (25, 36). The principal target organs of the acute and subchronic toxic action of cycasin and MAM are the liver, and the brain (in developing animals). The hepatotoxic effects observed in rats given cycasin or MAM acetate include loss of cytoplasmic basophilia, focal cellular necrosis, pyknosis of nuclei and cytoplasmic eosinophilia, progressing eventually to hemorrhagic centrilobular necrosis (13, 16, 36).

Table XXXIX
Acute Toxicity of Cycasin and Related Compounds

Compound	Species and Route	LD_{50} (mg/kg)	Reference
Cycasin	Mouse, oral	500; 1,000	(30, 31)
	Rat, oral	270; 562	(32, 33)
	Hamster, oral	<250	(30)
	Guinea pig, oral	≤20; 1,000	(30, 31)
	Rabbit, oral	30	(30)
	Fish, oral	LC_{50} = 20 ppm[a]	(34)
Macrozamin	Rat, oral	218	(26)
Methylazoxymethanol (MAM)	Rat, i.p.	LD_{lo} = 35[b]	(25)
MAM Acetate	Mouse, i.v.	25-30	(35)
	Rat, i.v.	35-50	(35)
	Rat, i.p.	90	(36)
	Chick embryo[c]	405 μl	(37)
MAM-β-D-glucosiduronic acid	Rat, i.p.	>1,000	(27)

[a]Lethal concentration causing 50% kill.

[b]Lowest lethal dose.

[c]Injected into yolk sac of the egg.

Newborn animals are extremely sensitive to the neurotoxic effects of cycasin and MAM acetate. Neonatal exposure of mice (38, 39), rats (40-43), hamsters (40, 44-46), ferrets (40, 47, 48), rabbits, cats and dogs (40) to cycasin or MAM acetate causes defects during the maturation of the cerebellum. The most notable abnormalities in rodents include misalignment or disorientation of Purkinje cells, deletion of granule cells and cerebellar dysfunction (41, 42, 49). The locomotive activity of the afflicted animals may be severely affected (43, 44, 48) resulting in gait disturbances and ataxia. In addition to cerebellar malformation, severe atrophy of the retina has been observed in hamsters (46) or rats (50) treated with MAM acetate and in mice and rats treated with cycasin (30) within a few days after birth.

Besides experimental animals, farm animals grazing on leaves or seeds of Cycas or Macrozamia plants (1) and humans consuming improperly prepared foodstuffs derived from Cycas plants (1, 51) were reported to develop various symptoms of acute intoxication. Outbreaks of cycad poisoning of cattle and sheep occurred frequently in Australia between 1879 and 1930 resulting in considerable livestock losses. The most commonly observed acute symptoms were severe gastrointestinal disturbances and partial paralysis of the hind legs (rev. 1). Cycad plants have long been known to be poisonous by natives who have developed methods of detoxifying cycad products. Cases of human poisoning and casualties usually occurred during periods of extreme food shortage or as a result of lack of knowledge when improperly prepared cycad products were consumed (1, 51). Hirono et al. (51) reported 17 cases of fatalities as a result of ingestion of cycad products on Miyako Island (Okinawa, Japan). The latent period for the appearance of symptoms was 12-24 hours. The majority of the patients died within several to 20 hours after the first sign of intoxication. The symptoms which occurred suddenly included headache, stomachache,

nausea, vomiting, convulsion and loss of consciousness. In most patients, swelling of the liver was observed.

Mutagenicity. The mutagenicity of cycasin and related compounds has been tested in a variety of systems ranging from bacteria, yeasts, plants, Drosophila, cultured mammalian cells to whole mammals. Table XL summarizes the results of the Ames Salmonella tests of these compounds. This subject has been reviewed in-depth by Morgan and Hoffman (18, 19). With one exception (56), cycasin was consistently nonmutagenic in in vitro studies using bacteria (52-55), yeasts (64) and cultured mammalian cells (65), regardless of the presence or absence of a metabolic activation system of mammalian hepatic origin (S-9 mix). However, cycasin is readily rendered mutagenic by enzymatic deglucosylation using β-glucosidase (52, 54, 58, 65), fecalase from human fecal extract (55) or rat intestinal flora (64), suggesting that the release of methylazoxymethanol (MAM) is a requisite for the expression of the mutagenic activity of cycasin. This is supported by the findings that neocycasin A and MAM-β-D-glucosiduronic acid (the glucuronide of MAM) are also nonmutagenic per se but are activated by incubation with fecalase or β-glucuronidase, respectively, which release MAM (27, 54, 55). In host-mediated assays using Salmonella typhimurium G46 strain as an indicator organism, cycasin is mutagenic when administered orally, but inactive when administered parenterally. The mutagenicity of cycasin is abolished by pretreating the host (mouse) with an antibiotic that kills the intestinal bacterial flora (57). Among higher plants, cycasin displays mutagenic activity in beans (Phaseolus vulgaris L.) inducing chlorophyll mutations and morphological alterations (66), and towards root cells of onion (67) or Zamia integrifolia (68) causing chromosome aberrations. Both bean seeds and Zamia plants contain β-glucosidase. In contrast, mutagenicity tests using Drosophila yielded

Table XL
Mutagenicity of Cycasin and Related Compounds in the Ames Test

Compound	With or without S-9	Preincubation with			Host-mediated
		β-glucosidase	fecalese[a]	β-glucuronidase	
Cycasin	- (52-55); + (56)[b]	+ (52,54)	+ (55)		+ (57)[c]
Neocycasin A	- (55)		+ (55)		
Macrozamin	- (55,56)		- (55)		
Methylazoxymethanol (MAM)	+ (52,54,58)				
MAM Acetate	+ (53,54,59-63)				+ (59,62)
MAM- β-D-glucosiduronic acid	- (27,54,55)		- (55)	+ (27,54)	

[a]Contains both β-glucosidase and β-glucuronidase activity.

[b]Positive in the presence of S-9.

[c]Negative if the host was pretreated with an antibiotic.

negative results (69, 70) consistent with the failure to detect β-glucosidase activity in homogenates of Drosophila (69). In experimental animals, orally administered cycasin induces strand breakage in rat liver DNA (71) and causes fragmentation in mouse liver DNA (72).

Consistent with the conclusion that MAM is the proximate or ultimate mutagen of cycasin, MAM and MAM acetate display mutagenic activity in a variety of test systems. In most cases, an exogenous metabolic activation system is not required for mutagenicity. Using the Ames tests, numerous investigators (52-54, 57-60, 62) have shown that MAM or MAM acetate induces base-pair substitution mutations in the absence of metabolic activation. Inclusion of S-9 mix has a variable effect ranging from enhancement of mutagenicity (54) to activation to a frameshift mutagen (63, 73) or to reduction of mutagenicity (60, 73). Methylazoxymethanol also induces base-pair substitution mutation in Escherichia coli WP2 trp^- in the absence of metabolic activation (74). In yeasts, MAM acetate is both mutagenic and recombinogenic (75-78). The yeast strains tested include Saccharomyces cerevisiae D3, D7, T2, and XV185-14C. With one exception (75), exogenous metabolic activation system is not required for expression of optimal activity of MAM acetate. In contrast to the lack of mutagenicity of cycasin in Drosophila, MAM acetate is clearly mutagenic in the fruit fly (69, 70, 79), indicating that the absence of β-glucosidase activity accounts for the lack of mutagenicity of cycasin in the fruit fly.

Evidence indicating the mutagenicity of MAM or MAM acetate in mammalian cells is abundant. In in vitro studies, these compounds produce a variety of genetic damages as indicated by the induction of mutation (80-83), chromosome aberrations (84, 85), sister chromatid exchanges (86-88), and unscheduled DNA synthesis (89-91). The ability of MAM acetate to induce mutation in V79

Chinese hamster cells is enhanced by tumorigenesis promotors, such as phorbol esters, and cocarcinogens, such as C_6 to C_{16} linear alkanes (80). The comutagenic activity of these compounds correlates with their promoting or cocarcinogenic activity. In in vivo studies, MAM acetate treatment was shown to cause DNA damages (92-94), chromosome aberrations (95, 96) and an increase in the frequency of sister chromatid exchanges (88) in liver, bone marrow, spleen and other cells.

Teratogenicity. Methylazoxymethanol (MAM) or MAM acetate is teratogenic in at least three animal species, with the central nervous system being the principal target tissue. Spatz et al. (97) demonstrated first the teratogenicity of MAM using the golden hamster. A single intravenous injection of 20-23 mg/kg MAM to pregnant hamsters on the 8th day of gestation led to malformations of the brain, eye and extremities in all of the living fetuses examined on the 12th day of gestation. The gross malformations observed include hydrocephalus, microcephalus, cranioschisis, exencephaly, spina bifida, rachischisis, anophthalmia, microophthalmia, and oligodactyly. No attempt was made to observe the development of the malformed fetuses beyond the 12th day of gestation. The finding of malformations of the brain and the spinal cord in this study suggests rapid action of MAM on embryonic development, since the neural tube of hamster fetus closes by the 9th day of gestation. A preliminary histological study of MAM-treated hamster fetuses by Laqueur (see 16) indicated necrosis of cells in the embryonic region known to be involved in the closure of the neural tube.

Cerebral malformation is the principal teratogenic effect of MAM or MAM acetate in the rat and in the ferret. Studies by various investigators (16, 98-101) have consistently shown that a single administration of 20-30 mg/kg MAM or MAM acetate to pregnant rats of various strains (Fischer, Osborne-

Mendel, Sprague-Dawley, Long-Evans) on the 15th day of gestation induces a high incidence of microencephaly in the offspring. The earliest recognizable lesion (necrosis of undifferentiated cells in the region destined to produce the final cerebral cortex) leading to microencephaly was visible within 24 hours after administration of MAM (see 16). Neurochemical studies (102-104) revealed that MAM is selectively cytotoxic to the cortical γ-aminobutyric acid (GABA)-containing neurons resulting in a severe loss of GABAergic neurons. In contrast, the noradrenergic neurons (102) and oligoendroglia (105) are minimally or not affected. Injections of MAM acetate (15 mg/kg) into pregnant ferrets on days 27-32 of gestation (the full gestation period of this species is 42 days) result in malformations of the cerebral hemisphere (microencephaly, lissencephaly, etc.), whereas similar treatment in later periods (days 37-42) produces malformations of the cerebellum (40, 47, 48). As adults, the ferrets with cerebral malformation (particularly those with lissencephaly) display learning difficulties, whereas those with cerebellar abnormalitites show no cognitive deficits despite being ataxic (48). It has been suggested that the MAM-induced lissencephalic ferrets may be a useful experimental model for the study of lissencephaly, which also occurs in humans as a severe birth defect with unknown etiology.

Besides prenatal exposure, perinatal or neonatal exposure to cycasin or MAM acetate can also cause abnormalities in the development of the nervous system in several species. The most notable effects are malformation of the cerebellum and atrophy of the retina (see above under Toxicity).

5.3.2.2.3 Carcinogenicity and Structure-Activity Relationships

5.3.2.2.3.1 CARCINOGENICITY OF CYCASIN AND CYCAD PRODUCTS

Since the first report in 1963 of carcinogenicity of crude cycad (Cycas circinalis L.) meal in rats, cycasin or cycad products have been found to be carcinogenic in 7 of 8 animal species tested. The cycad products thus far examined include nuts, kernel, husk, leaves, extract or flour derived from 10 different species of cycad plants belonging to 3 genera that grow in Guam, Japan, Australia and Central, East and South Africa. Most of the earlier carcinogenicity studies on cycasin and cycad products have been reviewed by Laqueur and Spatz (13, 16, 29) and by Hirono (17). The major findings of these and the more recent studies are summarized in Table XLI. Cycasin has also been used in several syncarcinogenesis studies which are discussed in Section 5.3.2.2.3.4.

Studies by Laqueur, Spatz, Hirono and various other investigators have clearly established cycasin as a potent carcinogen in the rat. The carcinogenicity of cycasin is dependent on the route of administration, the dosing regimen and the age of the animals. In virtually all studies using adult animals, the oral route is the only effective route of administration. As substantiated by the evidence analyzed below and in Section 5.3.2.2.3.2, enzymatic deglucosylation by the intestinal bacterial flora to the aglycone, methylazoxymethanol (MAM), is the requirement for the carcinogenicity of cycasin and cycad products. The principal carcinogenicity target organs of cycasin in the rat are the liver, the kidney and the intestines. Liver tumors are induced principally by long-term administration of relatively low doses of cycasin (9, 32, 106, 107, 109, 114, 115), whereas short-term administration of higher doses of cycasin predominantly induces kidney tumors (33, 110, 112-

Table XLI
Carcinogenicity of Cycasin and Cycad Products in Various Animal Species

Species and Strain	Cycad Products Tested[a]	Route	Principal Organs Affected	References
Rat, Osborne-Mendel or Sprague-Dawley	Crude cycad (*C. circinalis* L.) meal or cycasin	oral	Liver, kidney, intestines	(9, 106, 107)
Rat, Osborne-Mendel	Guamanian home-made cycad (*C. circinalis* L.) flour	oral	None	(108)
Rat, Sprague-Dawley	Cycad (*C. circinalis* L.) husks	oral	Liver, kidney	(109)
Rat, Sprague-Dawley	Cycad (*C. circinalis* L.) husks	oral	Kidney, liver	(110)
Rat, Sprague-Dawley (germ-free)	Cycasin	oral	None	(106, 111)
Rat, Sprague-Dawley	Cycasin	oral	Liver, kidney, intestines, mammary gland	(32, 112)
Rat, Osborne-Mendel	Cycasin	oral	Kidney, intestines, liver, lung, brain	(33)
Rat, Wistar	Cycasin	oral	Kidney	(113)
Rat, ACI	Cycasin	oral	Testis	(112)
Rat, Sprague-Dawley	Zamia (*Z. debilis*) leaves	oral	None	(110)
Rat, Osborne-Mendel	Zamia (*Z. floridana*) tubers	oral	Kidney, liver, colon	(16)
Rat, unspecified	Flour from *E. hildebrandtii* nuts[b]	oral	Liver, kidney, lung	(114, 115)

Table XLI (continued)

Species and Strain	Cycad Products Tested[a]	Route	Principal Organs Affected	References
Rat, Wistar	South African cycad (*Encephalartos* spp.)[b,c] cones	oral	Kidney, liver	(116, 117)
Rat, Fischer (newborn)	Cycasin	s.c.	Kidney, liver, lung, intestines, brain	(33)
Rat, Wistar (newborn)	Cycasin	s.c. or i.p.	Kidney, liver	(118)
Mouse, C57BL/6	Cycasin	oral	Liver, lung, kidney (low incidence)	(119)
Mouse, C57BL/6 (newborn or suckling)	Cycasin	s.c.	Liver, hematopoietic system	(119, 120)
Mouse, dd (newborn)	Cycasin	s.c.	Lung, liver	(121)
Mouse, C57BL	Cycad (*C. circinalis* L.) nut extract	skin painting[d]	Liver, kidney	(122)
Hamster, Syrian golden	Cycasin	oral	Liver, intestines, hematopoietic system	(123)
Hamster, Syrian golden (newborn)	Cycasin	s.c.	Liver	(123)
Guinea pig	Crude cycad (*C. circinalis* L.) meal	oral	Liver	(124)

Table XLI (continued)

Species and Strain	Cycad Products Tested[a]	Route	Principal Organs Affected	References
Rabbit	Crude cycad (C. revoluta) extract	oral	Liver	(125)
Chicken	Cycad (C. circinalis L.) nut husk or kernel	oral	None	(126)
Fish (Brachydanio rerio)	Cycad (C. circinalis L.) nut meal or cycasin	oral	Liver	(34)
Monkey (rhesus, cynomolgus or African green)	Combination of cycad nut meal, cycasin and synthetic methylazoxymethanol acetate	oral	Liver, kidney	(127)

[a]The genera of the cycad plants are: C. = Cycas; E. = Encephalartos; Z. = Zamia.

[b]Known to contain macrozamin (methylazoxymethanol- β-primeveroside)

[c]The species identified include: E. umbeluziensis; E. villosus, E. lebomboensis, E. laevifolius and E. lanatus.

[d]Topical application to chemically induced skin ulcers.

115). Intestinal tumors, which are almost exclusively located in the large bowel (cecum, colon, rectum), appear to be less dependent on the duration of exposure (29, 33, 107).

The dose-response relationship in the induction of tumors by cycasin has been studied by Hirono et al. (33). A single intragastric dose of 100 mg/kg cycasin was sufficient to induce tumors in 4 of 13 rats. Two higher doses (250 or 500 mg/kg) induced tumors in 100% of the treated animals whereas doses higher than 750 mg/kg were acutely toxic (LD_{50} = 562 mg/kg). The optimal dose for tumor induction consistent with the greatest number of survivors was 250 mg/kg body weight. Continuous administration of low doses (4 mg/kg) of cycasin failed to induce tumors in the liver, kidneys or intestines but accelerated the development of tumors known to occur spontaneously (e.g., mammary gland tumors in female Sprague-Dawley rats, testicular interstitial cell tumors of male ACI rats) in rats (112). Hepatic tumors were induced in Sprague-Dawley rats at a high incidence (80-90%) by continuous feeding of 10 mg/kg cycasin for the entire lifespan of the animals (32).

The carcinogenicity of orally administered cycasin in adult rats is greatly dependent on the presence of intestinal bacterial flora. Laqueur et al. (106, 111) showed that cycasin was not carcinogenic when administered to "germ-free" rats. This gnotobiotic protective effect is due to the absence of intestinal flora (such as Lactobacillus salivarius and Streptococcus fecalis), which contain β-glucosidase that can deglucosylate cycasin to MAM. Cycasin ingested by germ-free rats is excreted quantitatively and unchanged in the urine and feces (128, 129). The demonstration of potent carcinogenicity of MAM and MAM acetate in both conventional and germ-free rats (see Section 5.3.2.2.3.2) confirms the indispensable, key role of β-glucosidase in the activation of cycasin.

Studies with newborn rats further substantiate this mechanism. In contrast to the lack of carcinogenicity of parenterally administered cycasin in adult rats, subcutaneous or intraperitoneal injection of cycasin to newborn rats leads to the induction of a variety of tumors (33, 118). Consistent with this, the enzyme assay of skin homogenates from fetal, newborn and adult rats by Spatz (130) showed that neonatal rats (6 days before birth to 2 days after birth) contain significantly high levels of β-glucosidase activity; however, the activity of the enzyme falls sharply as the rats reach adulthood. Newborn rats are highly susceptible to the carcinogenic effect of cycasin. In one study (33), a single subcutaneous dose of 2.5 mg/rat was sufficient to induce tumors in 46/55 rats, whereas in another study (118) a single intraperitoneal or subcutaneous dose of 90 mg/kg cycasin induced tumors in 6/14 rats.

The cycad products which have been shown to be carcinogenic in the rat are derived from Cycas circinalis L., (9, 106, 107, 109, 110), Zamia floridana (16) and six different species of Encephalartos plants (114-117). Cycas circinalis L. is indigenous in Guam; the carcinogenic azoxyglucoside it contains has been identified as the glycoside of methylazoxymethanol, named cycasin. A sample of home-made cycad flour from Guam was found to be cycasin-free and was not carcinogenic in the rat (108). The processing procedure of the natives can, apparently, remove cycasin and eliminate the carcinogenic potential of the cycad product. Besides C. circinalis, rats fed diets containing 2% or 3% ground Z. floridana tubers had tumor incidences of 12% and 60%, respectively. The organotropism and the histological types of tumors induced by Z. floridana are similar to those of cycasin (16). The chemical structure of the carcinogenic azoxyglycoside(s) present has not been elucidated. Dried leaves of Z. debilis have been tested by Hoch-Ligeti et al. (110). Despite the presence of cycasin and macrozamin (at concentrations

higher than those of cycad husks), Zamia leaves were not carcinogenic in the rat. The reason for the inactivity is not clear. Various species of Encephalartos plants have been found in Central, East and South Africa. Mugera and Nderito (114) found that rats fed diets containing 5% or 10% flour derived from E. hildebrandtii nuts for 6 to 10 months developed a variety of tumors in the liver, kidneys and lungs; on short-term exposure (4-7 days), however, the kidneys were the only affected organ (115). Tustin (116, 117) fed rats the outer flesh and/or kernel of five species of South African cycad plants (E. umbeluziensis, E. villosus, E. lebomboensis, E. laevifolius and E. lanatus) and found them to be all carcinogenic, inducing mainly kidney and liver tumors. The principal azoxyglycoside present in E. hildebrandtii (131) and E. lanatus (26) has been identified as macrozamin. In view of the wide occurrence of azoxyglycosides in various cycad plants, it is suggested that all unprocessed cycad products should be considered potentially hazardous until proven otherwise.

In addition to the rat, cycasin and cycad products are carcinogenic in mice, hamsters, guinea pigs, rabbits, fish and monkeys. Cycasin is weakly carcinogenic in adult C57BL/6 mice; a single intragastric dose of 300-1,000 mg/kg induced tumors (hepatoma, fibroma, lung and kidney adenoma) in only 4 of 35 mice (119). Like newborn rats, newborn mice are much more susceptible; a single subcutaneous injection of 500 mg/kg cycasin was sufficient to induce tumors in 100% of both dd (121) and C57BL/6 (120) mice. This susceptibility decreases rapidly as the mouse ages. The tumor incidences of C57BL/6 mice given a single s.c. dose of 500 mg/kg cycasin on day 1, 2, 4, 7 or 14 after birth were 100, 100, 90, 73 and 15.7%, respectively (120). This age-dependence may reflect a rapid postnatal decline in β-glucosidase activity in subcutaneous tissue of mice. Such a change has indeed been demonstrated by Spatz (130) using rat skin homogenates.

Besides purified cycasin, in a preliminary report by O'Gara et al. (122), an aqueous extract of C. circinalis nuts was shown to induce liver and kidney tumors in 3 of 11 C57BL mice following repeated topical applications to skin ulcers artificially induced with 10% croton oil in mineral oil. This finding is of special interest because of earlier reports on medicinal use of cycad paste for the treatment of skin ulcers (1).

Regarding other animal species, Hirono et al. (123) showed that cycasin is a weak to moderately active carcinogen in Syrian golden hamsters. Single or repeated oral administration of 100-150 mg/kg cycasin to adult hamsters and single subcutaneous injection of 200-600 mg/kg cycasin to newborn hamsters induced mainly intrahepatic bile duct carcinomas; the incidence was in the range of 12-17%. In addition, a few colon tumors, malignant lymphoma and an occasional kidney or gall bladder tumor were observed. Spatz (124) maintained guinea pigs on diets containing 5% cycad (C. circinalis L.) meal for several 5-day periods. Nine of 27 guinea pigs which survived for more than 44 weeks developed liver tumors (4 hepatocellular carcinomas and 5 bile duct tumors). Watanabe et al. (125) administered to each of 15 rabbits 1 ml of cycad (C. revoluta) extract (containing 16.6 mg cycasin) once a week for 27-33 weeks; 7 of 9 rabbits that survived over 200 days developed malignant liver tumors identified as hemangioendotheliomas. White Leghorn chickens appear to be the only animal species thus far reported to be refractory to the carcinogenic effect of cycad products. Sanger et al. (126) found no tumors in chickens fed 0.5% cycad (C. circinalis) kernel for 68 weeks. In a preliminary communication, Stanton (34) reported that continuous feeding of a 50% dietary supplement of cycad (C. circinalis) nut meal to a species of aquarium fish (Brachydanio rerie) led to the induction of liver foci, many showing the histological characteristics of malignant neoplasms. The carcinogenic poten-

tial of cycasin has also been investigated in nonhuman primates (rhesus, cynomolgus and African green monkeys) by Sieber et al. (127). Eighteen monkeys were given a combination of oral administration of crude cycad meal for 10 months and purified cycasin for 3 years, followed by intraperitoneal injections of synthetic MAM acetate for 33-92 months. At the time of the report, 2 of 9 necropsied monkeys had developed hepatcellular carcinomas; one of them also had an intrahepatic bile duct adenocarcinoma, a kidney carcinoma, as well as adenomatous polyps of the colon. For comparison, only 4 of 143 historical controls developed tumors. In view of the proven carcinogenicity of MAM in monkeys (see Section 5.3.2.2.3.2), it is not possible to assess from this study (127) whether oral administration of cycasin alone is carcinogenic in monkeys.

5.3.2.2.3.2 CARCINOGENICITY OF METHYLAZOXYMETHANOL ACETATE AND RELATED COMPOUNDS

The evidence that methylazoxymethanol (MAM), the aglycone of cycasin, is the proximate carcinogen of the azoxyglycoside was first presented by Matsumoto and Strong (24). An ethylether-soluble fraction of cycad (C. circinalis L.) nuts, when fed to three rats for a period of 9 months, at a level equivalent to 2.5% cycad nut in the feed, induced hepatomas. The fraction was found to contain free MAM. Laqueur and Matsumoto (107, 132) provided further evidence that, unlike cycasin, MAM is carcinogenic when injected into rats. The carcinogenicity target organs of MAM are the same as those of cycasin (see Table XLII). Both conventional and germ-free rats are susceptible to the carcinogenic effect of MAM (111). Free MAM is also carcinogenic in Syrian golden hamsters and in guinea pigs (13). Of 64 hamsters that received a single or multiple (up to 5) i.p. or i.v. injection(s) of 10-20 mg/kg MAM, forty developed adenocarcinomas of the large intestine, nine had

Table XLII
Carcinogenicity of Free Methylazoxymethanol (MAM), Synthetic MAM Acetate and Related Compounds

Species and Strain[a]	Route	Principal Organs Affected	References
(A) Free Methylazoxymethanol (MAM)			
Rat, --	oral	Liver	(24)
Rat, Fischer	i.p.	Intestines, kidney, liver	(107, 132)
Rat, Sprague-Dawley (conventional or GF)	i.p.	Intestines	(111)
Hamster, Syrian golden	i.p. or i.v.	Colon, liver	(13)
Guinea pig	i.p.	Liver, nasal cavity	(13)
(B) Methylazoxymethanol Acetate (MAM Acetate)			
Rat, Sprague-Dawley (GF)	oral	Kidney, liver, intestines	(111)
Rat, Fischer 344	oral	Colon, stomach, kidney, Zymbal's gland, liver	(133)
Rat, Sprague-Dawley	i.v. or i.p.	Intestines, kidney, liver	(35, 134, 135)
Rat, Sprague-Dawley (GF)	i.p.	Intestines, liver, kidney	(111)
Rat, Wistar (newborn)	i.p.	Kidney	(118)
Rat, Buffalo	i.p.	Colon	(136)
Rat, Sprague-Dawley (GF)	s.c.	Intestines, liver	(111)
Rat, Sprague-Dawley or Lobund Wistar	s.c.	Intestines, liver	(137)
Rat, Donryu	intrarectal	Large intestine, kidney, liver	(138)

Table XLII (continued)

Species and Strain	Route	Principal Organs Affected	References
Mouse, CD-1	i.v.	None	(35, 134)
Mouse, SWR/J or C57BL/6J	s.c.	Colon, rectum, anus	(139)
Mouse, AKR/J	s.c.	Hematopoietic system	(139)
Mouse, BALB/c	Perianal painting	Anus, vascular system	(140, 141)
Guinea pig	i.p. or s.c.	Liver, vascular system	(13)
Medaka (Oryzias latipes)	oral	Liver	(142)
Monkey (rhesus, cynomolgus or African green)	i.p.	Liver, kidney, esophagus, intestines	(127)
(C) Methylazoxymethanol Benzoate (MAM Benzoate)			
Rat, Wistar (weanling)	oral	Intestines	(118)
Rat, Wistar (newborn)	s.c.	Kidney, colon	(118)
Rat, Wistar	s.c.	Kidney, liver	(118)
(D) Methylazoxymethanol- β-D-glucosiduronic acid (MAM glucuronide)			
Rat, Sprague-Dawley	oral	Colon, small intestine, liver, kidney	(143)
	i.p.	Small intestine (marginal activity)	(143)
Rat, Sprague-Dawley (GF)	oral or i.p.	None	(143)

[a]GF = germ-free

liver tumors, and five had gall bladder or intraorbital carcinomas. Among the 20 guinea pigs given a total dose of 9-19.5 mg MAM intraperitoneally, 14 developed tumors which included 10 liver tumors, 4 squamous cell carcinomas or hemangiosarcomas of the nasal cavity, 2 lung adenomas and 1 jejunal adenocarcinoma. In both experiments, none of the control hamsters or guinea pigs developed tumors.

The role of MAM as the proximate carcinogen of cycasin and related azoxyglycosides, as well as of 1,2-dimethylhydrazine and azoxyalkanes (see Section 5.2.1.3.4, Vol. IIIA), and its possible usefulness as a model experimental carcinogen have created considerable interest for the study of the compound. However, because of the instability of free MAM, most of the studies have been carried out with the much more stable synthetic compound, MAM acetate (see Table XLII). The synthetic MAM acetate has been shown to be carcinogenic in 6 strains of rats via 5 different routes of administration inducing tumors mainly in the large intestine, kidney and liver. Some minor strain and sex differences have been noted. Laqueur et al. (111) treated germ-free Sprague-Dawley rats with MAM acetate (total dose 12.5-13.7 mg administered over a period of 14 to 21 days) via oral, i.p., and s.c. routes. The i.p. route was the most effective, inducing colon carcinomas in 4 of 4 rats and hepatic bile duct adenomas in 2 of 4 rats. Rats that received MAM acetate in the diet developed more kidney tumors and less intestinal tumors than those treated parenterally. McConnell et al. (133) conducted a dose-response relationship study of MAM acetate in Fischer 344 rats of both sexes. The animals were given 5 doses each of 0, 0.2, 1.0, 4.0, 7.5, 15 or 30 mg/kg MAM acetate via gavage at 2-week intervals for 14 months; the resulting tumor incidences were 1.7, 1.9, 0, 3.7, 8.6, 37.5 and 75%, respectively. The male rats developed more colon tumors at lower doses than the female rats. Tumors of Zymbal's

gland and of the kidneys were found exclusively in male and female rats, respectively. Zedeck et al. (35, 134, 135) administered a single i.v. or i.p. dose of 35 mg/kg MAM acetate to Sprague-Dawley rats and induced tumors in 13/20 and 29/33 rats, respectively. Most of the tumors developed in the large intestine; however, some were found in the small intestine, kidney and liver. Hayashi et al. (118) found the kidney to be virtually the only target organ in Wistar rats that received a single i.p. dose of 20-30 mg/kg MAM acetate within 24 hours after birth. In Buffalo strain rats, however, the descending colon was the predominant carcinogenicity target organ of repeated i.p. injections of 20 mg/kg MAM acetate (136). In a comparative study by Pollard and Zedeck (137), all animals in groups of Sprague-Dawley and Lobund Wistar rats which received 10 weekly s.c. doses of 30 mg/kg MAM acetate developed colon tumors. However, the Sprague-Dawley rats were much more susceptible than the Lobund Wistar rats as indicated by the substantially greater tumor multiplicity (20.4 vs. 3.1). Narisawa and Nakano (138) infused 1 mg MAM acetate into the rectum of Donryu rats once a day for 7-21 days. Fifty-four weeks after the initiation of carcinogen administration, 22 of 24 treated rats developed multiple carcinomas in the large intestine. Seven rats also had nephroblastomas and two had hepatocellular adenomas.

Much greater strain differences in the response of mice to MAM acetate administration have been noted. Zedeck et al. (35, 134) found no tumors in CD-1 mice following injection of a single i.v. dose of 35 mg/kg of the compound. On the other hand, Diwan et al. (139) induced tumors in 20/21 SWR/J, 21/26 C57BL/6J and 22/28 AKR/J mice by injection of 10 s.c. doses of 20 mg/kg MAM acetate; the corresponding incidences in control mice were 2/20, 0/22 and 17/24, respectively. Most of the tumors in the SWR/J and C57BL/6J mice were in the large intestine. In contrast, AKR/J mice were resistant to the colo-

rectal carcinogenic effect of MAM acetate; the only type of tumor detected was leukemia, which is known to occur spontaneously in this strain. In BALB/c mice, topical painting of 0.5 mg MAM acetate at the anal region, three times weekly for 24 weeks, led to the induction of tumors of the perianal sebaceous gland in 23 of 24 (96%) male and 16 of 30 (59%) female mice. Vascular tumors of the liver and fat tissue of the abdominal cavity also developed in 16 of 24 male and 3 of 30 female mice. It was suggested that anatomical difference in the external genitalia in the two sexes contributed to sex differences described above.

Among other animal species tested, guinea pigs, medaka and monkeys are all highly susceptible to the carcinogenic effects of MAM acetate. Laqueur and Spatz (13) reported that in a group of 25 guinea pigs, given repeated s.c. or i.p. injections of MAM acetate (total dose 44-77 mg), all developed hepatocellular carcinomas, and 14 of them also had vascular tumors. None of the 59 controls bore tumors. In the study of Aoki and Matsudaira (142), who introduced MAM acetate into the aquarium water of medaka (Oryzias latipes) at levels of 0.1-3 ppm for periods ranging from 1 to 120 days, over 80% of the surviving fish developed liver tumors 2-5 months after the commencement of the treatment. No tumors were found in 74 control fish within the same period. Sieber et al. (127) dosed 10 old world monkeys (rhesus, cynomolgus, and African green) with weekly i.p. injections of 3-10 mg/kg MAM acetate for life beginning within 72 hours after birth. Six of the 8 monkeys, that died after 45-89 months of treatment, developed tumors which were identified as 5 hepatocellular carcinomas, 3 esophageal squamous cell carcinomas, 2 renal carcinomas, and 1 multifocal adenocarcinoma of the small intestine. Liver biopsy of one of the two remaining survivors revealed a tumor mass identified as a well-differentiated hepatocellular carcinoma.

Two other synthetic derivatives of MAM have been tested for carcinogenic activity (see Table XLII). Hayashi et al. (118) induced tumors in 7 (6 kidney, 1 colon) of 13 Wistar rats that survived a single s.c. dose of 40-60 mg/kg MAM benzoate. By oral route (4 x 5 mg via stomach tube), MAM benzoate induced tumors in both the small and the large intestine of 5 of 6 treated rats. Subcutaneous injections (2 x 5 mg) of the compound to young adult rats led to the development of one hepatoma and one nephroblastoma in the 2 surviving animals.

In an effort to test the hypothesis that biliary excretion may play a role in the intestinal carcinogenic action of cycasin and its aglycone (see 144), MAM-β-D-glucosiduronic acid was synthesized and bioassayed (143). Conventional and germ-free Sprague-Dawley rats were given a single or 4 weekly i.p. or oral doses of 70.5 mg/kg of the compound. Twenty-seven of the 30 orally treated conventional rats developed tumors, which were predominantly in the large intestine and occasionally in the liver and kidney. Among the i.p. dosed conventional rats, only two adenomas were found in the small intestine. The MAM glucuronide was completely inactive in germ-free rats by either oral or i.p. route. The study did not support the biliary excretion hypothesis. Mammalian β-glucuronidase was apparently unable to hydrolyze MAM-β-D-glucosiduronic acid to release MAM.

5.3.2.2.3.3 TRANSPLACENTAL CARCINOGENESIS BY CYCASIN AND ITS AGLYCONE

The transplacental carcinogenic action of cycasin was first shown by Spatz and Laqueur (145). Pregnant Sprague-Dawley rats were fed diets supplemented with 1, 3 or 5% cycad meal (which contained 3% cycasin) during the 1st, 2nd or 3rd week of gestation or throughout the gestation. Of the 81 offspring that survived more than 6 months, 15 (18.5%) had tumors. These included

mesenchymal tumors in the jejunum in 4 rats from the same litter (exposed *in utero* during days 2-5 of gestation), brain tumors (gliomas) in 5 rats (from 5 different litters) and a variety of other tumors (mostly exposed during days 14-18 of gestation). To determine the influence of the period of fetal development at the time of carcinogen exposure on the location and types of tumors, Laqueur and Spatz (13, 146) gave pregnant Fischer rats a single i.p. or i.v. dose of 20 mg/kg MAM or MAM acetate at various days of gestation. Among 340 offspring examined, 42 (12.3%) had tumors. Most of the tumors developed in the lungs, brain, kidneys and intestines. As many as 19 of these 42 tumor-bearing animals were among the progeny of 9 mothers who had received the carcinogen on the 21st day of pregnancy. These rats accounted for 10 of the 16 pulmonary tumors and 6 of the 7 gliomas found. Thus, the last day of gestation was not only the most susceptible period but also reflected a heightened sensitivity of the pulmonary and cerebral tissues to the carcinogenic action of MAM. In a more recent study by Kalter *et al.* (101), MAM acetate caused microencephaly (*see* Section 5.3.2.2.2.2) but produced no neurogenic tumors in Sprague-Dawley rats, when injected intraperitoneally at a single dose of 20 or 30 mg/kg on the 15th day of gestation. When administered together with N-ethyl-N-nitrosourea (ENU; a potent transplacental carcinogen, *see* Section 5.2.1.2.3.6), MAM acetate inhibited rather than potentiated the transplacental carcinogenic action of ENU. The site of tumor inhibition by MAM acetate was confined to the brain, coinciding with the site of teratogenic damage by the compound. The inhibitory effect of MAM acetate was attributed partly to the destruction of ENU-sensitive embryonic brain cells and partly to other mechanisms, such as blocking of molecular target sites denying access to ENU (101).

The evidence that cycasin and its aglycone, MAM, can indeed cross the placental barrier was demonstrated by Spatz and Laqueur (147). Both cycasin and MAM were detected in 15-day-old rat fetuses 3-24 hours after a single oral administration of cycasin to pregnant rats. Cycasin and MAM were also found in the maternal mammary glands and in suckling rats indicating that newborn animals can be exposed to cycasin through lactation. This finding is of particular importance because newborn rats (and possibly other newborn animals) contain β-glucosidase (130) and are, thus, highly susceptible to the carcinogenic action of cycasin. Up to 20% of the MAM can be recovered from hamster fetuses 10-30 minutes after a single i.v. injection of 2.5-20 mg MAM to pregnant hamsters on day 11 of gestation. Owing to the high chemical reactivity of the compound, only a trace was found after 3 hours and none after 4 hours (147). Using ^{3}H-labeled MAM acetate, Nagata and Matsumoto (148) showed that MAM crossed the placenta in rats and became covalently bound to fetal DNA, RNA and proteins.

5.3.2.2.3.4 MODIFICATION OF CARCINOGENESIS BY CYCASIN AND MAM ACETATE

Cycasin acts synergistically with several chemical carcinogens and tumorigenesis promotors. Mori and Hirono (149) fed Sprague-Dawley rats a coffee solution (equivalent to the amount normally consumed by humans) for 480 days and/or administered a single intragastric dose of 150 mg/kg cycasin on the 121st day of the experiment. Twelve of 35 rats that received both coffee and cycasin developed tumors at various sites with the colon and the rectum being the most affected sites. For comparison, only one of 18 rats treated with cycasin alone had a kidney tumor and no tumors were found in 18 rats given coffee alone. The investigators attributed the potentiating activity of the coffee solution to its cocarcinogenic constituents (e.g., chlorogenic acid). Uchida and Hirono (150) showed that chronic administration of pheno-

barbital (0.05% in diet) enhances the incidence of liver tumor (from 9% to 63.6%) in female ACI rats given a relatively low (marginally carcinogenic) dose of 100 mg/kg cycasin. The tumor incidences at other sites, or in male rats, were not significantly affected and phenobarbital alone was not carcinogenic. This finding gives further support to the body of evidence showing that phenobarbital is an effective promotor of hepatocarcinogenesis (see Section 5.2.1.7.11 in Vol. IIIA). Davis et al. (151) demonstrated that cycasin (160 ppm in cycad nut meal) acted synergistically with dipentylnitrosamine (1,500 or 2,000 ppm in diet) in inducing liver tumors in Fischer 344 rats. Davis and coworkers hypothesized that dipentylnitrosamine-induced hyperplastic nodules can resist the cytotoxic effect of cycasin and progress rapidly toward hepatomas while other areas of the liver are cytotoxically affected.

Methylazoxymethanol (MAM) acetate has been extensively used as a model chemical carcinogen in the study of various modifying factors and in the development of new chemotherapeutic agents. As may be anticipated, two antioxidants, butylated hydroxyanisole (152, 153) and selenium (154, 155), inhibit the carcinogenicity of MAM acetate toward the colon of rats and mice, while high-fat diet (156), marginally lipotrope-deficient diet (157), and surfactants (158) have the opposite effect. Colostomy (establishment of an artificial anus by an opening into the proximal end of the colon) had no significant effect of MAM acetate-induced carcinogenesis in the distal end of the colon of rats indicating that the carcinogen can reach the intestinal mucosa via the vascular system as well as via the fecal stream (159). Partial hepatectomy 24 hours prior to the administration of MAM acetate increases only slightly the incidence of liver tumors, suggesting that both dividing and resting liver cells are susceptible to MAM acetate carcinogenesis (160). Chronic admini-

stration of hydrogen peroxide (1.5% in the drinking water) before and after a single intraperitoneal injection of MAM acetate substantially increases the incidence of tumor in duodenum (161); the mechanism of this unusual potentiation remains to be studied. Pyrazole, an inhibitor of alcohol dehydrogenase, significantly inhibits MAM acetate-induced colon/intestine carcinogenesis (162, 163) in rats, implicating the involvement of the enzyme in the activation of the carcinogen. In contrast to the colon, pyrazole administration enhances MAM acetate-induced kidney and skin carcinogenesis (163). It has been suggested that rat kidneys contain a choline dehydrogenase which can activate MAM acetate and is not inhibited by pyrazole; furthermore, inhibition of liver and colon alcohol dehydrogenase by pyrazole causes a shunting of MAM acetate toward the kidneys and other organs (163, 164). Methylazoxymethanol acetate-induced colon carcinogenesis has been used as a model system for testing chemotherapeutic agents. Drugs which have been shown to be effective in inhibiting this tumor growth or causing tumor regression include indomethacin (165, 166), hydrocortisone (165), 4'-deoxyrubicin (167), 5-fluorouracil (167) and piroxicam (168). Dietary restriction also inhibits MAM acetate-induced colon carcinogenesis; however, it is effective only if it is implemented immediately after administration of the carcinogen (169).

5.3.2.2.4 Metabolism and Mechanism of Action

The metabolism and mechanism of action of cycasin and its aglycone, methylazoxymethanol (MAM) have been extensively studied. The known metabolic pathways are outlined in Fig. 10. Several lines of evidence concur that enzymatic deglucosylation of cycasin to MAM is the first and obligatory step in the metabolic activation of cycasin to genotoxic and toxic intermediate(s). Cycasin is toxic (31) and carcinogenic (9, 106, 107) in adult rats after oral administration but is apparently innocuous by intraperitoneal

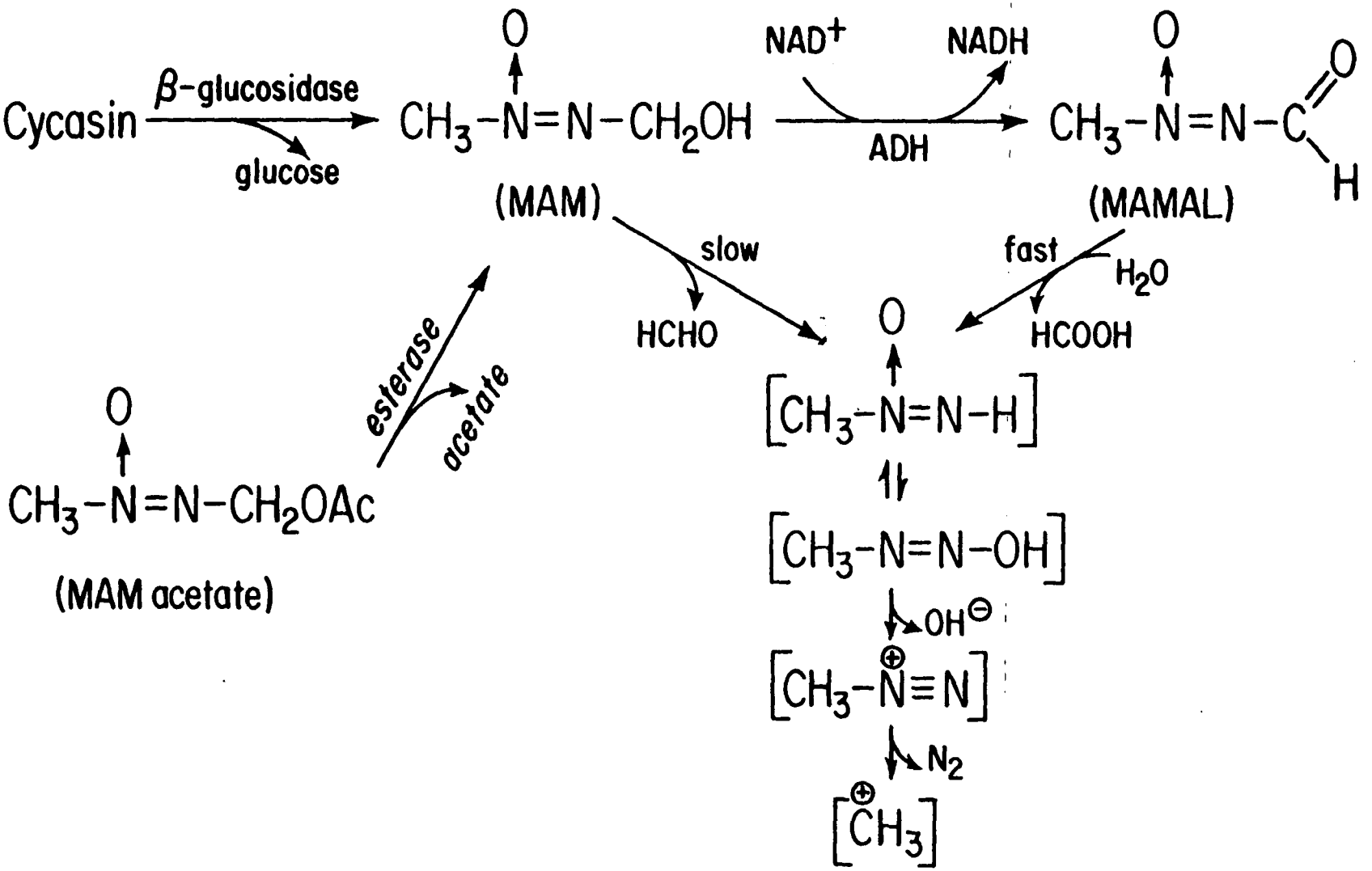


Fig. 10. Proposed metabolic pathways of cycasin and methylazoxymethanol acetate. Abbreviations: MAM, methylazoxymethanol; ADH, alcohol dehydrogenase; MAMAL, methylazoxymethanal.

injection. This dramatic difference in activity appears to be due to metabolism in the intestine, because almost 100% of the parenterally administered cycasin is excreted unchanged in the urine of rats, while only 30-60% is excreted by rats which received cycasin by intragastric administration (25). This is supported by the finding that germ-free rats, which are resistant to the toxicity and carcinogenicity of cycasin (106, 111), also fail to metabolize ingested cycasin (128). Establishing an intestinal bacterial flora in germ-free rats by oral administration with Streptococus fecalis (which has β-glucosidase activity) confers to the rats the ability to metabolize cycasin and susceptibility to the toxicity of the compound (129). Both conventional and germ-free rats are susceptible to the toxicity and carcinogenicity of MAM, the aglycone of cycasin, irrespective of the route of administration (see Section 5.3.2.2.3.2).

Studies by Hirono and associates (33, 119-121, 123) demonstrated that, unlike adults, newborn animals are susceptible to carcinogenicity of parenterally administered cycasin. Susceptibility rapidly decreases as the animal ages. Laqueur and Spatz (13, 146) found that rats are most sensitive to the transplacental carcinogenic action of cycasin when administered one day before birth. These observations can be best explained by the ontogenic development of the mammalian β-glucosidase. Spatz (130) detected -glucosidase activity in the skin (as homogenates) of fetal and neonatal rats from 15 days before birth to 30 days after birth; activity peaked around the time of birth (2 days prenatal to 6 days postnatal) and declined sharply afterwards. Matsumoto et al. (27) found that the β-glucosidase activity in the small intestine of preweanling rats reaches a maximum on the 15th postnatal day and decreases steadily thereafter.

Further support for the crucial role of enzymatic deglucosylation in the metabolic activation of cycasin is provided by various mutagenicity studies (see Section 5.3.2.2.2.2). Cycasin is nonmutagenic in virtually all *in vitro* studies using bacteria, yeasts or cultured mammalian cells, but is activated by β-glucosidase, fecalase or whole intestinal bacteria flora. In host-mediated assays, cycasin is mutagenic only by oral administration to the host; activity is abolished by pretreating the host with an antibiotic which wipes out or drastically curtails the intestinal flora. Among higher plants and insects, cycasin displays mutagenic or clastogenic activity only in cells or organisms which contain β-glucosidase activity. On the other hand, MAM or MAM acetate is mutagenic in a wide variety of test systems often without further metabolism.

Methylazoxymethanol is a relatively unstable compound which readily decomposes to yield formaldehyde, nitrogen and methanol (see Section 5.3.2.2.2.1). Under physiological conditions, MAM spontaneously hydrolyzes in aqueous solution with a half-life of about 11.5 hours (28). The chemically more stable synthetic derivative, MAM acetate, is expected to be readily converted to MAM after *in vivo* deacetylation by esterases. For example, serum cholinesterases have been shown to hydrolyze MAM acetate (170). Free MAM is a good alkylating agent upon decomposition. p-Chlorobenzoic acid, acetic acid, phenol and the guanine moiety of DNA and RNA are methylated by incubation with MAM at 37°C for several hours (28, 171). The biochemical action of MAM is strikingly similar to that of dimethylnitrosamine (172). Hydroxymethylmethylnitrosamine, the unstable, metabolically activated intermediate of the nitrosamine (see Vol. IIIA, p. 253) and MAM are isomers and it has been suggested (173) that the two compounds produce the same transient reactive intermediates. As in the case of dimethylnitrosamine, the idea that diazomethane

may be a possible reactive decomposition product of MAM has been discarded, because hydrolysis of MAM in the presence of D_2O does not yield the anticipated deuterated methanol (174). Methylcarbonium ion and methyldiazonium hydroxide are considered to be the probable methylating intermediates.

Investigations by Zedeck and associates indicate that further metabolism of MAM (see Fig. 10) to its aldehydic form, methylazoxyformaldehyde or methylazoxymethanal (MAMAL) may play an important role in the activation of the compound, in addition to the products generated by the spontaneous decomposition of MAM. Following the suggestion by Schoental (175) that MAM may be activated through oxidation to MAMAL by alcohol dehydrogenase (ADH), Zedeck and associates (28, 162, 176, 177) have demonstrated that MAM can indeed serve as a substrate for this cytosolic enzyme. The reaction requires NAD^+ or $NADP^+$ as a cofactor. Tissues which are sensitive to the toxic and carcinogenic effects of MAM (e.g., liver, colon, cecum) contain high levels of NAD^+-dependent ADH activity, whereas those which are relatively resistant to MAM (e.g., jejunum, ileum) contain little ADH activity. Furthermore, treatment of rats with pyrazole, an inhibitor of ADH, protects the animals against the toxicity (162, 176) and colonic carcinogenicity (155, 162, 163) of MAM. However, pyrazole treatment was found to enhance rather than to inhibit the renal and skin carcinogenicity of MAM (163). This apparent paradoxical effect can be partly explained by the finding of Tan et al. (164) that kidneys (and liver) contain another dehydrogenase (choline dehydrogenase) that oxidizes MAM and is not inhibited by pyrazole. The inhibition of ADH in the colon and liver by pyrazole is also expected to make more MAM available to act upon the kidneys and other organs. The exact role of MAMAL as a reactive intermediate of MAM and cycasin is not clearly understood. Feinberg and Zedeck (28) reported that MAMAL spontaneously decomposes to yield methylating inter-

mediates at a substantially faster rate than does MAM. Thus, MAMAL is likely to be a better alkylating agent than MAM. Furthermore, it has been suggested that MAMAL may act as a cross-linking agent (175) or directly react with the amino group of macromolecules to form a Schiff base or an acylated (N-formyl) derivative (28); however, evidence for such adduct formation is still lacking.

Biliary excretion of glucuronidated MAM was at one time hypothesized to account for the predilection of MAM for inducing intestinal tumors. This hypothesis has been tested by several groups of investigators, using different approaches yielding consistently unsupportive results. Matsumoto (144) injected synthetic glucuronide of MAM (MAM-β-D-glucosiduronic acid) into rats and was unable to recover the compound in the bile. Rats injected with free MAM also did not excrete the glucuronide in the bile. Cannulation of the bile ducts of rats had no effect on MAM-induced inhibition of DNA synthesis in the epithelium of various segments of the intestines (177). Yet, this site-specific biochemical event was well correlated with the eventual emergence of tumors. Matsubara et al. (159) showed that colostomy (see Section 5.3.2.2.3.4) had no significant effect on MAM acetate-induced colon carcinogenesis, indicating that the carcinogen can reach colon via vascular system as well as via fecal stream.

The mechanism of carcinogenic action of cycasin and its aglycone is not clearly understood but is generally believed to involve covalent binding of alkylating intermediate(s) to cellular macromolecules as the first step. 7-Methylguanine has been isolated from the hydrolysates of both DNA and RNA after incubation with MAM at 37°C for 16 hours (171). Like several other colon carcinogens, MAM acetate binds to cellular DNA and protein in explant cultures of rat colon (178). In vivo methylation of nucleic acids in several other target organs of cycasin and MAM -- the liver and kidney of adult rats (172) and the brain of rat fetuses (148) -- have also been demonstrated.

The early biochemical and cell morphological effects of MAM have been extensively studied. Inasmuch as a single administration of cycasin or MAM acetate can result in tumor induction, events occurring immediately after exposure may be of crucial importance to the initiation of carcinogenesis. Zedeck and associates (134) reported that MAM inhibited DNA synthesis in the three principal target organs (liver, kidney, intestine) of the carcinogen in rats within a few hours after treatment. No such activity was detected in CD-1 mice which are resistant to the carcinogenic effect of MAM acetate. Nucleolar structural alterations (occurring within 15 minutes after treatment and persisting for months thereafter), inhibition of RNA (both nuclear and nucleolar) and protein synthesis have also been noted in rat liver (35). Mitotic abnormalities were evident in hepatic cells 7 days after treatment when the rate of DNA synthesis had already returned to normal level. The number of polyploid cells increased significantly and remained elevated for as much as one month after treatment (95). Various segments of rat intestine displayed differences in acute response (inhibition of DNA synthesis and mitosis) to MAM acetate treatment. The segments which are the most acutely affected (e.g., colon, cecum, duodenum) are also the sites of eventual tumor emergence (177). It is of interest to note that MAM acetate causes inhibition of DNA synthesis in human colon mucosa in organ culture (179), suggesting that the human colon may be susceptible to the carcinogen.

The mechanism of inhibition of macromolecular synthesis by MAM has been investigated. Grab et al. (180) found no significant change in the template capacity of hepatic DNA chromatin from MAM-treated animals but noted conformational changes in some "aggregate" enzyme preparations. They suggested that the induction of such changes in hepatic nuclear protein may result in decreased RNA synthesis. The studies of Yu et al. (181), however, indicated

that MAM acetate inhibits RNA synthesis by both impairing the chromatin template function and selectively inhibiting RNA polymerase II. The impairment of template function may be a direct result of DNA methylation or a consequence of MAM acetate-induced chromatin condensation. The selective inhibition of RNA polymerase II appears to result from direct modification of existing enzyme to a catalytically deficient state. The possible mechanism of hepatic protein synthesis by MAM acetate has also been studied (182). No significant alterations of ribosomal subunits and of initiation factors were found suggesting that the inhibition may result from an alteration of cytoplasmic mRNA and its association with ribosomes.

5.3.2.2.5 Environmental Significance

The environmental occurrence of cycasin and related compounds, the human uses of cycad plants, and their potential health hazards have been discussed in a variety of review articles (1, 15-18, 144). Cycasin, macrozamin and other azoxyglycosides occur in the seeds, stems, roots and leaves of cycad plants which are indigenous in the tropical and subtropical regions around the world and occasionally in temperate zones such as in Florida, Japan and Australia. Among the 9-10 genera of cycads identified, the most widely distributed genus is Cycas. The growth zone of this variety extends from East Africa and Madagascar across the Indian Ocean to the Mariana Islands (Guam) and Japan. Zamia plants are located in Florida, the Carribean Islands, Mexico and the northern parts of South America. Macrozamia and Bowenia are the two most commonly encountered genera in Australia whereas Encephalartos and Stangeria are found in East, Central and South Africa.

The amount and the type of azoxyglycosides found in cycad products depend on the species of cycad, the condition of the products, and the method of

extraction. Cycasin occurs in two important Cycas species -- C. circinalis L. (in Guam) and C. revoluta Thunb (in Japan); its occurrence in Zamia plants has also been suggested (110). The reported yield ranged from 0.02 to 5% (24, 183, 184). In general, fresh and unwashed cycad nuts contain substantially higher amounts of cycasin than washed, fermented and dried cycad nuts. Boiling during the extraction procedure improves the yield by destroying the cycasin-destroying enzyme (emulsin) present in the cycad extract. Samples of dried cycad chips, prepared by Guamanians for human consumption, contained no detectable amount of cycasin (183). Small amounts of transglycosylated derivatives of cycasin (neocycasin A, B, C and E) have also been isolated from C. revoluta Thunb (20-23). Macrozamin was first obtained from Macrozamia spiralis (2) and has since been found in several different species of Microzamia (3), in Encephalartos species (26), and in Zamia leaves (110). The reported yield from one of these studies was 0.1% (26).

Various parts of cycad plants are still used as a source of food and medicine by local inhabitants in some parts of the world (1, 16-18). A major food use of cycads is as cycad flour or starch. After elaborate washing and fermentation procedures the final products appear to be free of cycasin. No cycasin was detected in samples of Guamanian homemade cycad (C. circinalis L.) flour (108) and dried cycad chips (183), and in Japanese homemade cycad bean paste "sotetsu miso" (185). Between 1845 and 1920, cycad starch was produced commercially in Florida using Z. floridana (1). Incidents of human poisoning have been reported (1, 51) during times of food shortage and famine when inadequately detoxified cycad products were consumed. In addition to their use as flour or starch, fresh cycad husk is eaten as candy by the natives of Guam. Seeds, gum, stems, leaves or roots of Cycas and Zamia plants were used medicinally to treat snake or insect bites, as emetic, laxative, aphrodiziac

and for various other purposes in India, China, Mexico and southeast Asia (1). Roots or barks of Encephalartos and Stangeria spp. were also used medicinally by African natives (117).

Despite the proven animal carcinogenicity of cycasin and various cycad products, there is no epidemiologic evidence in support of their human carcinogenicity. Mugera and Nderito (114) suggested that cycads (Encephalartos and Stangeria spp.) may play a role in the etiology of liver cancer among East African natives but provided no supportive documentary evidence. African natives are known to be exposed to other liver carcinogens such as aflatoxins (see Section 5.3.1.1.5.1). Between 1961 and 1965, Hirono et al. (51) carried out an epidemiologic study of the inhabitants of Miyako Islands, Okinawa. These natives were forced by circumstances to subsist on cycads (C. circinalis L.) during a period of food shortage, after a series of typhoons had struck the islands in 1959. An unusually high incidence of liver cirrhosis was observed among the natives. However, no significant increase in cancer mortality was found. It has been pointed out (17) that the results may be negative because of the short follow up time or because the cycad foods were well prepared. Judging from the absence of cycasin in well prepared cycad foods, it appears that the carcinogenic risk of consumption of cycad foods is low. However, human consumption of unprocessed cycad material should be totally avoided.

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