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Evidence for Transplacental Passage of the Natural Carcinogen Cycasin and Its Aglycone (32672)

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It was recently reported from this Laboratory that rats exposed *in utero* to cycasin, methylazoxymethyl-β-D-glucoside, developed tumors in later life (1) and that malformations of the central nervous system and extremities could be induced in fetuses when the aglycone of cycasin, methylazoxymethanol (MAM) was administered intravenously to pregnant hamsters on the eighth day of gestation (2).

These findings suggested but did not conclusively prove a transplacental passage of the compounds. Although the pattern of urinary and fecal excretion of cycasin was found comparable in pregnant and nonpregnant female rats (1), the possibility remained that small amounts of cycasin or MAM might be stored in the mammary glands and excreted with the milk postpartum. Such an event could have provided the mechanism by which the tumors were induced in view of the observations of Mugera *et al.* (3) who suggested that the toxic material was excreted with the milk when lactating animals were fed cycad materials. Similarly, the induced malformations in the fetuses of hamsters receiving the highly toxic MAM could conceivably have been the result of maternal injury without cycasin or MAM directly being involved.

To clarify the route of cycasin effect in fetuses, experiments were designed to (i) search for evidence of storage of cycasin or MAM in lactating mammary glands of rats fed crude cycad material during pregnancy, (ii) determine whether transplacental passage of cycasin or MAM occurred in pregnant rats and hamsters, and (iii) establish the presence of cycasin or MAM in secreting mammary glands when cycasin is administered to lactating rats.

Material and Methods. Experimental animals. Forty-seven female Fischer rats, 12 of which served as controls, and 10 female golden hamsters, including 3 controls, were used. The rats were obtained from the NIH animal breeding facilities and the hamsters from a commercial hamster colony in Newfield, New Jersey. Females of both species were mated in our laboratory and the day on which spermatozoa were observed in vaginal smears was designated as day 1 of pregnancy. The following groups of animals were used:

Group I. Ten pregnant rats were fed commercially available ground Purina chicken feed diet. Crude cycad meal containing 3% cycasin was added to the diet which was fed

TABLE I. Recovery of Cycasin and MAM from 15-Day-Old Fetuses After a Single Intragastric Dose of Cycasin to Pregnant Fischer Rats.

No. of pregnant rats	Interval[a] (hours)	No. of assays	Av cycasin intake (mg)	Av recovery (mg)	
				Cycasin	MAM
5	—	5	—	Neg	Neg
1	2	1	160	3.5	2.5
3	3	3	185	3.5	2.2
3	5	3	189	Neg	7.7
1	8	1	172	Neg	7.0
1	16	1	155	Neg	6.4
1	24	1	165	Neg	4.0
3	30	3	33.9	Neg	.25; Tr[b] in 2
2	40	2	21.2	Neg	.25; Tr in 1
2	48	2	18.3	Neg	Tr in 2
2	52	2	20.0	Neg	Tr in 1; 0.0
1	54	1	17.5	Neg	Neg

[a] Time between cycasin administration and assay.
[b] Tr = trace or ($<50\ \mu g$).

from days 16–19 of pregnancy to 6 of the 10 rats. The remaining 4 rats were maintained on the basal diet and served as controls. Food intake was measured and the amount of cycasin eaten during the 3-day period calculated. Three cycad meal-fed rats and 2 controls were killed on day 19 and their fetuses were assayed for cycasin and MAM. The remaining 3 experimental rats and 2 controls were returned to the basal diet on day 19 and allowed to litter after which they were killed. The newborns and the mammary glands were assayed for cycasin and MAM.

Group II. Twenty of 25 pregnant rats received a single dose of cycasin by stomach tube on day 15 of pregnancy after food was withheld during the preceding night. The animals were killed at intervals as indicated in Table I, the fetuses removed and assayed quantitatively for cycasin and MAM. The fetuses from each litter were pooled and the pools assayed separately. The fetuses of the 5 untreated rats served as controls. The dose of cycasin, dissolved in physiologic saline, was 750 mg/kg of body weight in the rats which were used for assays during the first 24 hours. This amount was drastically reduced to 125 mg/kg of body weight in rats used for the later assays because of marked toxicity resulting in early death with the larger dose. The amount of cycasin in mg given to each rat is listed in Table I, column 4.

Group III. Twelve pregnant rats were permitted to terminate pregnancy. On the second day of nursing, 9 lactating rats received cycasin in physiologic saline by stomach tube at the 2-dose levels described in Group II. The mothers and young were killed at the times indicated in Table II which also lists the amount of cycasin received by each rat. For each assay 4 newborns of each litter were used as well as the bilateral mammary glands of the mother rats. For controls, 4 newborns from each of the 3 controls and the respective mammary glands of the mother rats were used.

Group IV. Seven pregnant hamsters received MAM on day 11 of gestation in various amounts intravenously in 0.5 ml of physiologic saline as shown in Table III. The smallest dose was previously found to induce malformations in hamsters (2). Three pregnant hamsters served as controls. The animals were killed at various times after the MAM had been injected and the fetuses of experimental and control hamsters were quantitatively assayed for cycasin and MAM.

Tissue preparation for assay. Under deep ether anesthesia the animals were bled and appropriate tissues were removed quickly and

TABLE II. Qualitative Recovery of Cycasin and MAM from Maternal Mammary Glands and Sucklings of Rats After a Single Intragastric Dose of Cycasin at Second Day of Lactation.

No. of rats	Interval (hours)	Cycasin intake (mg)	Qualitative determination: Mammary gland Cycasin	Mammary gland MAM	Suckling rats Cycasin	Suckling rats MAM
3	—	—	Neg	Neg	Neg	Neg
1	2	149	Pos	Pos	Pos	Pos
2	3	168	Pos	Pos	Pos	Pos
1	8	160	Neg	Pos	Neg	Pos
1	16	95	Neg	Pos	Neg	Pos
1	24	163	Neg	Pos	Neg	Pos
1	30	10	Neg	Pos	Neg	Pos
1	48	18	Neg	Pos	Neg	Pos
1	52	17	Neg	Neg	Neg	Neg

kept under constant temperature of 4°C on ice. Small fetuses were homogenized *in toto* in 1–2 ml of double distilled water. The larger fetuses, newborn rats, and mammary glands were minced separately prior to homogenization in 2–10 ml of the same solution. The homogenates were kept on ice for 10 min, and centrifuged at 8000 rpm for 5 min using a SS 34 rotor at 4°C in a Sorvall refrigerated centrifuge. The aqueous supernatant was decanted and saved. The sediment was extracted with 1–10 ml of 95% ethanol for 10 min and recentrifuged. The alcoholic supernatant was saved and the sediment was reextracted with ether for 1 hour. The ether was evaporated and the residue was dissolved in 50–250 μl of double distilled water.

TABLE III. Recovery of MAM from Fetuses of Hamsters Receiving a Single iv Injection of MAM on Day 11 of Pregnancy.

No. of hamster	Interval (min)	MAM injected (mg)	MAM recovered (mg)	MAM recovered (%)
1–3	—	—	Neg	
4	10	13.7	.26	1.89
5	10	2.5	.05	2.00
6	30	20.0	4.0	20.00
7	30	20.0	2.5	12.50
8	30	2.5	.125	5.00
9	180	20.0	Trace[a]	
10	240	20.0	Neg	

[a] Trace (<50 μg).

Thin-layer chromatography, plate preparation and assay. Six grams of Cilic AR TLC-76F (Mallinckrodt) were quickly stirred with 13 ml of distilled water. The mixture was poured on 20 × 20 cm glass plate taped on two margins. A glass rod was rolled over the tape to form a 250 μ thick coating on the glass plate. After the plate was allowed to air dry it was activated at 100° temperature for 15 min.

The 25–100 μl portions of aqueous, alcoholic, and ethereal extracts together with a standard of cycasin and MAM were chromatographed on the silica gel thin-layer plates in a *n*-butanol–acetic acid–water solution (4:1:1) by the ascending method of Matsumoto[1] and Teas.[2] The standard MAM was prepared from cycasin by the method of Kobayashi and Matsumoto (4) without further extraction and purification procedures. Cycasin and MAM were identified by using (i) the chromatropic reagent and (ii) ultraviolet light fluorescence and checking the R_f values of the positive spots with control standards. Fluorescence in ultraviolet light was used also for confirming the presence of azoxy compounds. The aqueous and alcoholic extracts of fetuses, newborns, and maternal mammary glands, when chromatographed on thin-layer plates, revealed two additional fluorescent spots between cycasin and MAM with R_f values of .66 and .58, respectively.

[1] Matsumoto, H., personal communication.

[2] Teas, H. J., personal communication.

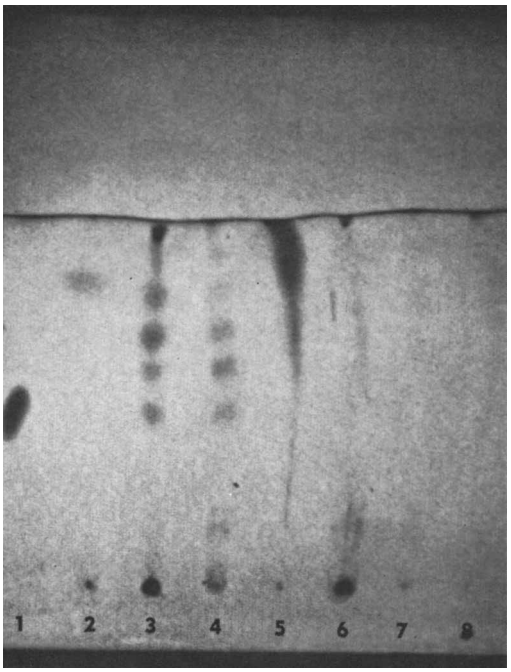

FIG. 1. Thin-layer chromatograph. Standards: cycasin, 1; MAM, 2; extracts of experimental tissues: aqueous 3; ethanol, 4; ethereal, 5; extracts of control tissues: aqueous, 6; ethanol, 7; ethereal, 8.

Their identity, at present, is unknown. The ether extract of the tissues showed the poorest separation of the substances (Fig. 1).

Appropriate spots were removed from thin-layer plates and reextracted in double distilled water. The silica gel was removed by centrifuging the samples at 20,000–30,000 rpm using 40.3 rotor at 4°C in a Spinco refrigerated centrifuge. Thereafter the specific ultraviolet absorption spectrum for azoxy compounds was verified in each sample. The presence of MAM was confirmed by obtaining the spectrum before and after boiling the samples for 10 min, a procedure which destroys MAM but not cycasin.

For the quantitative determination of cycasin and MAM, calibration curves were prepared from pure cycasin and MAM by measuring the changes in ultraviolet absorption spectrum at 215 mμ on a Beckman D4 spectrophotometer (5).

The values for cycasin and MAM from each extract were obtained by measuring the absorption spectrum before and after boiling the samples. The quantity of MAM present was calculated from the difference in readings between these two optical densities. The concentration of cycasin and MAM in each extract was calculated from the standard curve and the values were added to obtain total recovery of these substances.

Results. Group I. When cycad meal was added to the diet and fed to rats on days 16–19 of pregnancy MAM, but not cycasin, was found in the fetuses on day 19. Following discontinuation of cycad meal on that day, neither cycasin nor MAM were found in the mammary glands or fetuses assayed at termination of pregnancy on day 22. Neither MAM nor cycasin was found in the fetuses, newborns, and mammary glands of the control rats on days 19 and 22. Although the pattern of cycasin excretion was not examined in this particular experiment, data available in our laboratory indicate that under standard conditions Fischer rats metabolize cycasin uniformly better (85–97%) than either Sprague-Dawley (65–82%) or Osborne-Mendel (21–87%) rats. This may account for the observation that only MAM was found on day 19. No evidence for storage of cycasin or MAM in the mammary glands was obtained 3 days after discontinuation of feeding cycad material.

Group II. Transplacental passage of cycasin and MAM was observed in the fetuses of the group of pregnant rats receiving a single administration of cycasin by stomach tube on day 15 of gestation (Table I). The fact that cycasin as well as MAM were found in the fetuses within the first 3 hours of the tests was probably due to the large amounts of cycasin which the mothers received resulting in flooding of the tissues without adequate time for hydrolysis to the aglycone in the maternal intestinal tract to occur prior to absorption. Of greater importance is the demonstration of significant amounts of the reactive aglycone in the fetuses which quantitatively increased and later declined toward the end of the first 24 hours after cycasin administration. The changing values for MAM in the fetuses presumably reflect the progressive hydrolysis by bacterial enzymes as cycasin passes through the intestinal tract. If it can be assumed for the purpose of a

rough quantitation that hydrolysis of cycasin proceeds *in vivo* similar to that observable in *in vitro* studies (an assumption for which we have no proof as yet) the values of MAM can be multiplied by 3 to obtain a corresponding value for cycasin, since the yield of aglycone on hydrolysis of cycasin is about 30%. By this calculation, 21 mg or about 12% of the cycasin administered was recovered from the fetuses after 8 hours, a dose large enough to induce neoplasms. The very low values obtained within the second 24-hour period are partly due to the smaller dose of cycasin. They are also consistent with previously made observations that the greater part of a given dose of cycasin is excreted or metabolized within the first 24 hours following administration (4,6).

Group III. Table II summarizes the results of the experiment in which lactating rats received a single intragastric dose of cycasin. Cycasin and MAM were found in the maternal mammary glands and in suckling rats for 2 and 3 hours after cycasin administration, but only MAM could be demonstrated between 8 and 48 hours. The data indicate that cycasin and MAM were excreted through the milk when cycasin was administered during lactation. In one of the two rats killed after 3 hours quantitative data was obtained and the amount of cycasin and MAM in the mammary glands was found to be 6.72 mg and 8.76 mg, respectively. In the remaining rats only qualitative observations were made.

Group IV. The MAM was found within 10 min after its intravenous injection in the fetuses of golden hamsters. Its concentration markedly increased by 30 min but only a trace was found after 3 hours and none after 4 hours (Table III). Although the group of animals is small and the amount of MAM variable, a substantial part of this highly reactive compound appeared in the fetuses.

Discussion. The demonstration of cycasin and MAM in the fetuses of rats and of MAM in the fetuses of hamsters by thin-layer chromatography proves the transplacental passage of these compounds. A direct effect on fetal tissues may be considered, therefore, as the first step in the pathogenesis of malformations and tumor formation during intrauterine life. The nature of this interaction between chemical compound and fetal material remains to be elucidated more specifically in the future, but it should be recalled that MAM possesses properties of an alkylating agent through methylation of bases in RNA and DNA demonstrated both in *in vitro* (7) and in *in vivo* (8) experiments. The MAM has also been shown to act as a mutagen in a bacterial system in which the frequency of reversion to histidine independence of several histidine requiring mutants of *Salmonella typhimurium* was significantly increased above the spontaneous rate by MAM (9) and by a marked rise in sex-linked recessive lethal mutations in *Drosophila melanogaster* after addition of the aglycone to the nutrient medium (10). Lastly, chromosomal aberrations have been noted in significant numbers in onion roots exposed to the aglycone (11). There are available then several avenues for experimental approaches to the nature of interaction between MAM and fetal tissues.

Cycasin and MAM were also found in assays of whole, secreting mammary glands, provided cycasin was administered to lactating rats. This finding provides the explanation for previously made observations by Mugera *et al.* (3) who had noted liver damage in suckling young of various species when crude cycad material was included in the diet of the respective dams. On the other hand, no evidence for storage of cycasin and MAM in mammary glands was obtained when they were assayed 2–3 days after cycasin administration had been discontinued. It would seem unlikely, therefore, that the tumors found in rats exposed to cycasin as late as days 15–19 of intrauterine life, resulted from passage of the carcinogen through the milk.

It has recently been reported (12) that larvae of the insect *Serarctia echo* are capable of synthesizing cycasin when fed MAM in the diet. In this way, the dietary MAM is detoxified and converted to the nontoxic cycasin making it possible for the insect to live from and on cycad plants without ill effects. No evidence for the presence of a comparable mechanism was obtained in our studies in hamsters which received MAM and from which only MAM was recovered.

Although the purpose of the study was to

determine qualitatively the presence of cycasin and MAM in fetal tissues, a quantitative recovery of the two compounds at various times after a single intragastric dose of cycasin was performed in one group of rats (Table I) and in the hamsters (Table III). The data for fetal rats shows that cycasin was present at 2 and 3 hours after its administration and absent thereafter, whereas the amount of MAM increased reaching a peak between 5 and 16 hours after which it slowly decreased to zero levels by 52 hours. This probably means that some of the cycasin was absorbed from the gastrointestinal tract without prior conversion to the aglycone and that it circulated for a brief period before it was excreted. The MAM, on the other hand, was found much longer in the fetuses. Since MAM is a highly unstable compound, it must be assumed that its prolonged demonstration in the fetus resulted from continued deglucosylation of cycasin by bacterial enzymes in the intestinal tract and subsequent absorption of the MAM thus produced. That the MAM remained in tissues for only a short period of time may be seen from the data in hamsters which received a large dose of MAM intravenously (Table III). Whereas a substantial percentage of the injected MAM could be found after 30 min, only a trace of MAM was left after 3 hours and none after 4 hours.

Summary. The passage of cycasin and MAM through the placenta and mammary gland has been investigated in rats during pregnancy and lactation, respectively. Both cycasin and MAM cross the placenta when cycasin is given orally to pregnant rats. Both compounds can also pass the mammary gland and are excreted with the milk during lactation provided that they are administered within 1 day before littering or during lactation. MAM has also been found in the fetuses after its intravenous injection into the pregnant golden hamster. The recovery of MAM from the fetuses of rats and hamsters supports a direct action of the carcinogen and teratogen on fetal tissues.

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Protection of Mice by Postirradiation Treatment with a Cell-Free Component of Spleen* (32673)

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The existence of a chemical entity of tissue origin which can be administered after radiation exposure and still protect against radiation injury was first suggested by Ellinger (1), who demonstrated that cell-free extracts of spleen caused a 10% reduction in mortality. In subsequent studies, the postirradia-

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