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Toxicology of Cycasin

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INTRODUCTION

The purpose of this review is to summarize the toxicology of the naturally occurring glucoside cycasein, methylazoxymethanol-β-D-glucoside

$$(CH_3 \cdot \overset{\overset{O}{\uparrow}}{N}{:}N \cdot CH_2OC_6H_{11}O_5)$$

and of its metabolite, methylazoxymethanol (MAM)

$$(CH_3 \cdot \overset{\overset{O}{\uparrow}}{N}{:}N \cdot CH_2OH).$$

These compounds are extractable from seeds and roots of cycad plants

Cycads are ancient gymnospermous plants which are considered an intermediate form in plant evolution from ferns to flowering plants. Study of fossils indicates that cycads were distributed widely throughout the world in the early mesozoic period, i.e., about 200 million years ago. The cycads which can be found today are essentially limited to the tropical and subtropical zones around the globe (8, 51).

Although this review emphasizes recent developments in cycad research, it is important to realize that toxic properties of seeds and roots of cycads were known to populations which used them as food long before the toxic principles were identified. Strikingly similar methods for removing the poison were apparently employed by these populations. In the course of our studies with cycads, we have examined samples of cycad flour prepared by several families on Guam for human consumption and have found it nontoxic and noncarcinogenic in experimental animals (61). Apparently the methods employed by these people removed the greater part, if not all, of the poison from the seeds. An extensive literature review of the chemistry and toxicity of cycads is available and contains accounts of the uses of cycads as food and medicines (58).

The paper has been subdivided into five sections, reviewing the following aspects of cycad toxicity: (*a*) isolation of toxic principle, (*b*) neurotoxicity, (*c*) carcinogenicity, (*d*) metabolic conversion of cycasin to MAM *in vivo*, and (*e*) biologic effects. The review closes with several general comments, including suggestions for important work to be done in the future.

ISOLATION OF TOXIC PRINCIPLE

There are several communications which are historically important for present-day cycad research and deserve recall. Nishida and Yamada (34) found that formaldehyde in *sotetsu* (the Japanese name for *Cycas revoluta*) was a part of a new glucoside from which it was liberated by the action of an emulsion present in *sotetsu*. Formaldehyde resulted from enzymatic decomposition of a glucoside in *sotetsu* seeds, and *sotetsu* poisoning was considered to be due to its formaldehyde content (29).

The first biochemical isolation of a glucoside from cycads was reported by Cooper (1) who obtained a crystalline substance from seeds of *Macrozamia spiralis*, an Australian cycad, and named it macrozamin. It was toxic to guinea pigs when given by mouth, but nontoxic when injected subcutaneously. The carbohydrate component in macrozamin was later identified as primeverose, which was attached to the aglycone in a β-glucosidic link (21). The aglycone part of macrozamin was determined to have an aliphatic azoxy structure (15). Macrozamin was reported to be present also in seeds of cycads growing in Queensland, Australia (37) and in *Encephalartos barkeri*, an African cycad, according to Lythgoe as cited by Riggs (37). Toxic properties of seeds of several species of Encephalartos had previously been described (53).

The isolation of a glucoside from seeds of *Cycas revoluta* and the determination of its structure were accomplished by Nishida *et al.* (31), who named the compound cycasin. It was chemically closely related to macrozamin except for the sugar moiety, which was D-glucose in cycasin. It was toxic for mice and guinea pigs only when given enterically, whereas parenteral injections did not produce toxic symptoms; nor was it toxic in cold-blooded animals (33). Because of the similarity in toxic effects between macrozamin and cycasin, it was suggested that the toxicity of both glucosides resided in their identical aglycone part, and that hydrolysis of the glucosides occurred in stomach or intestines with the help of the digestive juices or enzymes. A method for the quantitative determination of cycasin was separately reported (32). In the same year, cycasin was found in seeds of *Cycas circinalis* L., a cycad indigenous to Guam (38).

The group headed by Nishida has reported during the succeeding years several new azoxyglucosides obtained from *Cycas revoluta* Thunb., which they named neocycasins. The aglycone, methylazoxymethanol, is common to all of them (30). The neocycasins can be expected, therefore, to produce similar biologic effects provided hydrolysis occurs in the *in vivo* systems.

Once the carcinogenic properties of the azoxyglucosides became known, the need for a readily available compound of this type for experimentation was obvious. A series of biochemical studies by Matsumoto and collaborators (12, 13, 27)

led ultimatelv to the synthesis of methylazoxymethyl acetate (26). This is now commercially obtainable (Mann Research Laboratories, Inc., New York, N.Y.).

NEUROTOXICITY

Information gathered by Whiting in 1954/55 on the frequency of paralytic conditions in cattle feeding on land where cycads grow and her survey in 1954 of the dietary uses of cycads by the Chamorros on Guam (59), among which amyotrophic lateral sclerosis is frequent, (14) led in 1961 to an exploratory study for possible neurotoxins in cycads at the National Institutes of Health.

In experiments with rats to which crude cycad meal was given as part of the diet, paralytic states were not observed. Since 1961 more than 500 brains and spinal cords have been examined, but no lesions have been found which conceivably could represent the counterpart to the lesions seen in amyotrophic lateral sclerosis or any other degenerative central nervous system disease. The laboratory rat has not been found suitable for this type of study in our hands.

It has only been recently demonstrated that distinct demyelination of at least two of the spinal columns occurred in cattle showing paralysis after ingestion of cycads (7). The tracts involved were the fasciculus gracilis and the dorsal spinocerebellar tract, which selectively showed deposits of osmiophilic material with the Marchi method. These observations, communicated to us in July 1965, have since been confirmed (24).

A new etiologic approach to the neurologic disorder has been suggested by Vega and Bell (57) who isolated a nonprotein amino acid, α-amino-β-methylaminopropionic acid (CH_3-NH-CH_2-CH(NH_2)-COOH), from seeds of *Cycas circinalis* which they found neurotoxic in chickens. They synthesized the compound which was as potent as the extracted natural compound. Further studies with this substance are very much needed in animals other than chickens, as are pathologic studies of the affected animals.

Lastly, we should cite a recent communication in which a neurologic disorder consisting of hind-leg paralysis was observed following a single subcutaneous injection of 0.5 mg of cycasin per gm of body weight in 80 percent of newborn mice of the G57BL/6 strain (11). Mature mice of the same strain did not develop a paralysis when equivalent amounts of cycasin were given by stomach tube. Further studies including those involving other strains of mice are necessary, particularly since this effect is not obtained in the newborn rat.

Whereas the experimental reproduction of degenerative disease appears difficult, cycasin and its aglycone have produced arrest either of normal development or of growth, as well as exaggerated growth responses in the brain and spinal cord of animals. Arrest of normal development was evident from a variety of malformations in the central nervous system of golden hamsters after intrauterine exposure to MAM (44). An example of arrested growth was observed in rats in which microencephaly was induced when exposure to MAM took place at the beginning of the third week of intrauterine development. Microencephaly was found in all littermates; it readily reproducible and inducible in more than one strain of rats. The reduction in size predominantly involved the cerebral hemispheres. Concomitantly, the width of the bony calvarium was diminished. This microencephaly was consistent with long life, but was recognizable early in the postnatal period (47).

Exaggerated growth responses of brain tissue to cycad materials were seen in the tumors which, with the exception of one meningioma, were gliomas (9, 45, 47). They were found between 13 and 15 months after the initial exposure to cycasin. Only one glioma was observed as early as 6.5 months. The smallest dose of cycasin which induced gliomas in several rats was 2.5 mg. It is probably of more than passing interest that gliomas were found in four of 44 microcephalic rats older than 1 year (47).

CARCINOGENICITY

Evidence that crude cycad material was carcinogenic was obtained early in 1962 when rats on a cycad meal diet were killed because of palpable abdominal tumor masses, ascites, and a rapidly developing anemia. At autopsy, tumors of the liver with and without pulmonary metastases and independent tumors of the kidney and occasionally of the intestine were found (20). The simultaneous occurrence of hepatic and renal neoplasms recalled to mind the reports by Magee and Barnes (22, 23) in which the development of such tumors had been described with dimethylnitrosamine (DMN). The chemical structures of DMN and cycasin showed a distinct resemblance, and within two years cycasin was established as the carcinogenic component in the crude cycad meal (16). A possible common metabolic pathway for DMN and cycasin resulting in formation of diazomethane has been suggested (28).

In reviewing our studies with cycasin with respect to carcinogenic effects, several summarizing statements can be made. (*a*) Whereas cycasin was carcinogenic only after passage through the gastrointestinal tract, its aglycone MAM induced tumors independent of the route of administration; MAM was, therefore, the proximate carcinogen (18, 19). (*b*) Sites of predilection for tumor development depended on the duration of feeding. Hepatomas, as a rule, required prolonged administration, whereas renal tumors developed after short periods of feeding. Intestinal neoplasms, which were almost exclusively located in the large bowel, were least dependent on the duration of exposure (17). (*c*) A single administration of cycasin was sufficient to induce single or multiple tumors in the majority of animals (9, 10). (*d*) The age of the animal when cycasin feeding was started influenced the relative frequency of various kinds of kidney tumors. Nephroblastomas, renal sarcomas, and the interstitial tumors of the kidney were considerably more common when immature rats were used, whereas renal adenomas appeared to develop with about equal frequency in immature and mature animals (10, 16). (*e*) The strain of rats influenced very little the carcinogenic effect of cycasin, and similar tumors were induced in Osborne-Mendel, Sprague-Dawley, Fischer, and Wistar rats (5, 6). (*f*) Cycasin-induced tumors were transplantable but showed a sex dependency for successful transplantation in the case of the nephroblastomas (10). (*g*) Cycasine and MAM readily passed the placenta in pregnant rats and hamsters, were demonstrable by thin-layer chromatography in the fetuses

(46), and induced tumors by this mechanism (45). (*h*) MAM induced all the tumors which were seen with cycasin and, in addition, carcinomas of the small intestines, particularly of the duodenum, provided it was administered by the intraperitoneal route (18, 19). (*i*) Tumor induction with cycasin was not limited to rats, but has also been demonstrated in mice (35), guinea pigs (41), fish (52), and hamsters (Spatz, unpublished data). (*j*) Regardless of the cycad material used, the age of the animal at first exposure, and the sex of the animal, tumors rarely developed before 6 months. This includes the group of rats with tumors which had been exposed to the carcinogen *in utero* (45). (*k*) The average rate of tumor induction with MAM was 100% but with cycasin only 85%. Studies of the animals which did not develop tumors strongly suggested that they had escaped liver injury. The possible causes for negative results in about 15% of the cycasin-treated animals were investigated, and these and related studies will be discussed in the following section.

CONVERSION OF CYCASIN TO MAM *IN VIVO*

In searching for causes for the lack of uniform response among animals fed cycasin, the literature indicated that azoxyglucosides were toxic only after ingestion and that the conversion of the glucosides to the active compound depended on hydrolysis in the gastrointestinal tract. Reports were available to indicate that the cells of the small intestine contained a β-glucosidase (2, 3). There were several reasons, however, to suspect that the bacterial flora might be of even greater importance for the hydrolysis of cycasin. One was that a variability of the intestinal flora seemed much more likely than a variability of the mucosal enzyme pattern. Another reason was the nearly exclusive localization of the intestinal neoplasms in the large intestine, suggesting a greater availability or higher concentration of the active carcinogen in that segment of the bowel. The question whether the small intestine was more resistant to neoplasia than the large intestine was left unanswered at this point.

We chose, therefore, to explore the importance of the bacterial flora in promoting cycasin hydrolysis by comparing the excretion pattern of cycasin in a germ-free rat with that in a conventional rat. The results indicated that germ-free rats nearly quantitatively excreted the ingested cycasin, whereas conventional rats excreted only a part of the intake, the difference having been matabolized (49). The importance of the bacterial flora for cycasin hydrolysis was thus established. The data also showed, however, that a considerable variation in the percentile excretion existed among the conventional rats, thus strengthening our suspicion that the bacterial flora could significantly influence the metabolism of cycads and thus their toxicity. Germ-free rats were then monocontaminated with organisms known to possess or to be free of a β-glucosidase as determined in *in vitro* assays; they subsequently were given cycasin by stomach tube, and it was found that the hydrolysis of cycasin depended, indeed, on microorganisms possessing a β-glucosidase. The presence of β-glucosidase-free bacteria in the intestine, moreover, failed to induce the enzyme in the mucosal lining in the presence of cycasin (50). It has already been shown that germ-free rats developed neoplasms when MAM or the synthetic MAM acetate was administered. Cycasin, when given in large doses for 20 days to germ-free rats, on the other hand, failed to do so over a two-year period of observation (19).

In conclusion, the bacterial flora largely determined the conversion of cycasin to MAM and through this mechanism the production of cancer in the experimental animal. Moreover, it would seem possible that the lack of uniform response noted in our early experiments might well have been due to differences in the bacterial intestinal flora.

In early 1965 we heard of the successful induction of kidney tumors in rats which had received a single subcutaneous injection of cycasin on the first day of life (Magee, personal communication). This observation, since then confirmed and extended by us to include also tumors of liver, intestine, lung, and brain (9), remained the exception to the rule that cycasin required hydrolysis by passage through the intestinal tract in order to be converted to the carcinogenically active aglycone. Magee's and our confirmative experiments required an explanation. The length of the postnatal period during which subcutaneously injected cycasin might be toxic to rats was first determined. All rats injected between the first and 17th day of postnatal life were dead within 3–4 days. Rats injected at 17 and 21 days after birth or later survived. Three of the uninjected mother rats were also dead within 72 hours after their young had been injected. They, like their young, had died with severe centrilobular liver cell necrosis. This suggested that the mother rats had ingested enough cycasin, presumably from licking urine while cleaning their young, and metabolized it. The aglycone was excreted through the milk while simultaneously subjecting the mothers to its hepatotoxic action. Further studies showed, however, that this mechanism could not be the only explanation. First, artificial nursing of the young after they had been injected with cycasin did not prevent their death. Secondly, germ-free rats treated identically also died even though hydrolysis of cycasin in the maternal intestinal tract did not occur. Thirdly, intraperitoneal injections of cycasin produced considerably less toxicity and all injected young survived for 14 days, whereas the survival time of subcutaneously injected rats was 2–4 days (Hirono, Spatz, unpublished data).

These observations suggested that enzymatic hydrolysis of cycasin occurred somewhere in the young rats independent of the mother rat and that this mechanism was transient in nature and independent of bacterial enzymes.

In order to determine whether local factors at the site of injection might play a role, the skin and subcutaneous tissue of conventional and germ-free Sprague-Dawley rats and those of conventional Fischer rats were assayed for β-D-glucosidase activity on various days during postnatal life. As substrates, two well-known synthetic glucosides such as *p*-nitrophenyl-β-D-glucoside and *o*-nitrophenyl-β-D-glucoside were used in addition to cycasin. The results indicated that the skin of newborn rats and of rats during early postnatal life contained a β-D-glucosidase capable of hydrolyzing the two synthetic substrates and cycasin. In the case of cycasin, the amount of MAM resulting from the hydrolysis was determined by ultraviolet spectrophotometry and thin-layer chromatography. The greatest enzyme activity was found during the first few days of

postnatal life. At 5–8 days it decreased, and it was no longer demonstrable by the 25th day of life (43, 48).

The demonstration of a β-D-glucosidase activity in the subcutaneous tissue of newborn and early postnatal rats provides the explanation for the toxic and carcinogenic effects of cycasin which had been observed after a single subcutaneous injection of cycasin in such animals. The disappearance of enzyme activity at 3–4 weeks after birth coincides with the time at which cycasin can be injected in relatively large amounts into the subcutis without producing ill effects.

Although our interest in this enzyme in the skin and subcutaneous tissue originated in the studies of cycasin toxicity, investigations of the significance of this enzyme in physiologic conditions now need to be undertaken.

BIOLOGIC EFFECTS

When small laboratory animals such as the rat are intoxicated with cycasin, the organ most strikingly and earliest involved is the liver. The degree of liver injury is dose dependent, and the light microscopic changes may vary from loss of cytoplasmic basophilia and glycogen in the mildest type of injury to diffuse centrilobular hemorrhagic necrosis in the most severe form (20). Concomitant with the loss in cytoplasmic basophilia is a decrease in glucose-6-phosphatase (42) and, with larger doses, of liver RNA and total liver phospholipids (60). An indication that protein synthesis is decreased was suggested by the studies of Rechcigl (36), who found that the rate of catalase synthesis in the liver of cycasin-fed rats was about one-fifth that of pair-fed controls. More recently it was shown that the incorporation of leucine-^{14}C into hepatic proteins was inhibited, but that into kidney, spleen, or ileum proteins was not (39).

We have recently made more and more use of the synthetic MAM acetate, thus avoiding variations due to differences in cycasin breakdown, and have reinvestigated early phases of liver cell injury with the electron microscope. Dr. Ganote of our laboratory, who has conducted this study, noted as the earliest prominent change, which was apparent two hours after an intraperitoneal injection of MAM acetate, a clumping and segregation of the granular and fibrillar components of the nucleolus which progressed to total loss of the granules and an aggregation of the fibrillar material. Cytoplasmic changes consisted of loss of polysomal aggregates of ribosomes, apparent depletion of the total number of free ribosomes, loss of dense particles from the Golgi cisternae, lipid accumulation, hypertrophy of the smooth endoplasmic reticulum, and formation of membrane whorls from the rough endoplasmic reticulum. Mitochondria showed little evidence of damage except at higher dose levels where mitochondrial swelling preceded frank necrosis. The changes just enumerated were found in cells in the centrilobular zone, while the liver cells in the periportal fields remained essentially unaltered. These structural changes probably offer the explanation for the biochemically noted alteration in protein synthesis already referred to (4).

Alkylation of liver RNA and DNA with cycasin and MAM was described in *in vitro* studies (25) and in *in vivo* experiments (39). In both experiments an additional purine base was found and identified as 7-methyl-guanine. The similarities in the biologic effects between cycasin and dimethylnitrosamine upon which we commented originally are well documented in these recent studies (4, 39).

In addition to its alkylating property, the aglycone of cycasin was shown to be a potent mutagen. This effect was demonstrated in a bacterial system in which the frequency of reversion to histidine independence of several histidine requiring mutants of *Salmonella typhirium* was significantly above the spontaneous rate with MAM (40). More recently, a marked rise in sex-linked recessive lethal mutations was noted in *Drosophila melanogaster* after addition of MAM or the synthetic MAM acetate to the nutrient medium (55).

Exposure to cycasin of Allium (onion) seedlings which show β-glucosidase activity was reported to result in as many chromosomal abberations as could be produced with 200 R of gamma rays (56). This observation was referred to as radiomimetic effect. Was there a possibility then to influence the late appearance of tumors by preventing the acute toxic phase with radioprotective agents? After the LD_{50} was established as 560 mg/kg body weight, a large number of rats received a single gastric instillation of 750 mg/kg body weight. As protective agents, β-mercaptoethylamine (cysteamine) and 3-amino-1,2,4-triazole were used and given intraperitoneally once to rats at different times relative to cycasin administration. It was found that rats could be protected from a lethal dose of cycasin by both compounds provided that the protective agent was given shortly before cycasin. Yet all rats which survived beyond 6 months had tumors (9). The mechanism of protection is still obscure. Available evidence suggests that the protective agent in some way decreased the effective dose of cycasin, perhaps by altering the rate of conversion of cycasin to the toxic MAM.

Although the conversion of cycasin to MAM has been emphasized throughout this review as the *sine qua non* for cycasin toxicity, there exists a fascinating observation describing the reverse phenomenon, namely, the ability of a living organism to detoxify MAM by converting it to cycasin (54). By feeding MAM to larvae of caterpillars (*Seirarctia echo*) in an artificial medium, analysis of the various body parts for β-glucosidase activity and for cycasin showed the highest enzyme activity in the gut, whereas the hemolymph being free of enzyme activity contained cycasin in substantial amounts. Studies along these lines might contribute significantly to our future understanding of the biosynthesis of this interesting glucoside, cycasin.

CONCLUSIONS AND OUTLOOK

We have attempted in the foregoing sections to summarize the important aspects of cycad toxicity. These included the biochemical studies which led to the isolation and elucidation of the chemical structures of the glucoside cycasin and culminated in the synthesis of the aglycone part. They dealt with the present status of a neurotoxic component in crude cycad material and described the carcinogenic properties of the crude cycad meal, of cycasin, and of its aglycone, methylazoxymethanol. The conversion of cycasin to MAM *in vivo* was covered separately, describing the mechanisms by which cycasin becomes toxic and carcinogenic in the living animal.

Finally a section was devoted to the biologic effects of cycasin and MAM which were observed in a variety of systems. The studies cited herein are most likely of fundamental importance for the production of cycasin toxicity.

In assessing the present status of cycad research, we have recognized cycasin as a general carcinogen and have recognized its profound effects on the central nervous system during embryonic and fetal development. New areas for detailed research into the nature of growth arrest of the brain and the significance of an enzyme in the subcutaneous tissue of fetal and newborn rats have been suggested by these studies.

There are three main areas thus far essentially uncovered which require attention in future investigations. They are (*a*) the biosynthesis of the glucoside within the plant, (*b*) a detailed study of the cellular events during the interval between exposure to the carcinogen and the appearance of neoplasms, and (*c*) the significance of the results obtained in animal experimentation in terms of human disease. Answers to these complex problems lie in the future and require the participation of many workers with different scientific backgrounds and interests. It may be well to keep in mind the broad spectrum of abnormalities which this natural compound can induce. Beyond the factual observations, one might also raise the question whether comparable situations may not exist in our environment by which an innocuously appearing compound can be changed into a potent toxin while being metabolized in the *in vivo* system.

REFERENCES

1. Cooper, J. M., Isolation of a Toxic Principle from the Seeds of *Macrozamia Spiralis.* Proc. Roy. Soc. New South Wales, *74:* 450–454, 1941.
2. Dahlquist, A., Bull, B., and Gustafsson, B. E. Rat Intestinal 6-Bromo-2-naphthyl Glycosidase and Disaccharidase Activities. I. Enzymic Properties and Distribution in the Digestive Tract of Conventional and Germ-free Animals. Arch. Biochem. Biophys., *109:* 150–158, 1965.
3. Dahlquist, A., Bull, B., and Thomson, D. L. Rat Intestinal 6-Bromo-2-naphthyl Glycosidase and Disaccharidase Activities. II. Solubilization and Separation of the Small Intestinal Enzymes, Arch. Biochem. Biophys., *109:* 159–167, 1965.
4. Ganote, C. E., and Rosenthal, A. S. Characteristic Lesions of Methyl-azoxymethanol-induced Liver Damage. A Comparative Ultrastructural Study with Dimethylnitrosamine, Hydrazine Sulfate and Carbon Tetrachloride. Lab. Invest., in press, 1968.
5. Gusek, W., Buss, H., and Krüger, Ch.-H. Morphologische und Histochemische Befunde au Experimentellen Nierentumoren der Ratte, Verhandl. Deut. Ges. Pathol., *50:* 337–343, 1966.
6. Gusek, W., Buss, H., and Laqueur, G. L. Histo logisch-histochemische Utersuchungen am "Interstitiellen Cycasin Tumor der Rattenniere," Beitr. Pathol. Anat. Allgem. Pathol. 135: 53–74, 1967.
7. Hall, W. T., and McGavin, M. D. Clinical and Neuropathological Changes in Cattle Eating the Leaves of *Macrozamia lucida* and *Bowenia serrulata* (Family Zamiaceae). Pathol. Vet., *5:* 26–34, 1968.
8. Hertrich, W. Palms and Cycads. Their Culture in Southern California as Observed Chiefly in the Huntington Botanical Gardens. The Henry E. Huntington Library and Art Gallery, San Marino, California, pp. 106–136, 1951.
9. Hirono, I., Laqueur, G. L., and Spatz, M. Tumor Induction in Fischer and Osborne-Mendel Rats by a Single Administration of Cycasin. J. Natl. Cancer Inst., *40:* 1003–1010. 1968.
10. Hirono, I., Laqueur, G. L., and Spatz, M. Transplantability of Cycasin-induced Tumors in Rats with Emphasis on Nephroblastomas. J. Natl. Cancer Inst., *40:* 1011–1025, 1968.
11. Hirono, I., and Shibuya, Ch. Induction of a Neurological Disorder by Cycasin in Mice. Nature, *216:* 1311–1312, 1967.
12. Kobayashi, A., and Matsumoto, H. Methylazoxymethanol, the Aglycone of Cycasin. Federation Proc., *23:* 1354–1355, 1964.
13. Kobayashi, A., and Matsumoto, H. Studies on Methylazoxymethanol, the Aglycone of Cycasin: Isolation, Biological, and Chemical Properties. Arch. Biochem, Biophys., *110:* 373–380, 1965.
14. Kurland, L. T., and Mulder, D. W. Epidemiologic Investigations of Amyotrophic Lateral Sclerosis. 1. Preliminary Report on Geographic Distribution, with Special Reference to the Mariana Islands, including Clinical and Pathologic Observations. Neurology, *4:* 355–378, 438–448, 1954.
15. Langley, B. W., Lythgoe, B., and Riggs, N. V. Macrozamin. Part II. The Aliphatic Azoxy Structure of the Aglycone Part. J. Chem. Soc., *46:* 2309–2316, 1951.
16. Laqueur, G. L., Carcinogenic Effects of Cycad Meal and Cycasin, Methyl-azoxymethanol Glycoside, in Rats and Effects of Cycasin in Germfree Rats. Federation Proc., *23:* 1386–1387, 1964.
17. Laqueur, G. L. The Induction of Intestinal Neoplasms in Rats with the Glycoside Cycasin and its Aglycone. Virchows Arch. Pathol. Anat. Physical./Clin. Md., *340:* 151–163, 1965.
18. Laqueur, G. L., and Matsumoto, H., Neoplasms in Female Fischer Rats Following Intraperitoneal Injection of Methylazoxymethanol J. Natl. Cancer Inst., *37:* 217–232, 1966.
19. Laqueur, G. L., McDaniel, E. G., and Matsumoto, H. Tumor Induction in Germfree Rats with Methylazoxymethanol (MAM) and Synthetic MAM Acetate. J. Natl. Cancer Inst., *39:* 355–371, 1967.
20. Laqueur, G. L., Mickelsen, O., Whiting, M. G., and Kurland, L. T. Carcinogenic Properties of Nuts from *Cycas Circinalis* L. Indigenous to Guam. J. Natl. Cancer Inst., *31:* 919–951, 1963.
21. Lythgoe, B., and Riggs, N. V. Macrozamin. Part I. The Identity of the Carbohydrate Component. J. Chem. Soc., *4:* 2716–2718 1949.
22. Magee, P. N., and Barnes, J. M. The Production of Malignant Primary Hepatic Tumours in the Rat by Feeding Dimethylnitrosamine. Brit. J. Cancer, *10:* 114–122, 1956.
23. Magee, P. N., and Barnes, J. M., Induction of Kidney Tumours in the Rat with Dimethylnitrosamine (N-nitrosodimethylamine). J. Pathol. Bacterial., *84:* 19–31, 1962.
24. Mason, M. M., and Whiting, M. G. Demyelination in the Bovine Spinal Cord Caused by Zamia Neurotoxicity. Federation Proc., *25:* 533, 1966.
25. Matsumoto, H., and Higa, H. H. Studies on Methylazoxymethanol, the Aglycone of Cycasin: Methylation of Nucleic Acids *in vitro.* Biochem. J., *98:* 20c–22c, 1966.
26. Matsumoto, H., Nagahama, T., and Larson, H. O. Studies on Methylazoxymethanol, the Aglycone of Cycasin: A Synthesis of Methylazoxymethyl Acetate. Biochem. J., *95:* 13c–14c, 1965.
27. Matsumoto, H., and Strong, F. M. The Occurrence of Methylazoxymethanol in *Cycas circinalis* L. Arch. Biochem. Biophys., *101:* 299–310, 1963.
28. Miller, J. A. Comments on Chemistry of Cycads. Federation Proc., *23:* 1361–1362, 1964.
29. Nishida, K. Biochemical Studies on "Sotetsu," the Japanese Cycad. VI. Removal of Poisonous Constituents in the Seeds and the Nutritive Value of the Seeds. J. Agr. Chem. Soc. Japan, *12:* 1106–1116, 1936.
30. Nishida, K., Azoxyglucosides (from *Cycas Revoluta* Thunb.), Japan J. Chem., *13:* 730–737, 1959.

31. Nishida, K., Kobayashi, A., and Nagahama, T. Studies on Cycasin, a New Toxic Glycoside, of *Cycas Revoluta* Thunb. Part I. Isolation and the Structure of Cycasin. Bull. Agr. Chem. Soc. Japan, *19:* 77–83, 1955.
32. Nishida, K., Kobayashi, A., and Nagahama, T. Studies on Cycasin, a New Toxic Glycoside, of *Cycas Revoluta* Thunb. Part V. Quantitative Determination of Cycasin in Cycad Seeds. Bull. Agr. Chem. Soc. Japan, *20:* 74–76, 1956.
33. Nishida, K., Kobayashi, A., Nagahama, T., Kojima, K., and Yamane, M. Studies on Cycasin, A New Toxic Glycoside of *Cycas Revoluta* Thunb. Part IV. Pharmacological Study of Cycasin. Seikagaku, *28:* 218–223, 1956.
34. Nishida, K., and Yamada, A. Biochemical Studies of "Sotetsu," the Japanese Cycad. III. Enzyme Chemistry of a Constituent Containing Formaldehyde. J. Agr. Chem. Soc. Japan, *11:* 357–364, 1935.
35. O'Gara, R. W., Brown, J. M., and Whiting, M. G., Induction of Hepatic and Renal Tumors by Topical Application of Aqueous Extract of Cycad Nut to Artificial Skin Ulcers in Mice, Federation Proc., *23:* 1383, 1964.
36. Rechcigl, M., Jr., Rates and Kinetics of Catalase Synthesis and Destruction in Rats Fed Cycad and Cycasin *in Vivo.* Federation Proc., *23:* 1376–1377, 1964.
37. Riggs, N. V. The Occurrence of Macrozamin in the seeds of Cycads. Australian J. Chem., *7:* 123–124, 1954.
38. Riggs, N. V. Glucosyloxyazoxymethane, a Constituent of the Seeds of *Cycas Circinalis* L. Chem. Ind. *September 8:* 926, 1956.
39. Shank, R. C., and Magee, P. N. Similarities between the Biochemical Actions of Cycasin and Dimethylnitrosamine. Biochem. J., *105:* 521–527, 1967.
40. Smith, D. W. E. Mutagenicity of Cycasin Aglycone (Methylazoxymethanol), a Naturally Occurring Carcinogen. Science, *152:* 1273–1274, 1966.
41. Spatz, M. Carcinogenic Effect of Cycad Meal in Guinea Pigs. Federation Proc., *23:* 1384–1385, 1964.
42. Spatz, M. Effects of Cycad Meal and Cycasin on Histochemically Demonstrable Liver Phosphatases and Nonspecific Esterases. Federation Proc., *23:* 1381–1382, 1964.
43. Spatz, M. Hydrolysis of Cycasin by β-D-glucosidase in Skin of Newborn Rats, Proc. Soc. Exptl. Biol. Med., *128:* 1005–1008, 1968.
44. Spatz, M., Dougherty, W. J., and Smith, D.W.E. Teratogenic Effects of Methylazoxymethanol. Proc. Soc. Exptl. Biol. Med., *124:* 476–478, 1967.
45. Spatz, M., and Laqueur, G. L. Transplacental Induction of Tumors in Sprague-Dawley Rats with Crude Cycad Material. J. Natl. Cancer Inst., *38:* 233–245, 1967.
46. Spatz, M., and Laqueur, G. L. Evidence for Transplacental Passage of the Natural Carcinogen Cycasin and Its Aglycone. Proc. Soc. Exptl. Biol. Med., *127:* 281–286, 1968.
47. Spatz, M., and Laqueur, G. L. Transplacental Chemical Induction of Microencephaly in Two Strains of Rats. I. Proc. Soc. Exptl. Biol. Med., in press, 1968.
48. Spatz, M., Laqueur, G. L., and Hirono, I. Hydrolysis of Cycasin by β-D-glucosidase in Subcutis of Newborns. Federation Proc., *27:* 722, 1968.
49. Spatz, M., McDaniel, E. G., and Laqueur, G. L. Cycasin Excretion in Conventional and Germfree Rats, Proc. Soc. Exptl. Biol. Med., *121:* 417–422, 1966.
50. Spatz, M., Smith, D.W.E., McDaniel, E. G., and Laqueur, G. L. Role of Intestinal Microorganisms in Determining Cycasin Toxicity. Proc. Soc. Exptl. Biol. Med., *124:* 691–697, 1967.
51. Sprone, K. R. The Morphology of Gymnosperms. The Structure and Evolution of Primitive Seed-plants, pp. 103–118. London: Hutchinson & Co. Ltd., 1965.
52. Stanton, M. F. Hepatic Neoplasms of Aquarium Fish to *Cycas Circinalis,* Federation Proc., *25:* 661, 1966.
53. Steyn, D. G., van der Walt, S. J., and Verdoorn, I. C. The Seeds of Some Species of *Encephalartos* (Cycads). A Report on Their Toxicity, S. African Med. J., *5:* 758–760, 1948.
54. Teas, H. J., Cycasin Synthesis in *Seirarctia Echo* (Lepidoptera) Larvae Fed Methylazoxymethanol. Biochem. Biophys. Res. Commun., *26:* 686–690, 1967.
55. Teas, H. J., and Dyson, J. G. Mutation in Drosophila by Methylazoxymethanol, the Aglycone of Cycasin. Proc. Soc. Exptl. Biol. Med., *125:* 988–990, 1967.
56. Teas, H. J., Sax, H. J., and Sax, K. Cycasin: Radiomimetic Effect. Science, *149:* 541–542, 1965.
57. Vega, A., and Bell, E. A. α-amino-β-methylamino propionic acid, a New Amino Acid from Seeds of *Cycas Circinalis.* Phytochemestry, *6:* 759–762, 1967.
58. Whiting, M. G. Toxicity of Cycads. Econ. Botany, *17:* 269–302, 1963.
59. Whiting, M. G. Food Practices in ALS Foci in Japan, the Marianas and New Guinea. Federation Proc., *23:* 1343–1345, 1964.
60. Williams, J. N., Jr., and Laqueur, G. L. Response of Liver Nucleic Acids and Lipids in Rats Fed *Cycas Circinalis* L. Endosperm or Cycasin. Proc. Soc. Exptl. Biol. Med., *118:* 1–4, 1965.
61. Yang, M. G., Mickelsen, O., Campbell, M. E., Laqueur, G. L., and Keresztesy, J. C. Cycad Flour used by Guamanians: Effects Produced in Rats by Long-term feeding. J. Nutr., *90:* 153–156, 1966.