CYCAD TOXICOSIS AND RELATED CARCINOGENESIS IN ANIMALS

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ABSTRACT

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by Gerald Munene Mugera

Cycads are used as a source of food by man and animals in many parts of the world. They are of particular importance as a source of sustenance during hard times or in areas where the food supply is naturally limited. They are also used for medicinal purposes in geographically widespread areas of the earth.

The impetus for research on cycads has been the recognition of a possible relationship between ingestion of cycad products and the occurrence of a severe paralytic condition in man. The possibility of other harmful effects from consumption of cycad products has also needed exploration.

Horses, cattle, swine and rats were used to study the pathologic effects of cycad nut flour obtained from the plant Cycas circinalis, indigenous to Guam. The flour was incorporated into natural grain rations, making up from 1% to 2% of the ration. The toxicity in animals was evaluated by its effect on the growth rate, symptomatology, hematology, changes in serum enzymes, gross pathology and histopathology. The transmission of the cycad toxic factor(s) through the mammary and placental tissues was evaluated by means of gross and histopathological lesions of suckling animals and fetuses of dams fed cycad.

Although the experiments failed to demonstrate effects on the nervous system, pathological changes were induced in certain organs in all species. In large animals, when trials were conducted for 3-4 months, the most significant gross and microscopic lesions were observed in the liver and kidney; also present were serous effusions in the peritoneal and pleural cavities. The pathologic changes in the livers of cattle and horses were portal fibrosis and slight to extensive bile duct proliferation. Varied degrees of degeneration and regeneration of hepatic cells were noted in all animals fed cycad flour. In swine, a high daily intake of cycad produced both portal and centrolobular fibrosis. The renal lesion was that of chronic interstitial nephritis of variable degree and extent.

In rats, when trials were conducted for 6-10 months, benign and malignant tumors developed, mainly in the liver and kidney. Most of the hepatic tumors were hepatocellular carcinomas or reticulum cell sarcomas, occurring either simultaneously or independently. In 2 rats, hemangiomas were seen in the liver occurring simultaneously with hepatocellular carcinomas. The renal tumors were adenomas, embryonal nephromas or fibrosarcomas.

The histopathologic lesions observed in suckling animals and fetuses of dams fed cycad were similar to those noted in the parent stock, and usually the lesions were more severe. This suggested that the cycad toxic factor(s) are transmitted through the milk and placental barriers and that suckling animals are more susceptible to the toxicosis than their mothers.

Rats which suckled mothers fed cycad, were then weaned and fed a control diet for 6 months, developed kidney tumors.

In rats fed a cycad-containing ration for a short period of time and then returned to a control ration, the liver showed complete or partial regeneration; but a few rats developed kidney tumors.

Pathologic changes were similar in rats fed (1) cycad flour, (2) cycasin, the glycoside isolated from cycads, (3) dimethylnitrosamine, and (4) diethylnitrosamine. The lesions were different from those induced by dimethylaminoazobenzene. This suggested that the cycad toxic factor yields in its metabolic breakdown a carcinogenic compound which may be similar to that produced by dimethyl- or diethylnitrosamine.

The transmission of carcinogens via the milk and across the placenta appears to be a significant discovery. The likely interrelationship of cycad and high incidence of liver cancer in trans-Saharan Africa and Southeast Asia, where cycads are used extensively as food and medicine, is discussed.

This study suggests the need for additional research on tropical herbal medicine, tropical poisonous plants in relation to human and animal diseases, and the extent to which these plants are consumed by man.

CYCAD TOXICOSIS AND RELATED CARCINOGENESIS IN ANIMALS

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INTRODUCTION

Cycad seeds or stems are used mainly as a source of food starch in many parts of the world. They are of particular importance as a source of sustenance during hard times or in areas where the food supply is naturally limited. They are also used as medicine in geographically widespread areas of the earth. In some areas cycads are used as fodder for livestock (Thieret, 1958).

The seeds or nuts have a coat differentiated into an outer, fleshy layer, usually some shade of red with a more or less orange cast, and an inner, stony layer. The bulk of the seed is a firm, starchy endosperm, containing the embryo. Seeds vary in length from 1.3 to 7.5 cm. The biggest are those of Cycas sp. (Chamberlin, 1919).

Cycads are palm-like plants with starch-rich stems which may be either aerial and cylindrical, as in Macrozamia reidlei, or subterranean and tuberous, as in Macrozamia spiralis. In either case the stem is surmounted by a crown of very large, coarse, stiff, featherlike leaves. Cycad leaves average perhaps 1 m. long. The extremes are those of Zamia pygmala, only 5-6 cm. long, and those of some species of Encephalartos, reported to reach a length of 5-6 m.

Cycads are thought to be the surviving remnants of an ancient line of plant families which probably predominated over other vegetation during the mesozoic era. They are thought to represent an intermediate evolutionary step from fern to flowering plants and are now restricted to tropical and subtropical regions, where they are well adapted to adverse conditions and survive when other plants are destroyed by

hurricane or drought. They are found in all continents except Europe. Cycads belong to the Cycadacal family of the plant kingdom, which has 9 genera. These genera and their approximate distributions are as follows:

Cycas: ranging from northern and northeastern Australia, New Caledonia, Samoa and Fiji north and west to the Ryukyu Islands, southern China, the Philippines, Indochina, Thailand, Malaya, Indonesia, Burma, India, Ceylon, south Pakistan, Madagascar, Zanzibar and the East African coast.

Encephalartos: ranging from the Union of South Africa, Mozambique, Southern Rhodesia and northeast Angola north to Kenya, southwest Sudan, Chad and Ghana.

Stangeria: eastern coastal region of South Africa.

Bowenia: northeastern Queensland, Australia.

Ceratozamia: southern Mexico and Guatamala.

Dioon: southern Mexico and Honduras.

Macrozamia: eastern, central and southwestern Australia.

Microcycas: Pinar del Rio and Cuba.

Zamia: Florida, West Indies, southern Mexico south to Brazil and Chile (Thieret, 1958).

Scientific interest in the role of cycads in disease is not new. Search for the plants' toxic ingredients began in the 19th century, but little progress was made until Copper isolated a glycoside from Macrozamia spiralis (Copper, 1941). Several glycosides have since been isolated and found to be lethal to animals.

The new impetus for research on cycads has been the recognition of a possible relationship between ingestion of cycad material and occurrence of a severe paralytic condition in man. On the island of Guam, the incidence of amyotropic lateral sclerosis has been found to be more than 100 times greater among the natives than among the residents of areas such as the Continental United States (Kurland and Mulder, 1963). In the search for causes of amyotropic lateral sclerosis, investigations have been focused on the toxic factors of Cycas circinalis, which Guamanians have utilized for a long time as a source of food starch.

The present experiments were conducted to investigate the possible relationship of amyotropic lateral sclerosis to the ingestion of products of Cycas circinalis. The research is presented in 3 parts: (1) cycad toxicosis in swine and horses, (2) pathology of cycad toxicosis and related carcinogenesis in rats, and (3) transmission of cycad toxic factor(s) through the mammary and placental tissue.

REVIEW OF THE LITERATURE

Cycads as Medicine

Whiting (1963) published a detailed literature review of cycad toxicity. According to her survey, cycad mixed with beverages and teas is prescribed as a purge, pain depressant and emmenagogue. The characteristics of the plant which may contribute to its therapeutic value for external as well as internal uses are the hardening of the exudate upon exposure to air and the astringent and mucilaginous properties of the plant sap. Cycad starch likewise has a mucilaginous property (Tryon, 1955). In the Dominican Republic the Zamia nut is fried in oil and mixed with mashed mamey and avocado seeds and the mixture is recommended for a therapeutic shampoo; and from Zamia stem gum is extracted which is used in treating ulcers.

Whiting (1963) reported that Cycas seeds pounded to a paste in coconut oil are used for sores, swellings, wounds, boils and various skin complaints in parts of southern and southeastern Asia, Indonesia and the Philippines. The sap squeezed from the nut of Zamia muricuta is described as a drastic purge in Venezuela.

Drury (1958) in his description of useful plants in India stated that the gum extracted from the stem of Cycas circinalis was used as an antidote for snake bite, the gum being applied to the wound, while the nut from the same plant was ground to fine powder, mixed with sugar and water and prescribed as a laxative.

Kirbikar (1933) reported that in Cambodia the mucilaginous terminal bud of Cycas circinalis crushed in rice water or in water holding in suspension fine particles of clay was used in the dressing of ulcerated wounds, swollen glands and boils.

Whiting (1963) described the treatment of tropical ulcers in Guam with Cycas circinalis nut as follows:

> "The mature nut is opened and the meat is scraped out. The meat is then squeezed out and the juice is allowed to run on the ulcer. This is said to cause great pain. After the juice has been put on the sore, the grated flesh is then put on it and the sore covered. This is done once a day for three or four days."

Okabe (1940) stated that Cycas revoluta nut was ground to fine powder and prescribed as an emmenagogue and astringent at the rate of 10-20 Gm. per day in the Ryukyu Islands.

Ondaatje (1862) stated that Cycas circinalis seed flour, in Ceylon, was highly esteemed as a remedy in bowel complaints and hemorrhoids, for which purpose the flour was boiled and eaten.

Cycads as Food

Cycad as food for man can be grouped into 4 categories: (1) uncooked, (2) boiled, (3) extracted dry starch, and (4) fermented starch.

Whiting (1963), in her survey, mentioned that in Guam the green outer husks of the seeds of Cycas circinalis are chewed to relieve thirst and when dried are considered a tasty sweet. Harvey (1945) reported similar use of husks of Cycas media in Australia by the Karawa tribe of Australian aborigines. Thieret (1958) reported that in several areas in Africa the raw gum or exudate from a species of Encephalartos is chewed by children. He also mentioned that succulent

leaves of several species of Cycas are cooked and eaten as vegetables in the Malay Peninsula, the Philippines and Indonesia; and the tender leaves of Cycas circinalis are said to be eaten by Ceylon natives as curries. On the Fiji Islands, Degener *1949) mentioned that the ripe nuts of Cycas circinalis are boiled until they are soft and the white flesh is eaten. In Ceylon, among the Vaeddas, Thieret (1958) mentioned that the Cycas seed kernels are cut into slices, dried and ground into a meal. A dough made from this meal is then baked into cakes.

Williams (1949) reported that on the East African coast sun-dried Cycas kernals were fermented in a tin with layers of banana leaves for about a week. They were next cleared of mold, soaked a day in water to soften and finally powdered and used as a porridge. He also mentioned that the spongy farinaceous center of the trunk gwede was used in Zanzibar as food in times of shortage. It was prepared by chopping small pieces, then heaping for about a week to allow fermentation to take place for neutralization of certain toxic substances. They were then washed, preferably in hot water, and dried in the sun, after which they were pounded and used in porridge; or the flour was placed in boiling water and cooked for some time, after which it was stirred into a thick paste known as "ugali".

Schweinfurth (1874) described the use of Encephalartos stem for preparation of intoxicating beer in Central and East Africa. Juritz (1915) described the preparation of Kaffir bread from starch extracted from stems of Encephalartos sp. in South Africa and Mozambique.

Maiden (1899) reported that one of the most important sources of food for the New South Wales aborigines was seeds of Macrozamia. Before the kernels could be eaten they were made innocuous by a process of

roasting, pounding and soaking in water. He also described the methods of preparation of starch from nuts of Macrozamia in Australia. The methods were similar to those used in East Africa.

Whiting (1963) reported that in Guam nuts of Cycas circinalis were split, soaked for varied lengths of time up to 18 days, with at least 4 changes of water, dried and ground, then used to thicken soup, mixed with coconut milk or fermented coconut sap and cooked or wrapped in banana leaves and steamed or boiled before eating. The dried powder or sliced seeds in Guam are kept from season to season. Whiting also mentioned that although cycad starch was no longer manufactured in the United States, it had been a pioneer industry in southern Florida as early as 1845. Most of this starch, which was marketed under the name "Florida arrowroot", was used for infant foods, biscuits, chocolates and spaghetti.

Cycad as Fodder for Animals

Chamberlin (1919) reported that while he was in Africa, gibbons interrupted his botanical collections of Encephalartos by carrying away the cones of the plant just as the seeds reached maturity. White (1928) mentioned that roots and piths of Macrozamia stems were ground and boiled to a thick jelly-like paste and used for feeding pigs, calves and poultry in western Australia. He also reported that cattle and sheep selectively grazed on young shoots of growing cycads.

Cycads as Poison to Humans

Accompanying the uses of cycads as food and medicine, several ill effects have been reported and have been attributed to the eating of inadequately prepared cycad products. All genera of cycads have been

implicated except Ceratozamia and Stangeria. Banks (1770) reported that a number of Captain Cook's men during their exploration in the Pacific developed strong fits of vomiting after eating nuts of Cycas media. Reitz (1930) in his book Commando related an incident wherein a number of men, including General Smuts of the Boer Commando, became violently ill from eating the nuts of Encephalartos altensteinii. Fitzgerald (1898) reported that in Madagascar it was well known that unless cycad starch were carefully prepared it would cause fatal diarrhea and vomiting. Safford (1905), of the United States Navy, wrote that Guamanians were well aware that cycad starch was injurious to health if consumed for any length of time. Maiden (1819), in Australia, reported children developing fever lasting a few days followed by constipation and paralysis of the lumbar region after eating Zamia nuts.

Standley (1931) reported that Zamia roots from Zamia furfuraceas were used in Honduras as a poison for criminals and in Costa Rica for both criminals and enemies. Whiting (1963) reported that the Guamanian, even today, believes that "Iytiko" - amyotropic lateral sclerosis - is caused by the handling and consumption of cycad plant products.

Cycads as Poison to Animals

Outbreaks of cycad poisoning have been known in Australia since the beginning of the vast sheep and cattle industry in the 19th century. Bryant (1900) reported loss of 200 head of cattle in the Saltash District in New South Wales between 1877 and 1884 after eating growing species of Macrozamia. Marks (1912) described the loss of 400 head of cattle from a herd of 900 after eating leaves of Macrozamia spiralis.

The animals developed paralysis of hind limbs and loosening of the horns before death. Cleland (1914) reported 2 effects in sheep and cattle after ingestion of Macrozamia spiralis material, one of which was an acute condition characterized by severe gastrointestinal disturbances and another of which was a partial paralysis involving the hind limbs. Seddon et al. (1931) noted that death occurred in sheep within 18 hours after eating leaves of Macrozamia spiralis. This was followed by 80 deaths daily for a week in 2 flocks of sheep. They reported total mortality of 2,200 sheep out of 6,000.

Chemical Research

Chemical studies on cycads were focused on the isolation and identification of the toxic constituents of the plants. Lauterer (1896) and other early researchers in Australia directed their investigations towards such substances as resin, mucin and oxalic acid.

Dongen (1903) isolated a toxic principle from nuts of Cycas circinalis. The substance was an amorphous glycoside, which was soluble in water and insoluble in alcohol. He named the substance Pakoein. Nishida (1939) produced formaldehyde from Cycas revoluta seeds by the action of emulsin which was present in the seeds.

Cooper (1941) isolated a crystalline compound from the seeds of Macrozamia spiralis which she named Macrozamin. Macrozamin was shown to be toxic to guinea pigs when administered orally but not when injected subcutaneously. Langley et al. (1951) isolated Macrozamin from other species of macrozamia and proposed a methyl azoxy-methanol $CH_3N{=}N{-}CH_2OH$ as the structure for the aglycone of Macrozamin. Riggs (1954) isolated Macrozamin from other genera of cycad, Cycas, Bowenia, and Encephalartos. All the compounds were similar except for the carbohydrate moiety.

Nishida et al. (1955) isolated a glycoside, which they named cycasin, from seeds and pith of a Japanese species of Cycas revoluta. Matsumoto and Strong (1963) isolated cycasin from Cycas circinalis nuts from Guam and confirmed the presence of methylazoxymethanol in cycasin.

$CH_3N{=}N{-}CH_2OH$

methylazoxymethanol

$CH_3{-}N{=}N{-}CH_2O\overset{O}{C}C_6H_{11}O_5$

cycasin

Kabayashi and Matsumoto (1964) demonstrated that methylazoxymethanol was the toxic factor in cycasin. This compound produced formaldehyde when heated with an acid, and cyanide when heated with a base. The compound decomposed about 12% in 5 days' standing at room temperature. It decomposed 75% when heated for 10 minutes at 75 C. and 100% in boiling water for 10 minutes. The substance was soluble in water and ethyl ether. Campbell (1964) found that the toxic factor in Cycas circinalis nut flour was readily destroyed by both moist and dry heat. When cycad flour was cooked for 1 hour with enough water to make a thin gruel and then dried at 37 C., all its toxicity was destroyed. This was also true when the dry flour was heated to 90 C. for 1 hour.

Animal Research

Farm Animals

Banks (1770) reported the first animal feeding experiment with cycad. After members of his party had become violently ill from eating cycad nuts during Captain Cook's voyage to Australia, they decided to test the nuts on hogs. When the hogs ate the nuts, 2 died within a week and the rest recovered, but with difficulty.

Turner (1893) recorded an experiment conducted by Norton, a grazier of Yeppoon, in Australia. Norton fed a heifer in a small paddock with Zamia leaves and fruits. The animal developed paralysis of the hindquarters. Edwards (1894) reported results after feeding cattle with Macrozamia fruseri in western Australia. The animals developed the following symptoms: the skin covering the ears, particularly at the tips, became bare and had a tendency to curl, just as in cases of poisoning by ergot on rye. In the more severe cases Edwards stated that the hair loosened on the whole body, especially on the extremities. Between 3 and 9 days, animals developed paralysis of the hindquarters. At necropsy Edwards reported paleness, hemorrhage and atrophy of muscles of the hindquarters, patchy congestion of the liver and kidney, constriction and occlusion of the terminal branches of arteries, hemorrhagic exudate in the spinal column, congestion of the arachnoid membrane of the spinal cord, and softening of the spinal cord just posterior to the middle of the loins.

Lauterer (1898) fed leaves of Macrozamia spiralis to 3 calves. The gait was not affected; however, all 3 died with severe gastroenteritis.

Hunt (1899) fed leaves, stem, bulbs and fruits of Macrozamia miquelii and Cycas circinalis to cattle at the rate of 2-4 pounds per day. The animals developed paralysis of the hindquarters after 14 days. Seddon et al. (1931) reported some experiments after a heavy loss of sheep fed nuts of Macrozamia spiralis. They used the starchy kernels and membranes. In some of their trials they used boiled and dried nuts. They gave drenches of powdered kernels and membrane to 451 adult sheep. One pound of the material was finely minced and

suspended in 16 oz. of water for each animal. Death usually occurred within 36 hours. When a smaller dose of the same material was given to 493 adult sheep, no ill effects were noted for 8 days. On the 9th day the feces were covered with blood-stained mucus and the appetite was depressed. On the 20th day the animals started eating well and gradually recovered. Seddon summarized the necropsy lesions as follows:

A. Gross lesions. Ecchymotic hemorrhages in the subcutaneous and intramuscular connective tissue were present in sheep which died acutely. Those that died later (had lived for more than 4 days) exhibited icterus; hemorrhagic fluids in the abdominal cavity; venous congestion, friable liver; edema, congestion, and petechiation of the mucosa of the stomach; hemorrhages, congestion of small intestine; hydrothorax and scattered subpleural hemorrhages on the lungs.

B. Microscopic lesions. Cloudy swelling and coagulative necrosis of the liver, congestion, coagulative necrosis of the tubules, with interstitial hemorrhages in the kidney, and marked congestion of the spleen were present. The gastrointestinal tract showed congestion and necrosis of the epithelium.

In experiments with cattle, Hall (1957) fed 3 groups different species of Macrozamia leaves. To the first group he gave fresh leaves of Macrozamia pauli guilielmi, to the second group he gave a mixture of fresh and dry leaves of Macrozamia douglasii, and to the third group he gave dry leaves of Macrozamia spiralis. Feeding the fresh leaves of Macrozamia pauli guilielmi produced an acute condition with ataxia terminating fatally between the 28th and the 29th day in 2 heifers. No symptoms were noted in the animals fed leaves exposed to the air for 2-3 weeks. At necropsy of the heifers, the subcutaneous tissue

and visceral fat were edematous, with many petechiae. The liver was swollen, yellow, hard to cut and apparently fibrosed. Microscopic sections showed advanced portal cirrhosis and some centrolobular fibrosis and vacuolated hepatic cells. The abomasal mucosa was edematous and showed a loss of nuclei of epithelial cells and ragged cell walls. No lesions were demonstrated in the spinal cord or brain sections when stained with hematoxylin and eosin and Marchi technique, even though ataxia was present.

Laboratory Animals

Walt (1944) drenched 3 rabbits with 15, 30, and 70 Gm., respectively, of finely mixed nuts of Encephalartos horridus suspended in water. The rabbits died in 1-3 days. The post-mortem changes were general icterus, severe hydrothorax, slight emphysema and severe hyperemia and edema of the lungs, regressive changes in the myocardium and kidneys, very severe regressive changes in the liver and orange-colored urine. Histological sections revealed fatty degeneration of the myocardium, with initial focal myocarditis and a few intramyocardial hemorrhages, severe hyperemia and catarrh of the bronchi, perilobular interstitial hepatitis, as well as necrotic hepatitis of central and sublobular distribution, desquamating reticulo-endothelial reaction and severe hyperemia of the spleen, and fatty degeneration of the renal epithelium of the proximal convoluted tubules and, to a lesser extent, of distal convoluted tubules. Similar lesions were observed in rabbits when fresh ripe nuts of Encephalartos lehmanii were fed.

Steyn et al. (1948) drenched rabbits with minced fresh nuts of Encephalartos lehmanii and Encephalartos horridus at the rate of 6.3 Gm./kg. body weight for 3 days. Before death of the rabbits he

observed the following symptoms: anorexia, diarrhea and apathy. The rabbits died on the third day, and the following post-mortem lesions were noted: general icterus, hydrothorax, hydroperitoneum, hyperemia and edema of the lungs with petechiae throughout the lung tissue, congestion of the gastrointestinal mucosa, myocardial hemorrhages, pronounced degenerative and fatty changes in the heart and liver, and pronounced congestion of the spleen.

They described the microscopic lesions as follows:

> "There were myocardial petechiae, fatty degeneration of the myocardium and an incipient focal myocarditis, pronounced congestion of the lungs and desquamative bronchial catarrh in the early stage; central and sublobular hepatitis and a perilobular interstitial hepatitis associated with fatty degeneration; pronounced congestion of the spleen; and fatty degeneration of the epithelium of tubuli contorti."

In guinea pigs, by Nishida et al. (1959), guinea pigs were given cycasin isolated from Cycas revoluta. They observed the following symptoms: anorexia, atonic paralysis of hind legs in 20 hours, and respiratory distress. The guinea pigs died in 2 days. They summarized post-mortem lesions as stagnation and bleeding in the lungs. Microscopically they noted degenerative changes in the lungs, liver, kidney and adrenals.

Rats were fed by Laqueur et al. (1963), 1% and 2% cycad meal obtained from Cycas circinalis nut indigenous to Guam. They reported the development of tumors in the rats after chronic ingestion of cycad nut meal as a part of their diet. They found hepatocellular carcinoma and reticulo-endothelial proliferation in the liver occurring simultaneously but independently, adenomas and undifferentiated tumors of the kidney, 1 primary pulmonary adenoma of the lung with foci of squamous metaplasia, and 2 primary mucinous adenocarcinomas of the

large intestines. They also reported peculiar vascular changes characterized by "subintimal hyalinosis" of capillaries, limited to the undifferentiated tumor of the kidney. With acute ingestion of cycad meal they noted edema of the pancreas, centrolobular necrosis of the liver lobules and marked fatty infiltration of the myocardium.

Nitrosamines

The pathology induced by ingestion of cycad products simulated in some respects that induced by nitrosamines and butter yellow. Therefore, studies were carried out to determine whether the lesions produced by these substances were similar to those produced by cycad.

Dimethylnitrosamine $(CH_3)^2N\text{-}NO$ is a mobile yellow liquid with a faint characteristic odor. It boils at 151 C., and its specific gravity is 1.815 at 20 C. It is soluble in all proportions with water, ethyl alcohol, methyl chloride or vegetable oil. Under ordinary conditions it is stable, but with zinc dust and acetic acid it can be reduced to dimethylhydrazine $(CH_3)^2N\text{-}NH_2$.

Diethylnitrosamine $(C_2H_5)^2N\text{-}NO$ is a mobile, slightly yellow liquid, soluble in water, ether and alcohol.

The nitrosamines and their derivatives are used in industries, primarily in Europe, in the vulcanization of rubber and preparation of textile fibers. The appearance of 2 cases of cirrhosis of the liver among 3 men working in an industrial research laboratory using dimethylnitrosamine led to suspicion of dimethylnitrosamine as the causative agent (Barnes et al., 1954). Barnes and Magee (1954) conducted an experiment with albino rats, mice, guinea pigs, rabbits and dogs. They gave a single oral dose of dimethylnitrosamine in water to 1 group of animals in each species. The other group was administered the chemical

by injection, either undiluted or in an aqueous solution. The third group was given the chemical in food first mixed in arachis oil before being added to the diet at the rate of 10 ml./kg. They reported a single oral lethal dose of less than 50 mg./kg. in rats, 15 mg./kg. in rabbits, 20 mg./kg. in mice, and 50 mg./kg. in dogs. They also reported extensive liver necrosis in all species which received a single lethal dose either by mouth or by injection. At necropsy they found various amounts of blood-stained fluid in the peritoneal cavity, a jelly-like edema of the pancreas and omentum, hemorrhages into the lumen of the upper part of the intestines, and swollen, dark and mottled livers. Histologically, they reported extensive necrosis of the liver.

In a second report, Magee and Barnes (1956) described development of primary hepatic tumors in rats after chronic ingestion of dimethylnitrosamine in their diet at the rate of 50 p.p.m. Tumors developed between 26 and 40 weeks. They classified the tumors as cystadenoma, fibrosarcoma and hepatoma, with a few metastases to the lung. They observed bile duct hyperplasia on several occasions.

In another report, Magee and Barnes (1959) described incidences of tumors in rats living 40 weeks or longer after dimethylnitrosamine (DMN) was given at various dose levels. They observed liver tumors when DMN was fed at 50, 20 and 10 p.p.m. No kidney tumors were present. When 50 p.p.m. of DMN was given for 4 weeks and then animals given a normal diet until the end of the experiment, there were no tumors; but when this level was fed for 2-3 months and then the animals fed a normal diet, both kidney and liver tumors were observed. When a higher dose (100 p.p.m.) was given for 1 week, no tumors were seen; but when it was given for 2-4 weeks, out of 16 rats they observed 10 kidney tumors and

1 liver tumor. When 200 p.p.m. of DMN was given for 1 week and then rats fed a normal diet, out of 4 rats there were 4 kidney tumors and no liver tumors.

In a recent study, Magee and Barnes (1962) described the condition in which high incidence of the kidney tumors could be produced by feeding dimethylnitrosamine in the diet. In this experiment a high dose for a short time produced tumors of the kidney, without liver tumors.

Schmal et al. (1959) reported development of liver tumors similar to those reported by Magee et al. (1956) from chronic ingestion of dimethylnitrosamine and diethylnitrosamine. Zak et al. (1960) reported renal and pulmonary tumors after chronic ingestion of dimethylnitrosamine. Argus (1961) reported tumors of the kidney, liver and lung after chronic ingestion of dimethylnitrosamine and diethylnitrosamine in the diet. From this work it appeared that diethylnitrosamine is a much more rapidly acting carcinogen than DMN for the liver, less active towards the kidney and inactive towards the lung. Magee and Hultin (1962) demonstrated methylation of rat liver protein and nucleic acid when rat liver slices were incubated with DMN in vitro. Magee and Farber (1962) described experiments in which they had injected C^{14}-labeled dimethylnitrosamine intraperitoneally to rats. Their experiments demonstrated methylation of protein and nucleic acids. The methylation of protein occurred in the histidine part of the protein molecule at positions 1 and 3 of the imidazole ring. Methylation in nucleic acids occurred in guanine, producing 7 methylguanine.

P - Dimethylaminoazobenzene
(butter yellow $C_{14}H_{15}N_3$)

This compound has yellow crystals, is insoluble in water, soluble in alcohol, benzene, chloroform, ether petrol, ether, mineral acids and oils. Its uses are for determination of free HCl in gastric juice, microscopic identification of fats (particularly margarine) and also as a pH indicator.

Yoshida (1932) described the first induction of tumors of the liver as a result of administration of azo dyes. Sasaki and Yoshida (1935) described the histology and histogenesis of the tumors induced by o-aminoazotoluene. They described the sequence of the changes they saw in the liver, and appeared to regard the process as starting with hyperplasia of the periportal parenchyma, which is continuously progressive until the stage of cancer is reached.

Heep (1936-37) described the sequence of the changes induced by o-aminoazotoluene and suggested that degenerative and regenerative changes play an important role in the induction of cancer by this compound. Kinosita (1937) reported that liver tumors were much more rapidly induced by p-dimethylaminoazobenzene than by o-aminoazotoluene and reported the primary change of the liver as hyperplastic, although degenerative changes also occur.

Orr (1940) described the sequences of histological changes in the liver of white rats induced by p-dimethylaminoazobenzene, or butter yellow. The butter yellow was added to the rats' food and the rats were killed at intervals throughout the experiments. He described the usual sequence as proliferation of connective tissue cells in the portal system, extension of granulation tissue from the latter into the parenchyma, with degeneration of the contiguous liver cells, atypical regenerative proliferation of bile duct and liver epithelium,

leading ultimately to nonarchitectural nodular hyperplasia and a macroscopically hobnailed liver, in which, in a certain proportion of cases, tumors arose. He described 3 types of liver tumors resulting from chronic ingestion of butter yellow in the food. The first tumor to be noted was bile-duct adenocarcinoma, which was noted after 111 days of feeding a diet containing butter yellow. Other tumors seen were hepatic-cell carcinomas and bile-duct cystadenomas. In several livers he observed a combination or occurrence of all 3 types of the tumor, or 2 types in 1 liver.

Edwards and White (1941) reported pathologic changes, with special reference to pigmentation and classification of hepatic tumors, in rats fed p-dimethylaminoazobenzene. They reported early changes as extensive fatty infiltration in hepatic cells within 2 weeks. Cirrhosis of the liver was noted in 100 days, and bile duct proliferation became apparent by the 60th day. Of 106 rats, 66 developed primary hepatic carcinomas. They divided the tumors into 2 major groups - hepatomas and adenocarcinomas. They divided hepatomas into 2 subtypes, designating the more highly differentiated form Type I. In this type the cells resembled the hepatic tissue very closely. The less differentiated form was designated as Type II. This showed the general structural pattern of liver, but the tumor cells had no striking resemblance to hepatic cells.

They described 3 types of visceral pigmentation. One was an iron-containing, granular, brown pigment found in the liver, spleen and lymph nodes. The second pigment was found in tubular cells and macrophages of the renal cortex. This was brown and granular and gave negative tests for iron. They suggested this pigment represented deironized

blood pigment. The third pigment, also iron-free, was deposited in the liver and lymph nodes as a canary-yellow material and stained with lipid stains.

Richardson and Borso-Nachtrebal (1950) reported development of liver tumors and histological changes in other organs in rats fed an azo dye, 3'-methyl-4-dimethyl-aminoazobenzene. They described 5 types of malignant neoplasms of the livers. Three types were parenchymal tumors, which they classified as hepatomas, both of small-cell and large-cell subtypes, adenocarcinomas and anaplastic carcinomas. The other 2 types were stromal types, classified as fibrosarcomas and angiosarcomas, respectively. In addition, they noted nonmalignant biliary adenomas on several occasions.

Price et al. (1951) described progressive microscopic alterations in the livers of rats fed the hepatic carcinogens 3'-methyl-4-dimethyl-aminoazobenzene (3-Me-DAB) and 4-fluoro-4-dimethyl-aminoazobenzene (4'-F-DAB). The carcinogens were fed in the diet at the rate of 0.064 mg./kg. of diet. Grossly, the first changes in the livers appeared at 18 days and consisted of pallor and a fine granularity of the surface. This was more conspicuous at 21 days, at which time ascites and pancreatic edema were also seen. At 48 days, the liver appeared very cirrhotic. At laparotomy on the 9th day, 3 of 17 rats fed 3'-Me-DAB and 6 of 19 fed 4'-F-DAB had small tumors. At 111 days, 9 of 14 animals fed 3'-Me-DAB and 10 of 13 fed 4'-F-DAB had gross lesions at autopsy. At 133 days, all survivors had tumors. Microscopically, the earliest changes in the livers were seen after 7 days of ingestion of the carcinogen, when small hyaline droplets appeared in the cytoplasm of some of the parenchymal cells. By 11 and 14 days

they were found in the cells of most liver lobules. As the size of the hyaline droplets increased, they assumed the shape of a sausage or horseshoe, and eventually some of these hyaline inclusions encircled the nuclei.

The second change in the livers in rats fed 3'-Me-DAB was an increased prominence and cytoplasmic basophilia of bile ducts. This occurred between the 11th and 14th days; with 4'-F-DAB, similar changes were noted at the 20th day. At 18-24 days a slight increase of bile-duct cells had occurred. At 26-28 days these cells had penetrated close to the central vein.

The malignancies that were produced were classified as hepatomas, cholangiomas and mixed tumors. In addition, bile-duct adenomas were noted on several occasions. The mixed tumors were often the larger ones and presented areas characteristic of hepatomas and cholangiomas, often with blending of one type into the other.

Richardson et al. (1959) reported that, for development of tumors of the liver, rats need only be fed 0.06% of dimethylaminoazobenzene for 7 weeks. They obtained their results by feeding the dye for several weeks, then discontinued the dye and placed the rats on a basal diet until the end of 16 weeks.

PART I. CYCAD TOXICOSIS IN SWINE AND HORSES

Experiment I. Swine

INTRODUCTION

Weanling pigs were used as the experimental animal in the first studies on cycad toxicity because (1) they were available, (2) they were of a size that would provide for collection of ample tissue material for pathologic studies, and (3) they facilitated clinical observations. The pigs were housed at barn number 5 at the veterinary research farm and maintained in individual metabolism cages.

The cycad flour used in this experiment and all later experiments was prepared as follows: the nuts of Cycas circinalis were harvested and collected at various times on the island of Guam and shipped to the National Institutes of Health. The cycad flour was prepared there in Dr. J. C. Kerestesy's laboratory by first removing the shells, cutting the kernels into small pieces, drying in a vacuum oven at 40 C., and then grinding into a fine powder in a Wiley mill. The cycad flour was shipped to Michigan State University and stored at -20 C. until needed.

EXPERIMENTAL PROCEDURE

Four Yorkshire pigs weighing approximately 50 pounds and 10 weeks of age were used. They were divided into 2 groups of 2 pigs each, as shown in TABLE 1. A balanced, growing type swine ration obtained from the swine unit of the Animal Husbandry Department was fed. The 2 control pigs were fed the basal ration, and the 2 experimental pigs were

fed the basal ration plus 2% unwashed cycad flour. The rations were fed on an ad libitum basis and fresh water was supplied continuously. The experiment was started November 7, 1962, and terminated February 5, 1963. The pigs were observed daily and weighed at weekly intervals. The calculated daily intake of cycad flour was 0.86 Gm./kg. body weight in each of the experimental pigs. At the end of 60 days, 1 pig on each treatment was killed, necropsied and selected tissues collected for histopathologic examination. At 90 days, the remaining 2 pigs were killed, necropsied and tissues collected for histopathologic studies.

At necropsy the tissues were collected and prepared for histopathologic examination in the following general manner: the sections were selected from all macroscopic lesions and routinely from the following organs - brain, liver, heart, pancreas, kidney, lungs, stomach, duodenum, and jejunum. They were preserved in acetate-buffered, 10% formalin. Sections of liver and kidney were fixed in Carnoy's fixative. Routine sections were stained with hematoxylin and eosin. The following special stains were used for selected materials: Heidenhain's aniline blue stain for connective tissue; Gridley's reticulum stain; periodic acid-Schiff reaction; crystal violet stain; Best's carmine; and Sudan IV. The histological and staining procedures followed were according to the Armed Forces Institute of Pathology Manual of Histological and Special Staining Technics (1957).

RESULTS

The weight gains during the experimental period and the weights of selected organs at the time the pigs were necropsied are given in TABLE 1. While the total number of animals was limited, the average weight gains were much less in the experimental pigs than in the controls.

Gross and microscopic lesions were very evident in the experimental pigs, while no significant lesions were noted in the control pigs. The lesions noted in the 2 experimental pigs are shown in Figures 1 to 11 and given in detail as follows:

Lesions

Liver. The livers of experimental animals killed at 60 and 90 days appeared enlarged, firm in consistency and had whitish patchy, apparently fibrotic areas.

Microscopically, the hepatic parenchymal cells showed a widely varied histological picture. Most of the cells were enlarged. Their cytoplasm was vacuolate. Many parenchymal cells had giant nuclei and some were multinucleated, with up to 5 nuclei. The cytoplasm of the enlarged cells showed many eosinophilic hyaline droplets.

Most of the lobules showed a generalized cirrhosis with bands of fibrous connective tissue connecting more solid masses of connective tissue in centrolobular and periportal areas. In the central areas of some lobules the central veins had been obliterated by the well formed fibrous tissue. In other cases the fibrous tissue was found in the form of a ring surrounding the central vein, where the wall of the central vein remained intact but collapsed due to fibrous tissue. In such cases the collapsed small slits of central veins were seen. The central fibrosis was equally extensive whether the central veins were occluded or not. The connective tissue connecting portal and central fibrous tissue varied in amount. In central areas within the proliferating connective tissue hemosiderin could be observed. In portal areas there were regenerating liver cells in the form of nodules. These consisted of small liver cells which in most cases were binucleate and very closely

packed without having the normal orientation pattern of hepatic cells. In addition, there was a varied proportion of mature and proliferating connective tissue and, in some areas, bile duct proliferation was very extensive.

Kidney. There was no visible gross lesion in the kidneys. Microscopically, there was a variable amount of fibrosis of the renal cortex. The fibrosis was mainly interstitial and was associated with mononucleated cell infiltration. In a few areas the glomerular tufts were hyalinized and only eosinophilic debris remained. The Bowman's capsule had proliferated at its parietal layer and was continuous with the interstitial connective tissue.

Lungs. Grossly the lungs were congested and edematous. The pig killed after 90 days on the experimental diet had more extensive edema of the lungs and a few ecchymotic hemorrhages of the lung parenchyma. A catarrhal bronchitis with a foamy exudate was observed in both pigs.

Peritoneum. Ascites with varied characteristics was seen in the 2 experimental animals. In the pig killed after 60 days on the experimental diet, 500 ml. of clear yellowish fluid were removed from the peritoneal cavity. In the animal killed after 90 days, 800 ml. of clear serous fluid were removed from the peritoneal cavity and about 100 ml. from the pericardial sac. Only a very small amount of fat was present in the fat depots.

Pancreas. Microscopically, slight interstitial edema and occasional vacuolation of acinar cells was seen.

Heart. Grossly, the heart appeared enlarged and flabby. Microscopically, there appeared to be a slight increase of heart muscle nuclei which had an appearance of nuclear beading in some areas.

Gastrointestinal tracts. The fundic and pyloric regions of the stomach revealed necrotic ulceration. Erosions were present in the duodenum. In the remainder of the small intestine a catarrhal enteritis was present. An exudate was seen in the submucosa and mucosa of the stomach and intestine. It was predominantly serocellular, including mainly eosinophils, macrophages and a few neutrophils.

Experiment II. Horses

INTRODUCTION

Horses were suggested as a desirable experimental animal to investigate further the possibilities of the cycad flour producing a specific neurologic lesion. The nervous system of the horse has been studied in some detail, and other researchers have mentioned that the horse is more susceptible to neurotoxins than other experimental animals.

EXPERIMENTAL PROCEDURE

Four male horses, approximately 2 years of age, were used in this experiment. They were maintained at the veterinary research farm. The experiment started June 19, 1963, and terminated October 26, 1964. Two horses were assigned to an experimental group to be fed 2% unwashed

cycad flour in an oat basal ration, and other 2 were the controls, fed only the basal oat ration. To improve the palatability of the ration containing the cycad flour and also to hold the cycad flour with the oats, 4 pounds of equal parts of water and molasses were added to 12 pounds of oats and cycad flour. The cycad flour was fed to the 2 horses at the rate of .1 Gm./kg. body weight for the first 15 weeks and then at the rate of .19 Gm./kg. body weight for 9 weeks. The oats were fed at the rate of .5 pound per 100 pounds body weight. Each horse was individually fed its assigned ration.

The horses were weighed and blood samples collected at weekly intervals for the first 3 months and thereafter at selected intervals. Blood samples were collected for analysis using ethylene diaminotetra-acetic acid as the anticoagulant. Two to five milliliters of blood were taken for hematologic examination. Five to ten milliliters of blood were taken without using an anticoagulant for serum enzume determinations. Hemoglobin was determined by the cyanmethemoglobin method. Packed cell volumes were determined by the micro method (capillary tube), white blood cell counts were done with Turk's diluting fluid and hemocytometer. Blood smears were made at the time of bleeding and then stained with Wright's stain and differential white blood cell counts made. Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were determined according to the methods of Reitzman and Frankel (1957), as outlined in Sigma Chemical Company's 1963 Technical Bulletin No. 505. Blood bilirubins were determined according to Benjamin (1961).

RESULTS

The data on weight changes for both experimental and control horses are presented in TABLE 2. TABLE 3 summarizes the blood SGOT, SGPT, bilirubin, hemoglobin and packed cell volume.

All animals gained weight at approximately the same rate. There was a rise in SGOT after 7 days of ingestion of the diet containing cycad flour. The rise reached its maximum after 28 days of ingestion of the diet containing cycad flour. After the maximum rise there was a steady fall of SGOT, but it remained higher in the experimental than in the control, even at the end of the experiment. Bilirubin began to rise after 35 days of ingestion of the cycad-containing diet and continued to rise steadily until the end of the experiment. There was no significant difference in either SGPT or hemoglobin between the control and experimental groups, although there were individual variations. Gross and microscopic lesions were noted in experimental horse number 3, which was fed cycad flour. No significant lesions were observed in the 2 control horses or in the horse fed cycad for 189 days and then fed a control diet for 301 days.

Lesions

Liver. Several light gray-colored depressed focal lesions were seen on the surface. The lesions were roughly spherical, with a diameter of approximately 6 mm. The liver was firm in consistency. The microscopic lesions are shown in Figures 12 to 17. The grossly apparent light-colored depressed areas histologically appeared as necrotic areas where necrotic hepatic cells had been replaced by macrophages, lymphocytes and a few eosinophils. In portal areas there was extensive fibrosis with leukocytic infiltration and bile duct proliferation. Bile duct

proliferation varied widely from one lobule to the other. In some lobules it was very extensive. In portal areas with extensive fibrosis and leukocytic infiltration, the hepatic arteries had hypertrophy of their media with some cases resulting in the obliteration of the lumen. A few vessels showed periarteritis with leukocytic infiltration in the vessel walls. Hepatic cells had different manifestations of degeneration, some having a vacuolate cytoplasm. Coagulative necrosis characterized by pyknotic nuclei was present in some lobules. A few bile canaliculi were distended with bile pigments.

Kidney. There was chronic interstitial nephritis with occasional extensive proliferation of fibroblasts and infiltration of mononucleate cells.

Lungs. Several patchy consolidated areas were seen. In addition, areas of compensatory emphysema were seen adjacent to the consolidated areas. The bronchiolar epithelium in some areas was hyperplastic. This was indicated by extensions of epithelial rugae into the lumens of the bronchioles, which appeared as small papillae. Many bronchioles and bronchi were filled with desquamated bronchial epithelial cells. There were peribronchiolar lymphoid hyperplasia, alveolar edema and emphysema in several areas.

Gastrointestinal tracts. The submucosa of the stomach and intestine was edematous. There were leukocytic infiltration and fibroblastic proliferation in mucosal and submucosal layers of the gastrointestinal tracts.

Pancreas. Slight vacuolation of cytoplasm of acinar cells was seen in several lobules of the pancreas.

GENERAL DISCUSSION FOR PART I

Australian workers who had observed several field cases of cycad toxicosis in farm animals described paralysis of hind limbs in various animals. In the present studies, paralysis was not observed in swine or horses, and no lesion in the nervous system was demonstrated which could be associated with cycad toxicity.

In these studies pigs fed cycad flour at daily intake of .86 Gm. per kg. body weight developed centrolobular and portal cirrhosis of the liver. Hall (1957) had described an advanced portal fibrosis and less extensive centrolobular fibrosis in heifers fed leaves collected from cycad plants. His histological findings, both in the liver and gastrointestinal tract, were very similar to observations recorded in this group of pigs, but there was no paralysis of hindquarters in the pigs, while Hall observed paralysis in half of his experimental animals.

In the horses, the most significant lesion was the portal fibrosis observed in horse number 3, fed cycad for 147 days. The other experimental horse showed no evidence of portal fibrosis.

In these studies there was an elevation of SGOT in the horses fed the cycad. However, there was no elevation of SGPT values. Elevation of plasma transaminases has been reported by Cornelius (1963) following carbon tetrachloride ingestion in dogs, horses, sheep and calves.

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While paralysis, without demonstrable lesions in the nervous system, has been associated with cycad feeding by previous workers, no signs of central nervous disturbances were noted in these studies with large animals. This might be partially accounted for by the lack of specific information as to the nature of compounds fed by earlier workers. Since edema of tissues was a consistent finding, it would be possible to have edema in areas of the central nervous system which would produce paralysis and yet be very difficult to demonstrate.

In the rat studies, the report of which follows, tumor formation was a constant finding in chronic studies. No tumors were observed in the larger species. However, the duration of cycad feeding was not as long in proportion to the life span of the large animals as in the rat studies.

TABLE 1. Body Weight Changes, Liver, Kidney, Spleen and Heart Weights of Pigs Fed 2% Cycad

Pig Identity	3M*	1F**	4F	2M
Treatment	none	none	cycad	cycad
Days on experiment	60	90	60	90
Initial wt. (lb.)	45	46	52	64
Final wt. (lb.)	130	138	64	131
Average daily gain (lb.)	1.41	1.03	.20	.98
Liver wt. (Gm.)	1352	1378	430	1649
Liver wt., % body wt.	2.3	2.2	1.5	2.8
Kidney wt. (Gm.)	129	---	112	141
Kidney wt., % body wt.	.22	---	.40	.25
Heart wt. (Gm.)	141	---	160	223
Spleen wt. (Gm.)	104	113	87	142

*M = male

**F = female

TABLE 2. Body Weight Changes, Liver, Kidney, Spleen and Heart Weights of Horses Fed Cycad

	Horse Numbers			
	1	2	3*	4*
Days on treatment	none	none	147	189
Days on experiment	490	161	147	490
Initial weight (lb.)	514	540	750	525
Final weight (lb.)	760	880	900	1120
Avg. daily gain (lb.)	0.5	2.11	1.02	1.21
Liver wt. (Gm.)	4540	4826	4800	6810
Liver wt., % body wt.	1.32	1.20	1.17	1.33
Kidney wt. (Gm.)	908	608	543	1362
Kidney wt., % body wt.	0.26	0.15	0.11	0.23
Heart wt. (Gm.)	---	2607	2300	---
Spleen wt. (Gm.)	---	885	830	---

*Fed cycad flour

TABLE 3. Transaminase, Bilirubin, Hemoglobin and Packed Cell Volumes in Horses Fed Cycad Flour

Days	Horse Number	SGOT* (Sigma-Frankel units)	SGPT**	Bilirubin (mg./100 ml.)	Hb. (Gm./100 ml.)	Packed cell Volumes %
1	1	160	8	0.0	10.48	37
	2	136	8	0.0	7.2	25
	3*	144	8	0.0	10.4	30
	4*	144	8	0.0	11.1	30
7	1	160	8	0.50	11.2	32.5
	2	134	8	0.50	8.0	---
	3	168	8	0.50	10.2	28.5
	4	194	9	0.56	9.2	28.5
14	1	160	8	0.5	8.1	24
	2	144	8	0.5	9.0	27
	3	234	9	0.75	11.4	32
	4	256	9	0.50	10.0	28
21	1	160	10	1.0	8.5	21
	2	172	8	0.5	10.8	28
	3	520	8	0.75	10.8	31
	4	440	8	0.5	9.5	26
28	1	144	8	0.75	8.7	27
	2	160	8	0.5	11.7	28
	3	680	8	0.5	11.0	33
	4	680	9	0.5	10.8	27
35	1	144	8	0.5	12.0	29
	2	144	8	0.75	11.7	31
	3	238	8	1.5	11.7	31
	4	264	8	1.0	10.0	27
42	1	144	8	0.5	12.2	34
	2	144	8	0.5	13.7	35
	3	240	8	1.5	14.7	37
	4	220	8	1.0	9.2	28
49	1	160	8	0.5	10.8	32
	2	166	8	0.5	11.2	33
	3	360	8	1.0	11.7	34
	4	320	3	0.75	9.2	28
56	1	160	8	0.5	10.0	33
	2	172	7	0.5	10.0	31
	3	400	8	1.25	9.7	31
	4	440	6	1.75	9.2	28

TABLE 3--continued

Days	Horse Number	SGOT* (Sigma-Frankel units)	SGPT**	Bilirubin (mg./100 ml.)	Hb. (Gm./100 ml.)	Packed cell Volumes %
63	1	168	8	0.5	12.5	38
	2	172	6	0.5	11.5	32
	3	240	8	1.25	11.2	32
	4	300	6	1.50	10.5	29
70	1	160	8	0.5	13.7	36
	2	148	6	0.75	11.3	29
	3	192	6	2.25	13.7	33
	4	208		1.50	9.9	27
			cycad increased to 4%			
77	1	172	8	0.5	11.3	32
	2	160	8	0.75	10.2	28
	3	380	8	2.25	12.3	34
	4	320	8	1.75	11.0	30
91	1	168	8	0.5	12.3	32
	2	140	8	0.75	11.6	29
	3	420	8	2.30	11.6	30
	4	340	8	1.75	10.5	28
105	1	174	8	0.75	14.0	35
	2	160	8	0.5	13.0	33
	3	300	8	3.4	14.8	38
	4	300	7	3.6	12.0	30
119	1	174	8	0.75	13.3	33
	2	---	---	---	13.7	35
	3	360	8	3.6	13.2	38
	4	310	8	3.95	12.6	34

*Fed cycad flour

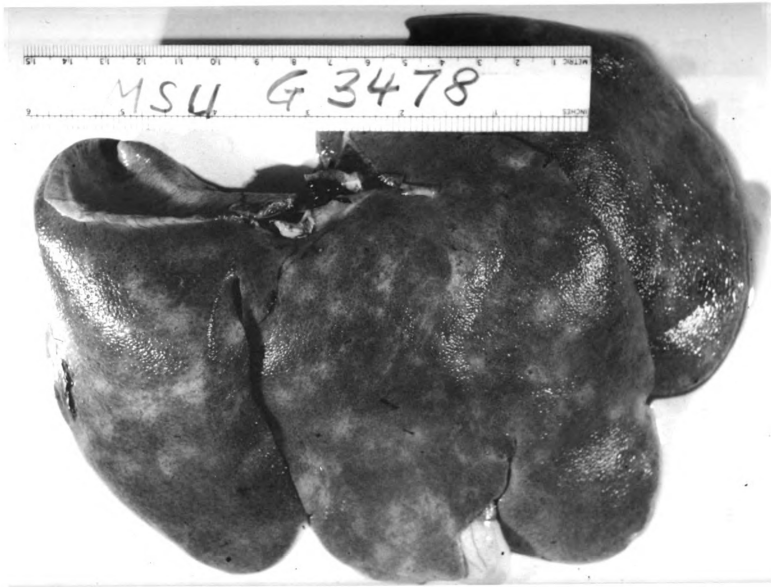


Fig. 1.--Fibrotic liver of pig 4, which had been fed a diet containing 2% cycad flour for 60 days.

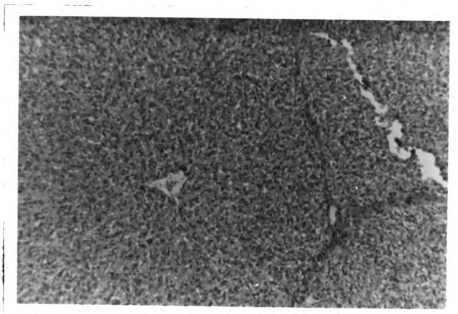


Fig. 2.--Liver of a pig fed control diet for 60 days. H & E stain; x 75.

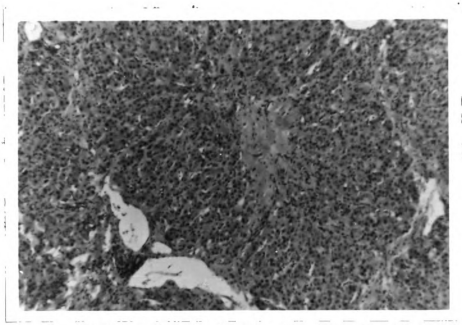


Fig. 3.--Portal and centrolobular fibrosis of a liver of pig fed 2% cycad for 60 days. H & E stain; x 75.

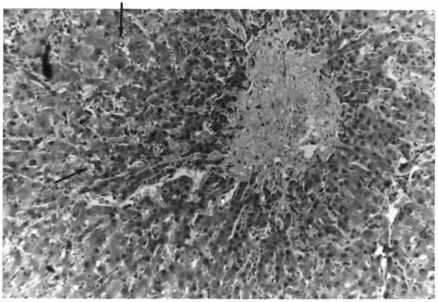


Fig. 4.--Centrolobular fibrosis. Higher power of Fig. 3. Arrows indicate globules in the nuclei. H & E stain; x 187.

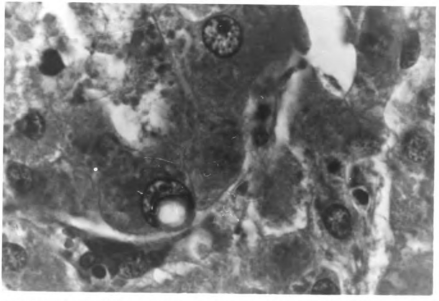


Fig. 5.--A small globule in the nuclei. Higher power of Fig. 4. H & E stain; x 750.

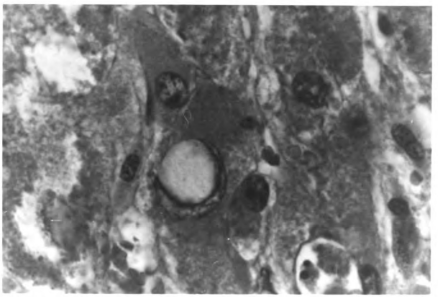


Fig. 6.--A large globule in the nucleus with nuclear content pushed into the periphery in a form of sickle-shaped structure. Higher power of Fig. 4. H & E stain; x 750.

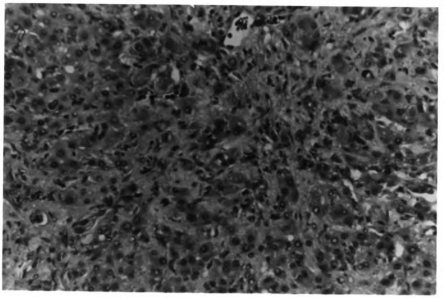


Fig. 7.--Diffuse fibrosis in the liver of pig fed 2% cycad for 90 days. Section around central vein. H & E stain; x 187.

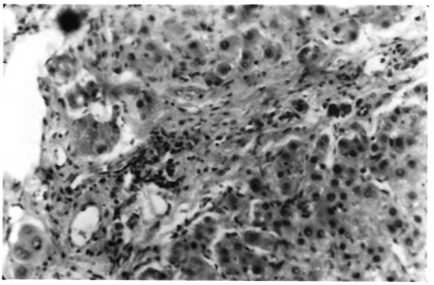


Fig. 8.--Portal fibrosis in the liver of pig fed 2% cycad for 90 days. Same liver as in Fig. 7. H & E stain; x 187.

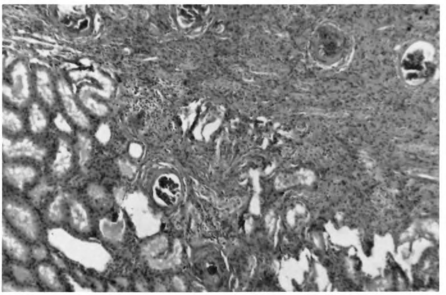


Fig. 9.--Fibrosis in renal cortex of pig fed 2% cycad for 90 days. H & E stain; x 75.

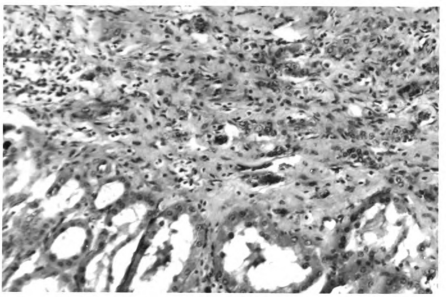


Fig. 10.--Fibrosis in the renal cortex of pig fed 2% cycad for 90 days. Higher power of Fig. 9. H & E stain; x 187.

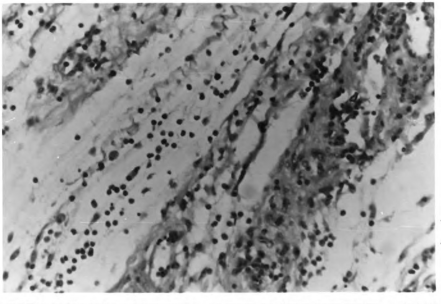


Fig. 11.--Edema of the submucosa of the stomach of pig fed 2% cycad for 60 days. H & E stain; x 187.

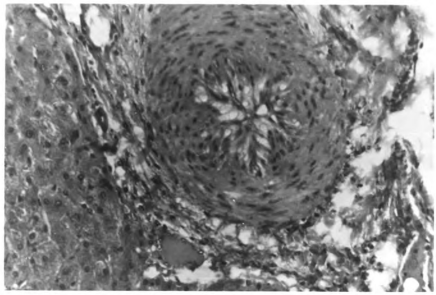


Fig. 12.--Occluded hepatic artery of a horse fed cycad for 147 days. H & E stain; x 187.

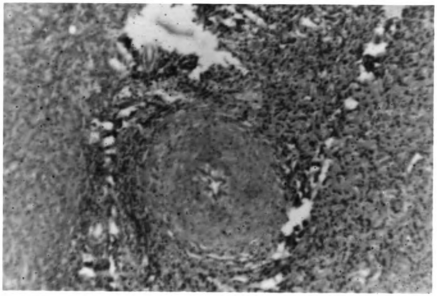


Fig. 13.--Hyalinized hepatic arterial wall in a liver of a horse fed cycad for 147 days. H & E stain; x 75.

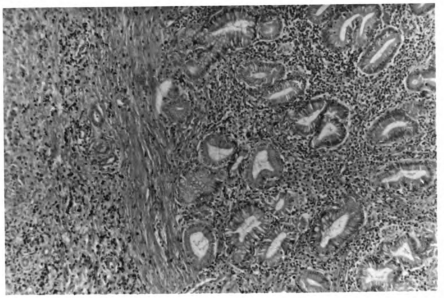


Fig. 14.--Portal fibrosis with bile duct proliferation and leukocytic infiltration in a liver of a horse fed cycad for 147 days. H & E stain; x 75.

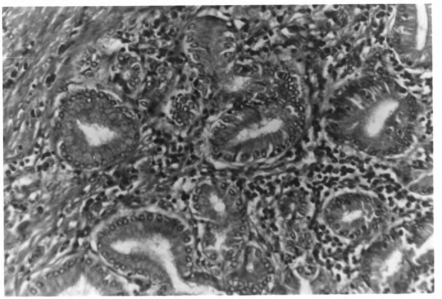


Fig. 15.--Bile duct proliferation with leukocytic infiltration. Higher power of Fig. 14. H & E stain; x 187.

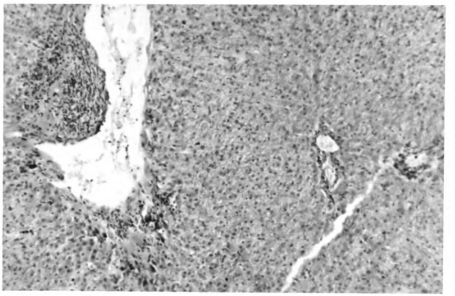


Fig. 16.--Fibrotic phlebitis of sublobular vein of a horse fed cycad for 147 days. H & E stain; x 75.

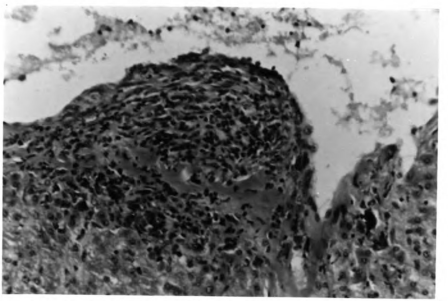


Fig. 17.--Fibrotic phlebitis of sublobular vein. Higher power of Fig. 16. Horse fed cycad for 147 days. H & E stain; x 187.

PART II. CYCAD TOXICOSIS AND RELATED CARCINOGENS IN RATS

Experiment I. Cycad Flour Toxicosis in Rats

INTRODUCTION

Rats were used as the experimental animal to explore the possible harmful effects from cycad, because large numbers of animals could be used and they could be maintained over a longer period of time more easily than larger species. The cycad preparation was from the same source as that described in PART I.

EXPERIMENTAL PROCEDURE

Animals and treatments

Weanling male and female Sprague-Dawley rats, 146 in number and divided into 7 groups, were used in Experiment I. The initial weights varied from 39 to 54 Gm. They were housed in groups varying from 2 to 4 in all-metal cages which were provided with coarse-mesh wire bottoms. For identification they were marked with notches in their right ears. The groups were fed varying percentages of cycad flour in a natural grain ration. The ration, designated as Michigan State University M_1 ration, is listed in TABLE 4.

The M_1 ration was mixed thoroughly in a Hobart food mixer with weighed cycad flour in the required percentage. The mixed diets were kept in tightly closed tin cans at approximately 4 C. until they were used.

Each morning weighed amounts of fresh food were placed in food containers designed so as to minimize scattering. Fresh water was

available at all times. The different groups, treatments, numbers of rats and specific details for each group are shown below.

Group	Number of Animals	% of Cycad Flour	No. per Cage
1	15 M.	5.0	3
2	30 M. & 30 F.	2.0	2 M. & 2 F.
3	30 M.	1.0	3
4	5 M.	0.5	3
5	6 M.	0.25	3
6	6 M.	0.125	3
7	15 M. & 15 F.	---	2 M. & 2 F.

The rats in group 1 were weighed daily and post-mortem examination done when they died. Those in group 2 were weighed daily and, during the first 7 days, 1 animal was killed each day - the one which had lost the most weight. From the 8th to the 21st days, 1 animal was killed at each 2-day interval, then 1 each week up to 3 months, then 1 each month until the experiment was terminated after 9 months. These rats were allowed to reproduce, and the data thus obtained on the toxicity to the newborn is reported in PART III of this thesis. In group 3 the rats were weighed and 1 killed at each weekly interval for 4 months, then at monthly intervals until the experiment was terminated after 10 months. In groups 4-6, the animals were killed at monthly intervals. In group 7, no cycad was included in the diet. The animals were weighed weekly and 1 killed at each monthly interval, all remaining animals being killed at the termination of the experiment. These rats were allowed to reproduce and were also the controls for later experiments on transmission of the toxic factors through the placenta.

Before the rats were killed at specified times, blood samples were taken by cardiac puncture under light chloroform anesthesia. Then the rats were killed by severing of the spinal cord.

Analysis

Blood samples were collected, using ethylene-diaminetetraacetic acid (EDTA) as the anticoagulant. One to two milliliters of blood were used for hematologic procedures. Three to four milliliters of blood were taken without an anticoagulant for serum enzyme determination. Hemoglobin was determined by the cyanmethemoglobin method. Packed cell volumes were determined by the micro method (capillary tube). White blood cell counts were made using Turk's diluting fluid and the hemacytometer. Blood smears were made at the time of bleeding, then stained with Wright's stain and a differential white blood cell count determined.

Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were determined according to the methods of Reitzman and Franket (1957), as outlined in Sigma Chemical Company's 1963 Technical Bulletin No. 505.

All the animals that were killed or died were examined by standard necropsy procedures. Thin sections from all macroscopic lesions, and routinely from the following organs, were preserved in acetate-buffered, 10% formalin for histological examination: brain, liver, heart, pancreas, kidney, lungs, stomach, duodenum, and jejunum. Sections of liver and kidney were also fixed in Carnoy's fixative.

Routine sections were stained with hematoxylin and eosin. The following special stains were used for selected tissues: Heidenhain's aniline blue stain, Gridley's reticulum stain, periodic acid Schiff reaction, crystal violet stain, Best's carmine, and Sudan IV. The histological and staining procedures followed were according to the Armed Forces Institute of Pathology Manual of Histological and Special Staining Technics (1957).

Selected liver and kidney sections were stained for nucleic acids, alkaline phosphatase and acid phosphatase, using cold microtome (Cryostat) methods. Sections of tissue approximately 0.5 mm. in thickness were flattened on aluminum foil. The foil was plunged into acetone chilled with crushed ice, taking care not to allow the acetone to come into contact with the tissue. The tissue was then frozen on the foil for 10 seconds and transferred into tightly closed bottles and kept at -20 C. until needed. The sections were cut at 8 microns thickness and while being maintained constantly at -20 C. and stained immediately. For nucleic acids, the tissue was stained using the methyl green-Pyronin method according to Brachet (1940a, 1942, 1944). For alkaline phosphatase, the Burstone method (Burstone, 1958) was used. The ester Naphthol AS-MX was used as substrate. For acid phosphatase, the Naphthol AS-B_1 phosphate method, as described by Pearse (1961) was used.

Procedures for preparation of the stains and staining were those described by Pearse in his Textbook of Histochemistry, Theoretical and Applied (1961).

RESULTS

Information on the growth rate of rats fed various levels of cycad is given in TABLE 5. The rats fed levels of .125, .25 and .50% cycad were essentially the same as the controls (not fed cycad) and are not included in the table. Levels of 1% and 2% cycad resulted in a slower growth rate than occurred in the controls. The 5% level of cycad flour produced such an acute toxicosis that growth rate information was difficult to evaluate and is not included.

The major pathologic findings and the serum SGOT, SGPT, hemoglobin and packed cell volume values are summarized in TABLE 6.

TABLE 7 summarizes the types of neoplasms in rats fed the various levels of cycad flour and other chemicals.

The control rats were maintained for 10 months on the basal ration and at no time were pathologic changes or any abnormalities noted in any of the animals, except a high incidence of murine pneumonia.

The rats fed .125, .25 or .5% cycad flour for 6 months were essentially normal in all respects, and pathologic changes were not observed in any of these rats.

Significant changes were noted in the rats fed the 1%, 2% and 5% cycad flour. These changes varied from acute deaths and severe lesions in rats fed 5% cycad flour to a more chronic course and chronic tissue changes, including a significant incidence of neoplasms in those fed lesser amounts. The description of the gross and microscopic lesions is given in the following pages in detail. The specific lesions are shown in Figures 18 to 56.

Group 1 (5% Cycad Flour)

Symptoms

On the third day after beginning feeding the experimental diet, the rats developed a loss of appetite. They consumed 3/4 as much food as they had consumed on the previous day. On the fourth day, 4 rats were found dead in the cage. On the sixth day, 2 others died. By the eighth day, all the rats in the group except 2 were dead. The remaining rats were in a moribund state, with difficulty in breathing. The breathing was exaggerated and abdominal. They were destroyed by severing the spinal cord.

Lesions

Liver. Scattered ecchymotic hemorrhages were noted over the surface of the liver parenchyma. The liver was also slightly yellowish in color and friable. Microscopically, there was centrolobular hemorrhage with pronounced leukocytic infiltration. No changes were noted in the vicinity of the portal triads.

Lungs. The lungs of the rats which died on the fourth day appeared congested. The congestion was primarily in the apical lobes. The lungs of the rats dying between the fourth and eighth days had scattered hemorrhages varying in size from small petechial to ecchymotic in character. Microscopically, the pulmonary alveoli of the lungs of the rats dying on the fourth day were filled with serous exudates, and the lung capillaries were very congested. The pulmonary alveoli of the rats which died later were filled with either blood or serous exudate with some leukocytes, mainly eosinophils and monocytes. Several atelectatic areas were noted.

Gastrointestinal tracts. Grossly, a catarrhal to hemorrhagic gastroenteritis was noted with severe congestion of the duodenum. Microscopically, there was a hemorrhagic duodenitis.

Group 2 (2% Cycad Flour)

Lesions

Liver. In the first 4 months there were no noteworthy changes in the liver, except for occasional congestion, exaggeration of the lobular pattern and pale depressed areas located centrolobularly. The first definite changes were seen in a rat killed at 121 days, in which the

liver appeared to be small in size, firm, with several scattered whitish nodular lesions. The nodules varied from 1 to 2 mm. in diameter and were firm in consistency. Thereafter, the incidence of the nodules increased, sometimes evenly distributed over the entire surface and at times limited to certain lobes. On no occasion did the median lobe of the liver not contain approximately 4 to 6 whitish nodules.

The second time gross lesions were observed on the liver was in a rat killed at 205 days. The liver had a distorted surface. There were many small, hard nodules 1 to 2 mm. in diameter and, in addition, several translucent, cystlike nodules were noted at the periphery of the lobules. They varied from 2 to 4 mm. in diameter. On section, colorless fluid oozed from these nodules. On the ventral surface of the median lobe there was a large nodule with a depressed surface. The nodule was approximately 7 mm. in diameter and whitish in color. On section it was softer than the rest of the liver tissue and greasy.

The animals killed after 205 days showed an increased incidence of cystlike nodules. In some livers they were very numerous and, on section, the liver had a honeycomb appearance. The incidence of grossly visible tumors also increased. Several rats had multiple tumors which varied in size. In 3 rats rupture of the tumors resulted in intra-abdominal hemorrhage. On cross section of a large tumor, necrotic and hemorrhagic patches, irregularly distributed, were noted.

In 2 rats killed at 240 days, in addition to the whitish nodules, reddish-pink nodules about 4 to 6 mm. in diameter were noted. On sectioning, blood-red fluid oozed out.

Microscopically, the earliest change noted in the liver was seen 3 days after ingestion of the cycad flour-containing diet. There was a

loss of cytoplasmic basophilia and demonstrable glycogen in the cells around the central veins. Some cells had eosinophilic condensed cytoplasm encircling the nuclei; others had well formed, round hyaline droplets. The droplets appeared as intracytoplasmic inclusions with a colorless rim between them and apparently normal cytoplasm. The droplets were noted in cells close to the central veins. Many hepatic cells showed fatty infiltration, as demonstrated with the Sudan IV stain. On the fourth day pyknosis and karyorrhexis were noted in the nuclei of hepatic cells. An increase of alkaline phosphatase activity was recorded on the fifth day, and there was a decrease of acid phosphatase activity. On the seventh day the number of hyaline droplets had increased and there was a change in their distribution. At this time they were noted mostly around the central veins and about the portal areas. A few were seen in the midzonal area of the liver lobules. The droplets stained magenta with the periodic acid Schiff reaction and purple with crystal violet. There was uniform centrolobular necrosis with hemorrhagic components in all lobules of the liver. In some lobules there was leukocytic infiltration, both in the center of the lobule and in the portal area. The leukocytes were mainly mononuclear cells, with a few neutrophils and eosinophils.

At 14 days the centrolobular areas consisted of necrotic debris mixed with erythrocytes and dying leukocytes. The rest of the liver lobules showed fatty infiltration and occasional necrotic cells in all areas, including cells around the portal triads. The fat vacuoles varied from small fine vacuoles to large ones twice the diameter of the hepatic cell nuclei.

Within 26 days most of the necrotic debris in the centrolobular area had disappeared. The central veins were obliterated in a few cases by collapsed reticulum framework and proliferating fibroblasts. Many macrophages were noted with ingested hemosiderin. The hepatic cells around portal triads were vacuolated, and there was slight proliferation of fibroblasts, with leukocytic infiltration. There were several isolated necrotic areas with mononuclear and neutrophil infiltration. Several individual necrotic cells characterized by pyknosis and karyorrhexis and eosinophilic cytoplasms were seen among large regenerating cells. The regenerating cells had hyperchromatic nuclei, enlarged nucleoli and vacuolate cytoplasm.

In rats fed the 2% cycad for 42 days there was marked destruction of liver cells. It was more evident in the central areas than in the periphery of the lobule. Most of the central veins were obliterated by proliferating fibroblasts. In the portal areas there were both mature and proliferating fibroblasts. Bile duct proliferation was seen at this time and there were macrophages with ingested hemosiderin and eosinophils surrounding the proliferating bile ducts. In rats killed between 60 and 112 days there was diffuse liver cirrhosis of varying degrees. In addition, the Kupffer cells of many dilated sinusoids were seen proliferating. In some cases a layer of dark staining cells 4-5 cells thick, was noted continuous with the wall of the sinusoid. In other instances similar cells were seen forming isolated islands. In these islands most of the cells had an elongated, spindle-shaped appearance with little cytoplasm, but there were some resembling those seen in the sinusoids. Similar cells were seen around the central veins and occasionally extensively infiltrating the portal areas. Nodular hyperplasia was

noted in animals killed at 121 days. Thereafter, it was seen in all rats. Microscopically, the nodular hyperplasia was in sharply defined rounded areas. They were composed of closely packed parenchymal cells, which lacked the normal trabecular arrangement. The cells were large, with enlarged nucleoli. The cytoplasm was acidophilic and very much vacuolated. In most cases the vacuoles contained no fat. Also characteristic of the lesions was the presence of many hyaline droplets in the cytoplasm of these enlarged cells. These droplets were sharply defined and often surrounded by a halo. These nodules appeared to be compressing the surrounding structures, with no capsule around them; but their acidophilic cytoplasm contrasted sharply with the smaller, slightly basophilic hepatic cells.

Bile duct proliferation was seen in all rats after 42 days on the experiment. Extent of this change was variable within each liver. In the earlier cases, clusters of bile ducts were lined with cuboidal or columnar epithelium and were associated with extensive fibrosis. In later cases of bile duct proliferation it corresponded with the gross translucent cystlike nodules. Microscopically, these were relatively large cavernous spaces lined by cuboidal epithelium in some cases. In others the spaces were lined by flattened endothelioid cells. The stroma between the cysts consisted of well formed, slender collagen fibers and, occasionally, normal columns of hepatic cells at different stages of degeneration. Intermediate types of cells between cuboidal and endothelioid cells were noted, lining adjacent cysts. This led to a conclusion that all were probably of the same origin. In some areas these cystic lesions were very extensive and resembled hemangiomas; but instead of blood, they contained serous fluid. Such cases were diagnosed as bile duct cystadenomas.

Nuclear changes were seen in the livers of some animals killed 60 days after ingestion of the cycad flour diet. At this time most of the liver cells were enlarged. They had large nuclei, irregular in shape, with one or more nucleoli and prominent chromatin granules. There were also 1-4 globules in a single nucleus. The globules were unstained with hematoxylin and eosin and appeared as a vacuole with a narrow basophilic rim. With periodic acid Schiff reaction, their staining properties varied. The nucleoli stained a green of varying shade. Some of the globules had colorless centers with red peripheries; others had small reddish granules which appeared as a precipitate. The number of the red-colored granules varied greatly.

Changes in blood vessels were rare. The central veins were seen occasionally obliterated by proliferating fibroblasts and later by formation of mature connective tissue. This lesion was very variable, as in many occasions the mature tissue formed around the central vein without a break in the intimal layer of the vein; occlusion resulted from compression. In some cases fibrous tissue proliferation was seen partially occluding the lumen of the vessel resulting from a break in the intimal layer. In a few cases sublobular veins were seen with organized thrombi. Proliferation of the intimal layer of the vein was also noted in vessels of the lungs in a few cases. Hepatic arteries were occasionally seen, with proliferated endothelial cells forming several layers and partially occluding the lumen.

The tumors of the liver were mainly of 3 types: hepatoma, hemangioma, and fibrosarcoma.

Hepatoma. Histologically, the cellular pattern varied greatly. Some tumors had large cells and other tumors were composed of compressed cells smaller than the normal liver cells but with an abnormal orientation into trabeculae. Where the trabeculae-like livers were seen, they appeared broader than normal. In others, the tumor had a tubular appearance with a blood sinus at the center surrounded by syncytial structures made up of hepatic-like cells. The width of the cellular column varied greatly. In all cases the hepatomas were associated with cirrhosis. Several rats had multiple tumors. In 2 cases metastasis had occurred in the lungs. Tumor emboli were seen locally in veins of other lobes of the liver. The tumor cells in some areas were very vacuolated and contained fat. Hyaline droplets similar to those seen in adjacent normal liver cells were very numerous in tumor cells.

Two cases of hemangioma, one associated with hepatoma, were seen. The fibrosarcoma type of liver tumor will be described with tumors seen in animals fed 1% cycad diet for 7 months.

Kidney. The first gross lesion seen on the kidney was a tumor found in a rat killed after 168 days. Twelve of 22 rats killed between 168 and 270 days had kidney tumors. Grossly, the tumors can be divided into 3 groups: (1) small whitish solid nodules, 3-4 mm. in diameter, localized in the renal cortex or at the junction of the cortex and medulla; (2) large tumors, whitish-gray in color and firm in consistency, sometimes occupying most of the abdominal cavity (The largest tumor of this group is shown in Figure 47. It had a constriction which divided a large mass from a smaller mass - both were continuous and weighed 70 Gm. The mass was was 8.6 cm. in longest diameter.); and (3) tumors varying from 6 to

30 mm. in diameter. The largest of these weighed 10 Gm. These tumors were reddish-white in color and of a fleshy, gelatinous or friable consistency. On cross section the tumors were cystic, and the cysts contained either serosanguineous fluid or blood. In 3 group-3 rats with large tumors, whitish elongated calculi, shown in Figure 49, were found in the urinary bladder. The calculi were resistant to cutting with a knife and had a few cavities.

Microscopically, the earliest lesions in the kidney occurred 3 days after ingestion of the cycad diet. These consisted of fatty infiltration in the ascending loops of Henle, coagulative necrosis of the proximal convoluted tubules, interstitial hemorrhages and congestion of the glomeruli. Thereafter, the lesions in the kidney progressed into chronic interstitial nephritis, characterized by proliferating fibroblasts.

The first sign of a neoplastic process in the kidneys was observed in rats fed the diet for 128 days. This consisted of a nodule which microscopically was composed of cords of large cells without lumen formation. The size of these solid cords of cells did not exceed that of a single tubule. The cells forming the cord were large and basophilic with rarefied cytoplasm, which was granular without vacuoles. The cell boundaries were very clear. They had vesicular or oval nuclei with rather scanty chromatin and basophilic nucleoli located eccentrically.

Histologically, the kidney tumor followed the same pattern as seen grossly. The small whitish nodules were solid renal tubular adenomas (Figures 41 and 42). Microscopically, these were well organized neoplastic masses. The tumors were sharply demarcated from the renal parenchyma by their basophilic cells, but they had no capsule. The adjacent tubules appeared compressed and narrow. The neoplastic structure was composed of very closely packed cells with round or oval

vesicular nuclei, hardly any chromatin and a basophilic nucleolus, which in most cells was centrally located and in others was just next to the nuclear membrane. The boundary of the cell was seen very clearly. The cytoplasm of these cells was more deeply basophilic than that of the adjacent tubular cells. There were fine intersecting fibrous strands with capillaries, giving a clear lobular structure to the mass.

The large tumors were typical fibrosarcomas (Figure 48). The cellular components were very anaplastic and pleomorphic, including cells fusiform and polyhedral in shape. The tumors showed many whorls and interwoven bundles of immature fibroblasts. The tumor cell nuclei were round and hyperchromatic. Mitotic figures were very common. In some areas there were undifferentiated cells which were light-colored and grouped together. In other areas secondary infection and necrosis were present. In addition, in a few areas tumorous cells were distributed in vacuolated basophilic mucinous stroma that resembled myxoma.

Tumors noted in the kidneys of the third group were diagnosed as embryonal nephromas. Histologically, these tumors varied from each other, and they also varied in different areas of the same tumor. One variation seemed to be a coexistence of 2 separately well formed tumors adjoining each other in the tumorous mass (Figure 44). One type was the well formed, typical solid adenoma, histologically almost identical to the tumors described previously. The modifications included occasional excessive necrosis, hemorrhages and a few areas within the adenoma of parts well differentiated into cystadenoma with eosinophilic staining material within the cyst. The other tumor was very anaplastic. The main cellular components were elongated spindle-shaped cells with hyperchromatic nuclei. In some areas these anaplastic cells were sheets and

cords of clearly differentiated epithelial cells surrounded by the spindle-shaped cells (Figure 45). A few of these epithelial cells were undergoing necrosis, and a few seemed to be forming glandular structures. The boundary between the adenoma and this anaplastic tumor was not marked. The anaplastic cells were invading the well differentiated adenoma, but in a few areas adenomas were clearly separated from the anaplastic tumor by bundles of collagenous fibers. There was no indication that the anaplastic tumor was derived from the well differentiated adenoma. Within these anaplastic tumors were dilated cavernous spaces lined with a single layer of endothelial cells. These spaces were either filled with blood or eosinophilic protein-like material with fibrin strands. In 2 cases a cluster of tumor cells was seen inside a cystic blood vessel. No metastasis was evident in any of the kidney tumors.

A variation of the above tumor was one that was composed mainly of closely packed anaplastic cells with no definite arrangement. The cells were spindle-shaped with elongated or oval nuclei. The boundary of the cell cytoplasm was not clear, but nuclei were clearly demarcated by sharply hyperchromatic nuclear membranes. The tumor mass was very cystic. These cysts were of two types - one filled with blood and the other filled with a granular eosinophilic deposit with fibrinous strands. In areas where the stroma was scanty, scattered tubular structures with a single layer of cuboidal epithelium were seen. Occasionally, partially formed glomeruli were present. These consisted of well formed Bowman's capsule with poorly organized glomerular tufts, associated with elongated fibrous cells. In some areas degenerating tubules were seen surrounded by concentrically arranged layers of elongated cells, giving an appearance

of whorls like those of hemangiopericytoma of the dog. In the centers of some of these tumors there appeared to be excess capillaries with hyalinized walls. The vessels appeared as hyalinized tubules with narrow lumens which contained blood. This resembled the lesion described by Laquer et al. (1963).

Peritoneum. Ascites with fluid excess ranging between 5 and 20 ml. in amount was found occasionally. The first ascitic case occurred in a male rat killed after 18 days in which 10 ml. of straw-colored fluid were removed from the abdominal cavity. Of the 47 rats killed between 18 and 270 days, 24, including both males and females, had ascites. Nine of the 24 ascitic cases had no neoplasm. Ascites was serous in character except in 3 cases, in which there was intraperitoneal hemorrhage as a result of rupture of the liver tumors.

Pancreas. In all cases of ascites, the pancreas had a jelly-like interstitial edema. In a few rats killed after 54 days a few patchy areas, whitish-gray in color, firm in consistency and raised above the surface of the pancreas, were noted. These usually involved one or two adjacent lobes and were irregularly scattered in the organ. These lesions were not noted in the rats which were killed after 3 months on the experiment.

Microscopic lesions are shown in Figures 50-53. The earliest lesion noted in the pancreas was interstitial edema seen in the rat after 6 days of ingesting the cycad-containing diet. Rats killed after 8 days had vacuolation of the cytoplasm of the acinar cells with eosinophilic staining of the nonvacuolated cytoplasm (Figure 50). No nuclear changes were seen until the 17th day, and at this time a few nuclei were pyknotic. A characteristic lesion in the pancreas was seen in rats

which died after 18 days of ingestion of the cycad-containing diet. This was extensive interstitial edema with infiltration of leukocytes, mainly lymphocytes, polymorphonuclear leukocytes and eosinophils (Figure 51). A rat killed at 26 days had proliferating fibroblasts in the interstitial areas with some infiltrating into the degenerating acinar tissue (Figure 52). A rat killed at 54 days, in addition to intralobular fibrosis, had hyperplastic pancreatic ducts and periductal fibrosis. At this time the inflammatory cells were essentially eosinophils. One rat killed at 70 days had most of the acinar cells completely destroyed and replaced by fibrous tissue in many of the lobules (Figure 53); this area corresponded with the gray areas noted grossly. In the rats killed between 70 days and 8 months, the incidence of pancreatic lesions was low and when present consisted of microscopic focal fibrotic areas.

Heart muscle. The earliest lesion in heart muscle was noted in a rat killed after 3 days. This consisted of fatty infiltration and progressed into patchy hyalinization. After 22 days of ingestion of cycad-containing diet, a few Anitschkow cells were noted in these patchy necrotic areas. Fibrosis of these areas was noted after 31 days. These lesions were very patchy and could be easily missed. They had no uniform distribution.

Lungs. The earliest lung lesion appeared in a rat killed on the 6th day. The lung was dark red and firm in consistency, with patchy areas of atelectasis and emphysema. Thereafter, the lungs had either areas of consolidation without signs of acute inflammation or typical lesions of chronic murine pneumonia.

Microscopically, the earliest lesion seen in the lung was noted 4 days after ingestion of the cycad-containing diet. The lung capillaries

were very congested. A rat killed on the 6th day had serous to hemorrhagic exudate in the pulmonary alveoli with several emphysematous alveoli. Rats killed after 18 days on the cycad diet had various lesions. Some had thickening of interalveolar septa due to increases of fibroblasts and septal cells. Several lung alveoli were atelectatic with mononuclear and eosinophilic infiltration and edema of varying degree. Several animals had chronic murine pneumonia with marked foamy cell infiltration in the alveoli.

Gastrointestinal tract. In most rats no lesions were demonstrable in the digestive tract. Occasionally, catarrhal to ulcerative enteritis was noted. In one killed at 270 days, a slightly rough elevation, whitish-gray in color, was noted at the ileocecal junction.

Histologically, this nodule was of glandular components. It was lined by columnar epithelium, and some had a mucous exudate. Between the glands were infiltrations with neutrophils and a few monocytes.

Other lesions. Two rats killed at 240 days had small nodules on the diaphragm. In one case the nodule was on the middle of the abdominal surface of the diaphragm and clearly demarcated. In the other case the nodule was on the diaphragm and was adherent to the liver capsule. The nodules were whitish-red in color. On sectioning, a few cavities with blood were noted, separated by distinct trabeculae of white-colored tissue. The consistency of the nodule was firm in the white part and rubbery in the reddish parts. The part adjacent to the muscle appeared to be continuous with the muscle without a definite capsule.

Histologically, these were tumors made up of vascular elements in a connective tissue stroma. The vascular elements were cavernous spaces

filled with blood, and in some portions the components of the blood in these spaces resembled those of normal blood. The spaces were lined with a single layer of endothelium and a wall which in some areas was very thin; but in others the central areas, especially, were very thick and made up of fibrous connective tissue. This varied greatly in its cellular elements. In some cases fibroblastic proliferation was very extensive, but there was neither mitosis nor anaplasia present. The skeletal muscle was found in all parts of the tumor at different stages of degeneration. The degenerative changes seen in the muscle were atrophy, loss of striation, and hyaline degeneration. Between the muscle bundles were lines of fatty vacuoles and histiocytes with ingested pigments. Several areas had localized hemopoietic centers with undifferentiated, darkly staining nucleated round cells. Occasionally, these dark staining cells were seen forming a ring around the necrotic muscle fibers and appeared as though they were the origin of the endothelium lining the vascular element. These lesions were diagnosed as hemangioma of the striated muscles (Figures 55-57).

Group 3 (1% Cycad Flour)

Lesions

In this group of rats, with the exception of lesions of chronic murine pneumonia, no gross lesions were demonstrable until the 8th month of ingestion of the cycad-containing diet.

Liver. Six of the 10 rats killed between the 8th and 10th month had neoplasms of the liver. In one rat there was metastasis to the lungs and peritoneum. Grossly, the tumors could be divided into 2 groups: (1) large, firm and whitish in color, and (2) small, slimy and pinkish-white in color, which on cross section had small cavities. The tumor of

the latter type was 1.8 cm. in diameter and was found in 3 livers. These tumors were similar in appearance to the 2 tumors seen in 2 rats fed 2% cycad and killed after 270 days. Occurring with the large tumors were smaller, discrete, whitish and shiny nodules noted when the liver was cut on cross section.

Microscopically, the earliest changes were noted 14 days after ingestion of the cycad flour-containing diet. These consisted of slight fatty infiltration in the cells around the central veins and absence of glycogen.

After 28 days of feeding the cycad ration, hyaline eosinophilic droplets with similar staining characteristics as those described in Group II rats were noted. At 56 days, ballooned hepatic cells with gigantic nuclei and occasional mitotic figures were noted. Among the enlarged cells were individual necrotic cells characterized by a contracted cytoplasm that surrounded the nuclei. The contracted cytoplasm left a halo between it and the cell membrane. At 84 days, proliferation of Kupffer cells lining the hepatic sinusoids were seen. In addition, isolated accumulations of cells resembling reticuloendothelial cells were seen in different locations, forming scattered islands of cells.

In 2 of the 4 rats fed the diet for 8 to 10 months, reddish-white nodules were noted in the liver. These nodules were 5 mm. in diameter. Histologically, they were localized in the portal triads and consisted of proliferating reticuloendothelial cells, with dark staining nuclei and scanty cytoplasm. Among these cells were dilated vessels filled with blood. These vessels were lined by endothelial cells with similar nuclei. The nuclei were dark staining and elongated. Similar lesions were seen in the livers with large tumors. In some areas these lesions

were associated with bile duct proliferation. Occasionally, islands of reticuloendothelial cells were seen in the dilated sinusoids.

Four of the 6 grossly visible liver tumors were hepatomas with a similar histological pattern as those described in Group II. The small, pinkish-white, slimy tumors had a sarcomatous cellular component. Histological appearance of this tumor was varied (Figures 37-39). The cellular components showed great pleomorphism. In some areas the cells had scanty cytoplasm and round hyperchromatic nuclei with prominent nucleoli. These cells were closely packed. In less dense areas the cells had spindle-shaped nuclei with slightly eosinophilic cytoplasm and prolonged fibers. Among these spindle-shaped cells were cells with hyperchromatic nuclei and no cytoplasm, resembling the cells seen in the dilated sinusoids. A few contained mitotic figures. There were many dilated blood vessels in the less dense areas with cells lining the vessel resembling the dark staining areas in the sinusoids. There were other vessels lined with endothelial cells and eosinophilic staining exudate in the lumen. In less dense areas, remains of portal tracts surrounded in a few areas by 2 or 3 cords of atrophic liver cells were noted. Reticulum stain demonstrated reticulum fibers continuous with the cytoplasm of the cells. These tumors were classified as reticulum cell sarcomas originating from cells lining the sinusoids or from the reticuloendothelial cells with the liver stroma.

Kidney. No gross lesions were seen in this group of rats. Microscopically, most of the rats killed after the 3rd month of ingestion of the cycad diet had slight chronic interstitial nephritis.

Pancreas. No gross lesions were noted. Microscopically, a few patchy microscopic fibrotic areas were noted near the interlobular ducts.

Lungs. More than 50% of the rats had chronic murine pneumonia. One rat had metastatic tumors in the lung. These were scattered in all the lobes. They were nodular and about 2 mm. in diameter. The nodules had a translucent, reddish center surrounded by a hemorrhagic periphery. The hemorrhagic line clearly demarcated the lesion from the rest of the lung tissue. Histologically, the metastatic tumors were hepatomas with similar cellular patterns as those found on the liver of the same rat.

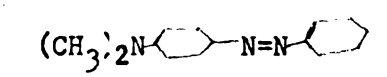
Other lesions. The rat with the largest whitish slimy tumor on the liver had a whitish mass 1 cm. in diameter adhered to the inner wall of the abdominal cavity. The mass was firm in consistency. Histologically, it was composed of thick fibrous sheets oriented in different directions. Among these fibers were glandular structures lined with cuboidal epithelium with a prominent basement membrane. The lumens of these glands contained a mucous exudate. Reticulum stain demonstrated reticulum fibers. The tumor was diagnosed as a fibroadenoma. Two rats had small oval whitish growths at the ileocecal junction. These were glandular structures with a cuboidal desquamated epithelium lining. The fibrous stroma had leukocytic infiltration, mainly monocytes. The lesion appeared more or less like a granulomatous reaction.

Experiment II. Carcinogenesis in the Rats Induced by Specific Chemicals

INTRODUCTION

A number of chemical compounds have been known to induce carcinogenesis in animals. Miller et al. (1953) demonstrated that at least 1 n-methyl group is required for carcinogenic activity of dyes related to dimethylaminoazobenzene. Both dimethylaminoazobenzene and dimethylnitrosamine have n-dimethyl groups, as shown.

$(CH_3)_2NNO$
dimethylnitrosamine


P-dimethylaminoazobenzene

Schoental (1960) suggested that dimethylnitrosamine owes its carcinogenic activity to its metabolic breakdown in the form of diazomethane. Argus et al. (1961) suggested that a similar mechanism may exist by analogy with diethylnitrosamine $(C_2H_5)_2NNO$.

A toxic constituent in the cycad nuts has been identified as cycasin (methylazoxymethyl-B-glucoside) (Matsumoto and Strong, 1963). The compound is nontoxic when administered orally. Matsumoto et al. (unpublished) have demonstrated that the toxic component of cycasin is the aglycone methylazoxymethanol, or its metabolic product. Laqueur et al. (1963) suggested that a similar metabolic breakdown to diazomethane could result from cycasin or its aglycone ($C_6H_{11}O_5$-O-$CH_2NNO{=}CH_3$).

If all these compounds produce their toxic reactions only after being converted to diazomethane, it would suggest that they should produce similar lesions. This experiment was designed to test this theory.

EXPERIMENTAL PROCEDURE

Dimethylnitrosamine, diethylnitrosamine and P-dimethylaminoazobenzene were purchased from Eastman-Kodak Company. Cycasin was obtained from the National Institutes of Health.

Weanling male Sprague-Dawley rats, 50 in number, were used. The rats were divided into 5 groups of 10 each and housed in individual metal cages. The dimethylnitrosamine, diethylnitrosamine and P-dimethylaminoazobenzene chemicals were first dissolved in olive oil before mixing with the basal M_1 ration. Cycasin was dissolved in water.

Small portions of M_1 ration were placed in trays. The dissolved chemical was sprayed over the ration and premixed by hand. The weighed M_1 ration was then added to make the required percentage and was mixed in a Hobart mixer. The diets were kept in tightly closed tin cans, as described in Experiment I. The different groups and treatment for each are as follows:

Group A, 125 p.p.m. dimethylaminoazobenzene

Group B, 125 p.p.m. diethylnitrosamine

Group C, 1000 p.p.m. dimethylaminoazobenzene

Group D, 400 p.p.m. cycasin

Group E, controls, basal M_1 ration

The feeding routine, as described in Experiment I, was followed. In this experiment, the rats were weighed weekly and killed (1 from each group) at 3, 7, 14, and 21 days. The remainder were kept until found dead, in a moribund condition, or were killed after 6 months, when the experiment was terminated.

RESULTS

Information on the growth rate of rats fed various levels of carcinogenic chemicals is given in TABLE 5. All of these chemicals caused some reduction in the normal growth rate of the rats, and it was most noticeable in the rats fed dimethylnitrosamine.

The major pathologic findings and serum SGOT and SGPT, hemoglobin and packed cell volume values are given in TABLE 6. No significant changes are noted in hemoglobin or packed cell volumes that could be attributed directly to the feeding of a specific chemical. However, significant increases are noted in SGOT and SGPT values, which varied somewhat with the chemicals. Most noticeable are the increased values associated with dimethylnitrosamine.

The incidence and types of neoplasms associated with the various chemicals are summarized in TABLE 7. The results on the group of rats fed cycasin are not included, as most of the rats in this group were killed and sent to Dr. Laqueur at the National Institutes of Health for his examination. The results in this group will be given in another report.

The dimethylnitrosamine caused deaths in the rats before neoplasm formation would have occurred. Diethylnitrosamine and dimethylaminoazobenzene appear to be stronger hepatic carcinogens than cycad flour. The cycad flour appeared to be a specific liver carcinogen, and the ability to induce kidney neoplasms appeared to be related to the concentration of that fed. A higher concentration of cycad appeared to induce a higher incidence of kidney tumors. No kidney tumors were found in the rats fed 1% cycad, while liver tumors were noted in 60% of the rats fed the same level. Two per cent cycad flour induced an incidence of 60%

kidney tumors. The description of the gross and microscopic lesions in the rats fed the different chemicals is described in detail in the following pages. The specific lesions are shown in Figures 57 to 68.

Dimethylnitrosamine

Lesions

All the rats in this group except one died or were killed, when moribund, after 96 days on the diet. Deaths started after feeding the diet for 70 days. The one which survived longer than 96 days lived for 150 days. The only pathological lesion seen grossly in animals killed before 70 days was edema of the pancreas. After 70 days of experimentation, the rats had various lesions in the viscera.

Liver. In all animals killed or dead after 70 days, the liver appeared small and firm in consistency. There were hemorrhages on the surface varying from ecchymotic to large blood splashes. In one animal killed at 96 days, the liver had several grayish-white nodules similar in size and appearance to those described in rats fed 2% cycad flour. In the rat killed at 150 days, the liver had both firm and cystlike nodules.

Microscopically, the earliest lesions seen were noted in the hepatic cells around the central veins. These consisted of congestion of sinusoids, loss of cytoplasmic basophilia and accumulation of fine fatty vacuoles. This occurred on the 3rd day of the experiment. At the 7th day, there was a contraction of the cytoplasm of these cells and a characteristic eosinophilic staining. In a few lobules there was centrolobular necrosis with hemorrhagic components. A few cells had eosinophilic cytoplasmic droplets. The distribution of these droplets was very varied. At 14 days there was an accumulation of leukocytes, mainly in the necrotic

central areas of the lobules and occasionally in the portal areas. At 21 days, fibroblast proliferation was noted in the necrotic debris in central areas of the lobules. At the portal areas there was a slight proliferation of fibroblasts and large oval nucleated cells resembling those of the bile duct. At 28 days many hepatic cells were enlarged and had large nuclei. A few were binucleated. The cytoplasm of these cells was eosinophilic and vacuolated. In the necrotic debris there were many macrophages and a few neutrophils.

In those rats killed or that died between 70 and 96 days, the main histological lesion was extensive centrolobular hemorrhage. Many lobules appeared homogeneous, containing only necrotic debris. In the midzonal and portal areas of the lobules were apparently normal cells with very vacuolated cytoplasm. Some had 1 to 4 intracytoplasmic eosinophilic hyaline droplets. In the portal areas there was proliferation of both fibroblasts and bile ducts. Mature fibrous connective tissue was seen winding around regenerating hepatic cells. Accumulation of reticulo-endothelial cells was noted in a few scattered areas, together with macrophages containing ingested pigment.

The rat killed at 96 days had nodular hyperplasia similar in histological pattern to those described in rats fed 2% cycad flour. The rat killed at 150 days had dilated bile ducts, corresponding to gross cyst-like nodules. The histological picture was similar to that seen in animals fed 2% cycad.

Kidney. No significant gross lesion, except paleness, was seen in this organ. The earliest histological lesion was seen after the rats had been fed the diet for 3 days. This consisted of fatty infiltration of

the cells of the ascending loop of Henle. At 14 days coagulative necrosis of the proximal convoluted tubules was noted. In animals killed after 14 days, different lesions were noted. In some, the kidney appeared normal, and in others there was slight interstitial nephritis with brown pigments in the cells of the proximal convoluted tubules.

Peritoneum. Ascites in amounts between 20 and 45 ml. occurred in all animals killed or dying after 21 days. The ascites was hemorrhagic in character.

Pancreas. In all rats killed after 21 days, the pancreas was edematous, with a jelly-like appearance. Microscopically, the edema was interstitial. All rats killed after 21 days had edema in varying degrees. There was no inflammatory cell infiltration in the edematous exudate, but several macrophages with ingested pigment were seen in the interstitial areas. There was very little precipitated material in the edematous areas. The acinar tissue had vacuolation of cytoplasm, and in 2 rats coagulative necrosis of the acinar cells, but no fibrosis, was seen.

Lungs. Lesions in the lung varied from congestion to extensive hemorrhage, where every lung alveolus was filled with erythrocytes.

Gastrointestinal tract. Hemorrhagic gastroenteritis was observed in the anterior portion.

Diethylnitrosamine

Lesions

The chronic pathological lesions in this group, in general, were similar to those reported by Argus et al. (1961), with the exception of the development of tumors of the vascular system, which are reported in this thesis for the first time (in rats) as far as known.

Liver. The first gross liver change was seen in a rat killed after 139 days on the diet. This consisted of a great distortion in the shape of the liver. One large whitish tumor on the median lobe, measuring 2.5 cm. in diameter, was present. In addition, there were many other smaller tumors varying between 0.6 and 1 cm. in diameter. The large tumor had metastasized into the peritoneum and lung. On the peritoneum the lesions were mainly granule-like nodules, whitish in color and firm in consistency. The whole peritoneum was edematous. Forty milliliters of serous fluid were removed from the abdominal cavity.

Four more rats were killed between 139 and 180 days, and they all had tumors on the liver. The tumors varied greatly in size, location and number in any one particular lobe of the liver. All the lobes contained tumors. Most of the tumors were whitish in color and of firm consistency. In 2 rats, tumors of different types were seen. In one rat a reddish mass was noted on the right lateral lobe of the liver. On cross section, blood oozed out. The other rat had a large tumor, 2.6 cm. in diameter, which was whitish-red in color and firm in consistency. In addition, there were 2 small nodules, reddish in color and of soft consistency. On cross section they had cavities filled with blood.

Microscopically, in the rats killed during the first 2 weeks on the diet, the liver cells appeared essentially normal, with the exception of a very slight rarefication of the cytoplasm. At 14 days, a few hyaline droplets were seen around the central veins. A few liver cells had fatty vacuoles. At 21 days a few cells had a contracted eosinophilic cytoplasm and intracytoplasmic droplets. Many enlarged hepatic cells were seen with enlarged nuclei.

Three types of neoplasms appeared in the liver. The large tumors measuring 2-4 cm. in diameter, together with many smaller ones, histologically were hepatomas. In 2 rats, an adenocarcinoma occurred adjacent to a hepatoma. The reddish tumors which appeared in 2 rats were hemangiomas.

Hepatoma. These tumors varied considerably in histological pattern. Some were composed of small cells resembling hepatic cells with hyperchromatic nuclei. Others had a cell type which appeared larger than normal liver cells. The cells were arranged in cords alternating with endothelial-lined blood sinuses. The sinuses were filled with neutrophils and macrophages. The hepatomas were associated with cirrhosis of the adjacent liver tissue. Extramedullary hematopoietic centers were noted in most of the tumors. The cytoplasm of the tumor cells had many hyaline droplets, with staining characteristics similar to those described in rats fed 2% cycad flour. The adjacent liver tissue occasionally had fatty infiltration in the hepatic cells.

Adenocarcinoma. The 2 cases of adenocarcinoma were similar in histological pattern to those described for dimethylaminoazobenzene fed rats.

Hemangioma. These tumors were composed of large blood spaces, lined by a single layer of epithelial cells. Between the blood spaces were atrophied hepatic cells. Most of the stroma was made up of mature collaginous fibers, among which were proliferating fibroblasts. Within the stroma there was a yellowish pigment.

Histologically, the metastasized nodules in the peritoneum and lungs noted in one animal were similar to the adenocarcinoma in the liver of the same animal.

Lungs. Chronic murine pneumonia and consolidation of lung parenchyma were seen in all rats. In one rat the bronchial epithelium showed metaplasia of the epithelium into a squamous type of epithelium with keratinization.

Peritoneum. In 2 rats, ascites amounting to 40 and 50 ml. was present. The fluid was hemorrhagic in both cases.

Kidney. The epithelium of the proximal convoluted tubules contained a yellowish pigment.

P-dimethylaminoazobenzene

The pathological changes in the livers of the rats fed dimethylaminoazobenzene and liver tumors induced by this chemical have been studied in detail by Edwards et al. (1941-42), Kinosita (1937), Opie (1944), Orr (1940) and Price (1949). Changes seen in this group of animals were similar to those described by the above authors.

Lesions

Liver. The first gross changes were seen in a rat killed after 120 days on the experiment. These were tumors on the liver. All 5 rats killed

between this time and 180 days had tumors. In addition to the large solid tumors, the livers had multiple cysts, 2 to 3 mm. in diameter. In some animals the cysts were very numerous, forming localized clusters. The cysts contained clear fluid on cross section. All rats had multiple tumors. In some, all lobes of the liver were involved. The tumors varied in consistency, size and color. Their colors were whitish-gray and pinkish-red. The whitish-gray tumors had a firm and fibrous consistency. The largest of these tumors had a diameter of 4.9 cm. Others varied from 2 to 4.6 cm. in diameter. In addition, there were smaller firm nodules, about 0.6 to 1 cm. in diameter, which gave the external surface of the liver a rough and nodular appearance. Histologically, these were nodular hyperplasias. The large tumors contained necrotic and hemorrhagic centers.

The pinkish-red tumors had a soft to rubbery consistency, and on cross section they were hemorrhagic. The largest of these was seen in the rat killed after 120 days. In most cases the tumors were adherent to the adjacent diaphragm, stomach, pancreas, spleen and omentum. Grossly, metastatic tumors were seen in the lung, peritoneum, spleen, pancreas, and intestinal lymph nodes.

The earliest microscopic change in the liver was present in rats after 3 days of ingestion of the diet containing dimethylaminoazobenzene. This consisted of a characteristic eosinophilic staining of the cytoplasm of cells at the periphery of the lobules. A few cells had fine fat vacuoles, as demonstrated with Sudan IV stain. At 7 days, small hyaline droplets similar in staining characteristics to those described in rats fed 2% cycad flour were present.

The third change in the liver was seen at the 21st day of the experiment. This consisted of proliferating basophilic oval-shaped cells around the portal areas. Next to the bile ducts many of the proliferating cells were seen arranged in a glandular pattern with narrow lumens. Extending away from the portal areas towards the central veins the proliferating cells were arranged in a form of cell cords or sheets. Among these cell sheets neutrophilic leukocytic infiltration was noted in a few instances. The surviving hepatic cells were very enlarged, with rarefied and vacuolated cytoplasm and an enlarged nuclei.

Five types of neoplasms appeared in the livers in these rats. They were classified as bile duct adenomas, cystadenomas, adenocarcinomas, hepatomas and hemangioendotheliomas.

Cystadenomas and hepatomas were generally similar in histological pattern to those described in previous animals.

Bile duct adenomas. These tumors were grossly small - about 1 cm. in diameter. Histologically, they were composed of glandular structures with their lumens filled with mucous coagulated exudate. The epithelium lining these glands was atrophied and in some was completely desquamated. Associated with these glands were wide bands of connective tissue which separated the glands. These tumors in some areas were adjacent to a hepatoma or associated with liver parenchyma with excess fatty infiltration and many hyaline droplets within their cytoplasm.

Adenocarcinoma. The large tumors were generally adenocarcinomas or adenocarcinomas associated with hepatomas. They were composed of columnar epithelium several layers thick forming glandular structures.

On some occasions no clear lumen was formed. The cells of the gland were hyperchromatic and had lost the normal gland cell polarity. Numerous atypical mitotic figures were seen. Some tumors had papillary projections in a cystic glandular structure. There were marked inflammatory reactions, necrosis, and hemorrhages within and around some of the large tumors. Within the glandular structure was some connective tissue metaplasia (Figures 63 and 64). This was seen as spindle-shaped cells proliferating in the lumen of the glandular structures and in some cases connected to the stroma of it. In 2 rats, hyaline cartilage metaplasia was seen in the adenocarcinomas (Figures 65 and 66). In these rats similar cartilages were seen in the metastatic tumors on the peritoneum. All grossly visible metastatic tumors in different organs histologically were adenocarcinomas.

Hemangioendothelioma. Histologically, these tumors were composed of spindle-shaped cells with hyperchromatic, oval, or elongated nuclei. These cells in some areas had anastomosing processes and tended to form variable cell sheets. There were many blood spaces lined with a single layer of endothelial cells. The blood spaces varied greatly in size and were filled with blood. Mitotic figures were quite common in most of the cells. In areas with the cell sheets there was infiltration of a few macrophages with ingested pigment.

Kidney. Microscopically, the epithelium of the proximal convoluted tubules of the kidney was filled with a yellowish pigment.

Peritoneum. Serous to hemorrhagic ascites, measuring from 20 to 50 ml., occurred in all rats with tumors.

Pancreas. Interstitial edema of the pancreas was seen in allrats with liver tumors. In 2 rats, metastatic adenocarcinoma was present.

Spleen. The spleen appeared enlarged and reddish-purple in color in most rats. Microscopically, the splenic sinusoids were engorged with erythrocytes. Within the splenic stroma there was a lot of hemosiderin and macrophages with ingested pigments. In one case the splenic lymphoid tissue had been replaced with fibrous tissue.

Cycasin

The acute lesions in animals fed 400 p.p.m. of cycasin were similar to those described for rats on 2% cycad flour. The incidence and type of tumor on rats fed cycasin for a long period are still under study by Dr. Laqueur.

Experiment III. The Effects of Feeding Cycad Flour for a Short Period of Time on Tumor Formation

INTRODUCTION

This experiment was conducted to determine if cycad flour fed to rats for a short period of time would result in a progressive lesion which might result in formation of liver tumors.

EXPERIMENTAL PROCEDURE

Twenty-five weanling Sprague-Dawley rats, 38 to 62 Gm. in weight, were used. The rats were put into 2 groups, 10 females and 15 males. The rats were housed in groups of 4. The housing and feeding techniques were the same as those described in Experiment I.

The females were fed M_1 ration containing 1% cycad flour for 3 weeks. They were then fed the basal M_1 ration for 6 months and killed.

RESULTS

TABLE 7 summarizes the incidence and types of tumors observed in this experiment.

Lesions

Liver. The first gross lesions noted in this experiment were seen in 2 of the 10 rats. The lesions were translucent, cystlike nodules at the periphery of the liver lobules. One had 3 large nodules, and the other one had 2 small nodules. On cross section, serous fluid was noted.

Microscopically, the nodules were dilated bile ducts similar in histological structure to those described in Experiment I as cystadenomas. Histologically, in the male rats fed the diet containing 2% cycad for 2 weeks and then normal diet for 6 to 10 months, regeneration of hepatic cells was noted occasionally. The regenerated areas were patchy in distribution and consisted of large hepatic cells very closely packed with no columnar pattern. The cytoplasm was vacuolated, but no fibrosis was seen.

Kidney. In the male group of rats, the gross lesions were noted in 3 rats after consuming the 2% cycad flour for 2 weeks and then the basal ration for 10 months. One had a large oval whitish mass on the left kidney. The tumor was firm in consistency and on cross section extended from cortex to the pelvis. The other 2 had small round nodules localized in the renal cortex.

Histologically, all rats in this group had chronic interstitial nephritis which varied greatly from one rat to the other. In some, marked fibroblastic proliferation was demonstrated. The tumors seen

grossly in the 3 rats were diagnosed as embryonal nephromas. The tumors had in general cellular components similar to those described in Experiment I, in which rats were fed 2% cycad for a long period of time.

GENERAL DISCUSSION FOR PART II

The earliest histological change induced by all the carcinogens was similar. This consisted of development of hyaline droplets, or what has been referred to in the literature as hyaline inclusion bodies, in the cytoplasm of the parenchymal cells. This histological alteration occurred between 3 and 7 days after the first ingestion of 3 of the carcinogens. In the case of diethylnitrosamine, it occurred by the 2nd week. All the hyaline droplets had similar histochemical staining characteristics. Initially, they differed in distribution. With cycad flour and the nitrosamines, the droplets were of centrolobular distribution and later they occurred around the portal areas. With dimethylaminoazobenzene they were of peripheral distribution in the first week but later were found centrolobularly. These droplets probably represented a degenerative change, as they were followed by coagulative necrosis in the regions of their earliest appearance, either centrolobularly or peripherally. The appearance of hyaline droplets was accompanied by fatty infiltration of varying degrees. The distribution of fatty infiltration followed that of the hyaline droplets and was more marked with dimethylaminoazobenzene than with the other carcinogens. This alteration was followed by necrosis, with or without hemorrhagic components, and infiltration of leukocytes.

The differences in histological alteration were noted in animals killed on the 21st day after ingestion of the carcinogens. In rats fed

cycad flour, cycasin or the nitrosamines, the centrolobular areas were composed of collapsed central veins with leukocytes and proliferating fibroblasts within the necrotic debris. In animals fed the diet containing dimethylaminoazobenzene, the zone of necrosis was being replaced by extensive hyperplasia of oval basophilic cells and leukocytic infiltration. All livers of the animals fed any one of the carcinogens had hepatic cell regeneration in the necrotic areas. The regenerating hepatic cells were characterized by their large size, with enlarged nuclei, and were occasionally binucleated. The nuclei had enlarged nucleoli. The cytoplasm was vacuolated.

The sequence of histological changes up to this point in rats fed diets containing cycad flour is similar to that described by Laqueur (1963), who described the sequence of changes for 2 weeks. He also described in general the chronic lesions. The sequence of histological changes in rats fed the dimethylaminoazobenzene diet is similar to that described by Kinosita (1937), Orr (1940), Opie (1944) and Price et al. (1952).

In rats fed the diet containing nitrosamine, the lesions are similar to those described by Barnes et al. (1954).

Fibrosis of the liver of the rats fed the 2% cycad flour started at the central veins. This was seen as a proliferation of fibroblasts in the necrotic debris. Almost at the same time, fibrosis was developing around the portal areas. In rats, diffuse fibrosis was not observed as regularly as in pigs, where tissues occluding the central veins were connected by thick fibrous strands with portal fibrosis.

In some rats, hyperplasia of the arterial intima and lesions simulating a chronic thrombus were seen in the sublobular veins and in the

venules. They were in areas with extensive chronic inflammation. These are probably processes of chronic irritation, as they could not be demonstrated elsewhere. Magee et al. (1956) described intravascular ante-mortem thrombosis in the vessels of the liver of rats following chronic ingestion of dimethylnitrosamine. They were unable to identify the vessel due to the distortion of the liver.

Laqueur et al. (1963) described a lesion which they called reticuloendothelial proliferation. With use of reticulum stains they could not decide the derivation of these cells. They considered the possibility of it being an angiomatous tumor due to the large cystic blood spaces.

This lesion was followed in these animals from early development of dark staining cells to formation of large tumors of similar cells. The cells were of reticuloendothelial origin. Several were seen proliferating from the Kupffer cells of the hepatic sinusoids. Others were seen developing around the portal areas, where they formed localized lesions. In more chronic lesions composed of similar cells, reticulum fibers were easily demonstrated with reticulum stain as projections from these spindle-shaped cells. Gillman et al. (1949) have described proliferation of reticuloendothelial cells lining the sinusoids of the rat liver and also reticuloendothelial cell proliferation around central vens and portal areas after repeated injection of trypan blue. They followed the development of this lesion to the formation of a high incidence of what they diagnosed as reticulum cell sarcoma.

The reticulum cell sarcoma tumors produced morphologically resemble human tumors described as Kupffer cell sarcoma or hemangio-endothelioma of the liver (Gray, 1929, Puhr, 1931, White, 1933, Videback, 1946, and Baker et al., 1956), as summarized by Baker et al. (1956).

Nodular hyperplasia of hepatic cells was common in rats after chronic ingestion of all the carcinogens. It was characterized by changes in staining and arrangement of cells. This lesion was more frequent with other carcinogens than dimethylaminoazobenzene. This lesion appears to be the one which develops into a hepatoma, although no gradual differentiation from one to the other was noted. Its frequency in the rats fed the carcinogens producing a high incidence of hepatomas supports this theory. The proliferating oval cells around portal areas are the possible origin of the adenocarcinomatous tumors, which occurred in high incidence in the rats fed dimethylaminoazobenzene and not in those fed low levels of cycad flour, and in only 2 cases after the chronic ingestion of diethylnitrosamine.

The adenocarcinomas and hepatomas observed were morphologically similar to the tumors produced by different carcinogens which have been described in the literature. Cartilage and bone formation had been noted previously in hepatic adenocarcinoma after chronic ingestion of dimethylaminoazobenzene by Edward and White (1941). Firminger et al. (1952) have described islands of cartilage in adenocarcinoma induced by chronic ingestion of dimethylaminoazobenzene.

In these studies 2 rats had cartilage islands in the adenocarcinoma. In one, sarcomatous metaplasia occurred inside the glandular part of the adenocarcinoma. Cartilage islands were found in the metastatic lesion in the peritoneum in both rats, with cartilage in the adenocarcinoma of the liver.

Richardson et al. (1951) reported angiosarcoma in rats fed 3-methyl-4-dimethylaminoazobenzene. In these studies, hemangioendotheliomas and hemangiomas were seen only in rats fed p-dimethylaminoazobenzene.

Hemangiomas have not been reported after chronic ingestion of nitrosamine in rats. In this study, hemangiomas of the liver were seen in rats fed 2% cycad flour and in those fed diets containing diethylnitrosamine. Hemangiomas of diaphragm muscle were seen in 2 animals fed a diet containing 2% cycad flour. Morphologically, these hemangiomas were not different from those seen in rats injected at birth with 9.10-dimethyl-1-2-benzanthrene (Howell, 1964).

Kirsten et al. (1962) produced similar hemangiomas in rats by injecting polyoma virus into rats at birth. Furth (1933, 1934) induced hemangioma and hemangio-endothelioma in mice following treatment with carcinogenic hydrocarbons. Andervont (1950) had a high incidence of hemangio-endothelioma and sarcoma after treating mice with o-aminoazotoluene.

Cystic ducts and cystadenomas consisted of dilated tubules lined by endothelial-like or cuboidal epithelium. Those termed cystadenoma had lumens filled with homogeneous material which grossly had a serous appearance. This type of lesion was very extensive in animals which had been fed a diet containing dimethylaminoazobenzene. The number of cysts and their size assembled in one spot varied widely. Histologically, all lesions were similar in all groups of animals fed diets containing the different carcinogens. The periphery of the cysts was lined by a single layer of cuboidal epithelium, while there was progressive flattening of the epithelial lining towards the center, resembling the endothelial cells.

Renal adenomas, with the exception of the lack of cystic appearance, in this study were in general similar to those described by Boyland et al. (1962) and Van Esch et al. (1962) after chronic lead intoxication. They were also similar, in general, to those described by Morris et al.

(1957) in rats after treatment with N-4-(4-fluoro)-biphenylacetamide. Mathews and Walpole (1958) described similar adenomas in rats fed 4-fluoro-4-aminodiphenyl. They had a similar histological appearance to what Magee et al. (1962) described as well differentiated tumors in rats after chronic ingestion of diethylnitrosamine. Laqueur et al. (1963) fed rats cycad flour and observed adenomas morphologically similar, except for the cystic part, which was not seen in this study. However, cysts were observed in adenomatous parts of the embryonal nephromas in all animals with this tumor.

The embryonal nephromas observed in rats after chronic ingestion of 2% cycad and in rats after ingestion of 2% cycad for 2 weeks then control diet for 9 months were in general similar in histological morphology to what Magee et al. (1962) described as anaplastic tumors after chronic ingestion of dimethylnitrosamine. However, no cartilage was seen in the embryonal nephromas in this study, while in their study they observed cartilage metaplasia in a few cases.

In this study, no kidney tumors were seen in any group other than that fed a diet containing 2% cycad flour.

Laqueur et al. (1963) described 2 cases of sarcomas of the kidney, with one case having metastasized into the lung. No metastases were seen in this study in animals fed diets containing less than 2% cycad flour up to 9 months, but the 3 fibrosarcomas seen were very large, and the invasive character and high number of mitotic figures leaves no doubt of their malignancy. The failure of metastasis may be related to the lack of renal vessel involvement and early killing of the animals.

Most of the rats fed diets containing one of the carcinogens developed ascites of varying amounts and edema of the pancreas. Only 8 rats

fed the diet containing 2% cycad flour showed generalized degeneration of acinar tissue of the pancreas with extensive inflammatory reaction or fibrotic pancreatitis. Generalized pancreatic lesions were normally associated with a large amount of edema and more damage of hepatic parenchymal tissue than have been seen in the other animals.

The relationship between fatty changes and cirrhosis of the liver, on the one hand, and pancreatic disease, on the other, have been reported in man by several investigators. Davis (1948) stated that pancreatic lesions in kwashiorkor occur first and are responsible for hepatic changes. Hartz (1949) expressed a similar opinion when describing pancreatic atrophy in infants with fatty liver. Gillman et al. (1949), however, stated that fatty liver was the first lesion, followed by pancreatic lesion. In our work (unpublished), when weanling rats were fed milk from a cow ingesting cycad, fatty liver appeared first, followed by pancreatic lesions.

In man, Steinhaus (1902) described interstitial fibrosis of the pancreas in all of 12 cases of cirrhosis of the liver. Kirshbaum et al. (1943) studied 356 cases of alcoholic cirrhosis and found a 36.2% incidence of pancreatic fibrosis. Stinson et al. (1952) compared 75 cases of cirrhosis of the liver with 75 control cases without any liver lesions. In 72 out of the 75 subjects with cirrhosis of the liver, there was some inflammatory reaction of the pancreas. The reaction was characterized by interstitial infiltration of leukocytes. In the controls they observed 37 cases of interstitial pancreatitis, and most of the cases were mild.

Netik (1957) described a high frequency of pancreatic fibrosis in liver cirrhosis among Africans. No definite conclusion can be drawn as to the pathogenesis of the pancreatic lesions, but there are 3 possible contributory factors: (1) the same factors that produced the hepatic injury could have produced the pancreatic injury; (2) edema and accumulation of metabolic products around the interstitial areas of the pancreas could have produced the injury; and (3) damage of the liver could have reduced the ability of the liver to perform its normal synthesis of particular amino acids which indirectly would produce a deficiency of the particular amino acid important for maintenance of the integrity of pancreatic acinar tissue. Veghelyi et al. (1950) produced degenerative changes and fibrosis of the pancreas in rats by feeding yeast or gelatin as the only source of protein. The pancreatic lesions preceded by many weeks the changes in the liver.

The complete regeneration of liver cells after short-term feeding of rats with 2% cycad flour and development of kidney tumor was similar to that described by Magee et al. (1959) with dimethylnitrosamine. The incidence of the kidney tumors was very low in our series in comparison with their report.

The finding of a naturally occurring carcinogen in a plant so widely used by man and having such wide distribution raises a theoretical consideration of the possible association of this plant and cancer in man. Available information suggests a high liver carcinoma in all areas where cycads are indigenous. Higginson (1963) summarized epidemiological data on primary liver carcinoma. This summary indicates a high incidence of primary liver cancer in Africa south of the Sahara and in southeast Asia. Higginson (1963) states:

> "The highest incidence of primary liver cancer is reported in Bantu males from Lourenco Marques, Mozambique, where in the 25 to 34 year age group, the rate is nearly 500 times that in the United States and 15 times that in South African Bantu males."

Kwashiorkor has been suggested, but it has been demonstrated that kwashiorkor itself does not lead to cirrhosis of the liver; and the liver of the young African adult is histologically normal (Broek, 1957, Higginson et al., 1957, and Trowell, 1960). Higginson (1963) indicated that liver cancer is rare in India and Latin America, where kwashiorkor is common.

There is no other single agent other than the cycad which has been shown to be common in these 2 communities of widely differing cultures in Asia and Africa. Cycad is widely used in these areas both uncooked and cooked. Campbell (1964) demonstrated that both dry and moist heat destroy cycad toxic factors. A map of the world (Figure 68) shows the areas of known or suspected high incidence of primary liver cancer, distribution of kwashiorkor and distribution of 2 cycads which are used often uncooked. This distribution suggests a possible connection between the ingestion of uncooked cycad products and a high incidence of liver cancer.

TABLE 4. Composition of Basal Ration (M_1) Fed to Rats

Ingredient	Pounds per 100 lb.*
Yellow corn, ground	60.70
Soybean oil meal, 50% protein	27.98
Dehydrated alfalfa meal, 17% protein	2.00
Fish solubles, condensed (50% solids)	2.50
Dried whey product, 50% lactose	2.50
DL methionine	0.50
Calcium Carbonate (38% Ca)	1.60
Dicalcium Phosphate, 20% P, 24% Ca	1.75
Salt	0.50
Vitamin A, stabilized (4000 IU/Gm.)	0.20
Vitamin D_3 (1500 IU/Gm.)	0.05
Choline Chloride	0.07
Total	100.00

*To each 100 lb. of ration the following vitamins or additives were added: Riboflavin, 150 mg.; calcium pantothenate, 250 mg.; Niacin, 1.5 Gm.; vitamin B_{12}, .3 mg.; Alpha Tocopherol acetate, 200 mg.; vitamin K (menadione), 100 mg.; and Arsanilic acid, 4.5 Gm.

TABLE 5. Average Body Weights (Gm.) of Weanling Rats Fed a Basal Ration Plus Various Levels of Cycad Flour, Cycasin, Dimethylnitrosamine, Diethylnitrosamine or Dimethylaminoazobenzene. (Numbers in parentheses indicate number of rats.)

		Weeks of Study											
		1	2	3	4	5	7	9	11	15	19	23	26
Control M1	Male	90 (10)	141	188 (9)	216	251	320	367	397	438	457	472	478
	Female	81	123	152	161	180	210	226	236	255	264	272	276
Cycad Flour 2%*	M	88 (10)	110	137	143	168	225	262	311	339	361	386	391
	F	70 (10)	95	119	126	134	153	177	208	217	239	258	250
Cycad Flour 1%*	M	90 (10)	139	181	205	238	291	340	372	410	415	446	478
Cycasin 400 p.p.m.	M	90	108	126	144	176	240 (18)	275	306 (17)	352 (16)	374	392 (15)	396 (13)
	F	81	92	100	118	133	168	183	196	218	235	236	244
Cycasin 200 p.p.m.	M	90 (20)	121	144	173	206	273	316 (19)	316	403	421	442	446
	F	81 (20)	101	121	134	157	185 (18)	200	210	229 (17)	243	259	257
Dimethyl-nitrosamine 125 p.p.m.	M	120 (8)	149 (7)	173 (6)	209	211 (5)	235	244	248	279 (4)	278	280	
Dimethylamino-azobenzene 1 Gm./kg.	M	120 (8)	124 (7)	153 (6)	197	157 (5)	167	166	183	210	237	244	237
Control	M	120	167	193	131	255	276	316	354	390	402	425	432

*The body weights for rats fed 2% and 1% cycad flour are only for the rats which survived more than 10 weeks.

TABLE 6. Summary of the Major Pathological Findings, Serum SGOT and SGPT, Hemoglobin, and Packed Cell Volumes in Experiments I and II

Days on Expt.	Ingredient	Amount	SGOT*	SGPT*	Liver Lesion	Hemoglobin (Gm./100 ml.)	PCV %
3	Control		62	22	No lesion	16.0	48
	Cycasin	400 p.p.m.	120	36	Cloudy swelling	15.0	42
	$(CH_3)_2NNO$	125 p.p.m.	120	38	Cloudy swelling	14.0	43
	DAB**	1000 p.p.m.	124	38	Cloudy swelling	16.0	45
	$(C_2H_5)_2NNO$	125 p.p.m.	72	24	No lesion	16.0	46
7	Cycasin	400 p.p.m.	148	48	Early necrosis	15.6	44
	$(CH_3)_2NNO$	125 p.p.m.	146	42	Early necrosis	13.0	42
	$(C_2H_5)_2NNO$	125 p.p.m.	120	32	Cloudy swelling	15.6	46
	DAB	1000 p.p.m.	220	72	Early necrosis	15.2	42
14	Cycasin	400 p.p.m.	672	154	Necrosis	15.0	43
	$(CH_3)_2NNO$	125 p.p.m.	740	182	Necrosis	12.3	37
	$(C_2H_5)_2NNO$	125 p.p.m.	488	152	Necrosis	16.0	45
	DAB	1000 p.p.m.	642	176	Necrosis	14.0	47
21	Cycasin	400 p.p.m.	440	131	Necrosis	15.6	44
	$(CH_3)_2NNO$	125 p.p.m.	984	158	Necrosis	13.0	42
	$(C_2H_5)_2NNO$	125 p.p.m.	---	---	---	14.8	44
	DAB	1000 p.p.m.	680	150	Necrosis	14.8	42
70	Cycasin	400 p.p.m.	390	68	Regeneration with fibrosis	13.7	41
	$(CH_3)_2NNO$	125 p.p.m.	973	174	Hemorrhagic hepatic necrosis with portal fibrosis	3.6	12

TABLE 6--continued

Days on Expt.	Ingredient	Amount	SGOT*	SGPT*	Liver Lesion	Hemoglobin (Gm./100 ml.)	PCV %
70	$(C_2H_5)_2NNO$	125 p.p.m.	340	51	Regeneration, nodular hyperplasia	14.4	44
	DAB	1000 p.p.m.	380	87	Regeneration, portal fibrosis	13.7	42
90	$(CH_3)_2NNO$	125 p.p.m.	1020	191	Extensive centrolobular necrosis with hemorrhages	3.5	9
180	Control						
	1		183	83	No lesion	16.0	47
	2		160	57	No lesion	14.4	41
	3		286	89	No lesion	15.6	35
	4		217	58	No lesion	17.5	52
	5		220	49		13.9	40
180	DAB						
	1	1000 p.p.m.	440	102	Adenocarcinoma, hepatoma	15.3	45
	2	1000 p.p.m.	360	86	Adenocarcinoma, hepatoma	16.0	46
	3	1000 p.p.m.	520	89	Adenocarcinoma, hepatoma	15.2	46
	4	1000 p.p.m.	840	149	Adenocarcinoma, hepatoma	11.9	36
180	Cycasin						
	1	400 p.p.m.	580	191	Slight fibrosis with nodular hyperplasia	14.8	41

TABLE 6--continued

Days on Expt.	Ingredient	Amount	SGOT*	SGPT*	Liver Lesion	Hemoglobin (Gm./100 ml.)	PCV %
180	Cycasin						
	2	400 p.p.m.	292	59	Slight fibrosis with nodular hyperplasia	14.8	41
	3	400 p.p.m.	350	76	Slight fibrosis with nodular hyperplasia	15.2	43
270	Cycad flour, 2%						
	1		1341	269	Hepatoma of liver, fibrosarcoma of kidney	7.7	21
	2		840	208	Hepatoma of liver, embryonal nephroma of kidney, with hemorrhage from the kidney	8.3	37
	3		257	61	Slight fibrosis	15.2	35
	4		610	197	Hepatoma	14.8	43
	5		270	66	Nodular hyperplasia	15.4	45

*Sigma-Frankel units/ml. serum 37 C.
**DAB - dimethylaminoazobenzene.

TABLE 7. Summary of the Incidence and Classification of Neoplasms Observed in Rats Fed Various Levels of Cycad and Specific Chemicals

Ingredient and Experiment	Number of Rats and Period of Observation, days*	Kidney		Liver		Other Organs, Incidence and Types
		Incidence	Types	Incidence	Types	
Cycad flour 2%, Expt. I	(20) 160-270	12/20	3 adenoma 3 fibrosarcoma 6 embryonal nephroma	9/20	2 hemangioma 8 hepatoma 2 sarcoma	2/20 on diaphragm cavernous hemangioma 1/20 on ileocecal junct. adenoma
Cycad flour 1%, Expt. II	(10) 240-310	0/10	---	6/10	4 hepatoma 4 sarcoma	2/10 on ileocecal junct. adenoma 1/10 abdom. muscle reticuloadenoma
$(CH_3)_2NNO$ 125 p.p.m. Expt. II	(1) 150	---	---	---	---	---
$(C_2H_5)_2NNO$ 125 p.p.m. Expt. II	(5) 120-170	0/5	---	5/5	5 hepatoma 2 adenocarcinoma 2 hemangioma	---
DAB** 1000 p.p.m. Expt. II	(5) 120-180	0/5	---	5/5	5 adenocarcinoma 4 bile duct adenoma 2 hemangioma 2 hepatoma	---

TABLE 7--continued

Ingredient and Experiment	Number of Rats and Period of Observation, days*	Kidney		Liver		Other Organs, Incidence and Types
		Incidence	Types	Incidence	Types	
Cycad flour 2%, Expt. III	(15) 14 days on cycad, then control diet 180-300 days	3/15	3 embryonal nephroma	0/15	---	---
Cycad flour 1%, Expt. III	(10) 21 days on cycad, then control diet 180 days	0/10	---	0/10	---	---

*The total number of rats ( ) examined after the first observation of neoplasms.
**Dimethylaminoazobenzene.

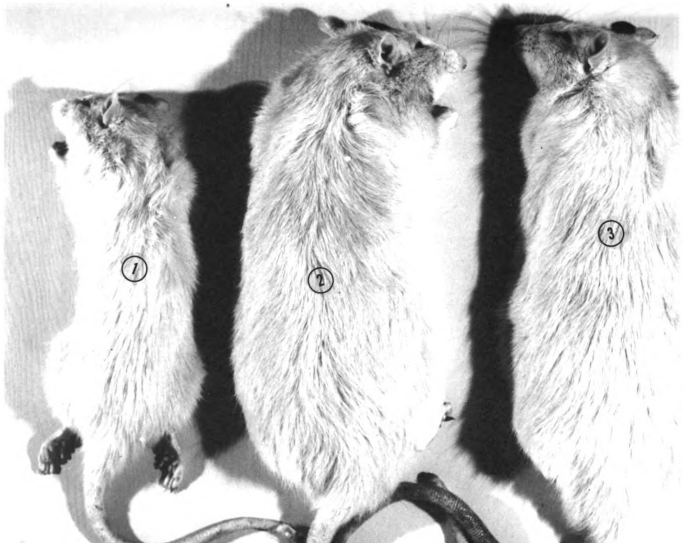


Fig. 18.--(1) Male rat fed 2% cycad for 8 months; (2) Male rat fed control diet for 8 months; (3) Male rat fed 2% cycad flour for 2 weeks, then control diet for 7 months.

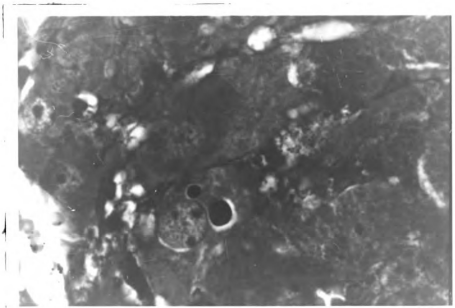


Fig. 19.--Intracytoplasmic hyaline bodies in hepatic cells of a rat fed 2% cycad for 6 days. Periodic acid Schiff: x750.

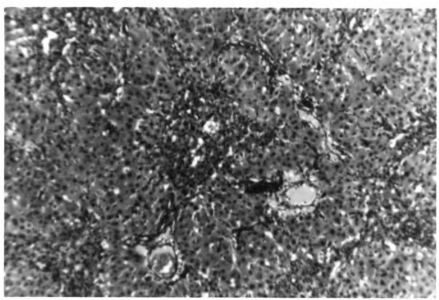


Fig. 20.--Centrolobular hemorrhage in the liver of a rat fed 5% cycad for 4 days. H & E. stain: x75

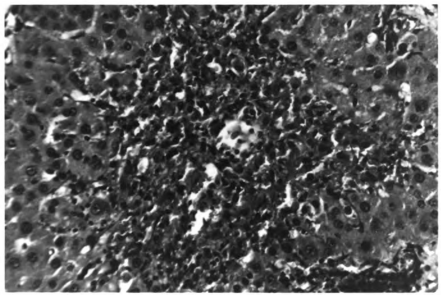


Fig. 21.--Higher power of Fig. 20. Rat fed 5% cycad for 4 days. H & E stain: x 187.

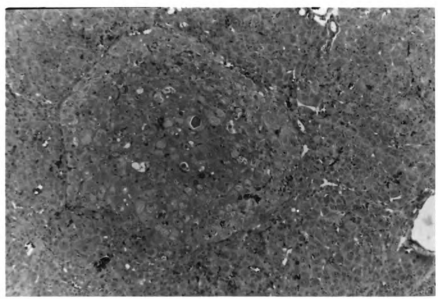


Fig. 22.--Nodular hyperplasia in liver of a rat fed 2% cycad for 70 days. H & E stain: x75.

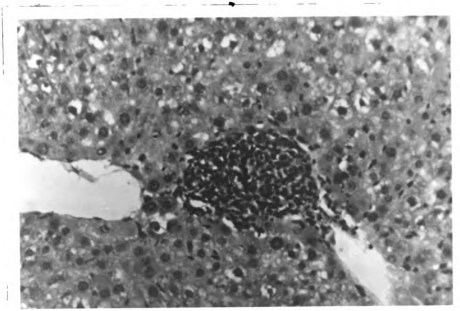


Fig. 23.--Reticuloendothelial cell proliferation adjacent to the sublobular vein, in the liver of a rat fed 1% cycad for 90 days. H & E stain: x187.

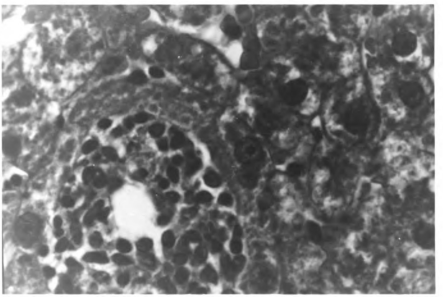


Fig. 24.--Proliferation of Kupffer cells in a dilated sinusoid of the liver of a rat fed 1% cycad for 90 days. H & E stain: x750.

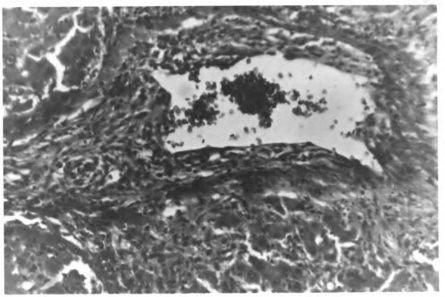


Fig. 25.--Fibrotic phlebitis of sublobular vein in the liver of a rat fed 2% cycad for 180 days. H & E stain: x187.

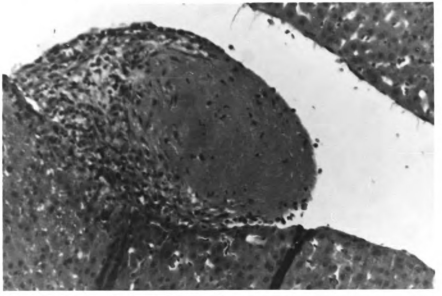


Fig. 26.--Organized thrombus in sublobular vein in a liver of a rat fed 1% cycad for 180 days. H & E stain: x187.

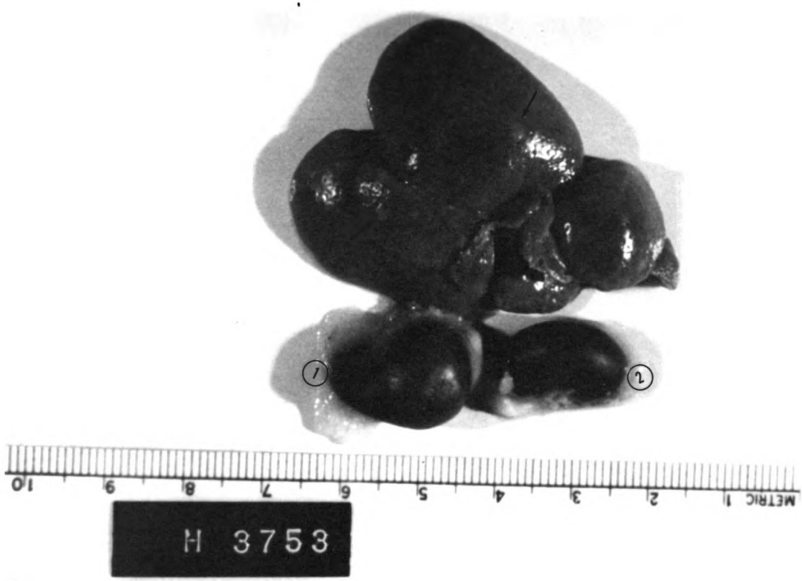


Fig. 27.--Liver and kidneys of a rat that had been fed 2% cycad flour for 180 days. Arrows show nodular hyperplasia of the liver. (1) Tumorous kidney; (2) Normal kidney.

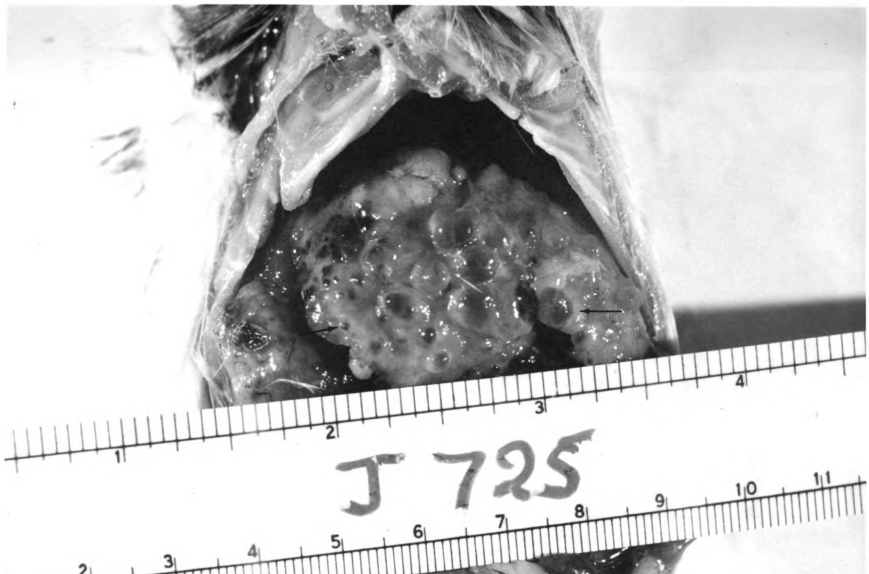


Fig. 28.--Liver of a rat that had been fed 1000 p.p.m. of dimethylaminoazobenzene for 150 days. Arrows show cystadenomas.

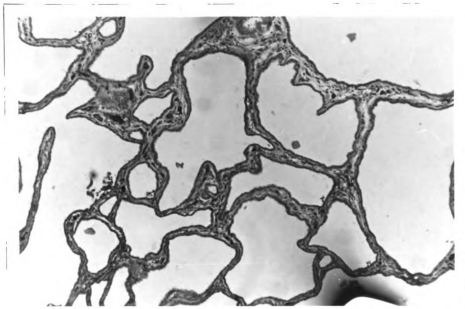


Fig. 29.--Cystadenoma. A section taken from the liver in Fig. 28 of a rat fed 1000 p.p.m. of dimethylaminoazobenzene for 150 days. H & E stain: x75.

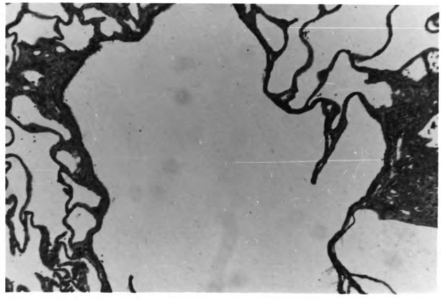


Fig. 30.--Cystadenoma. A section taken from a liver of a rat fed cycad (2%) for 180 days. H & E stain: x75.

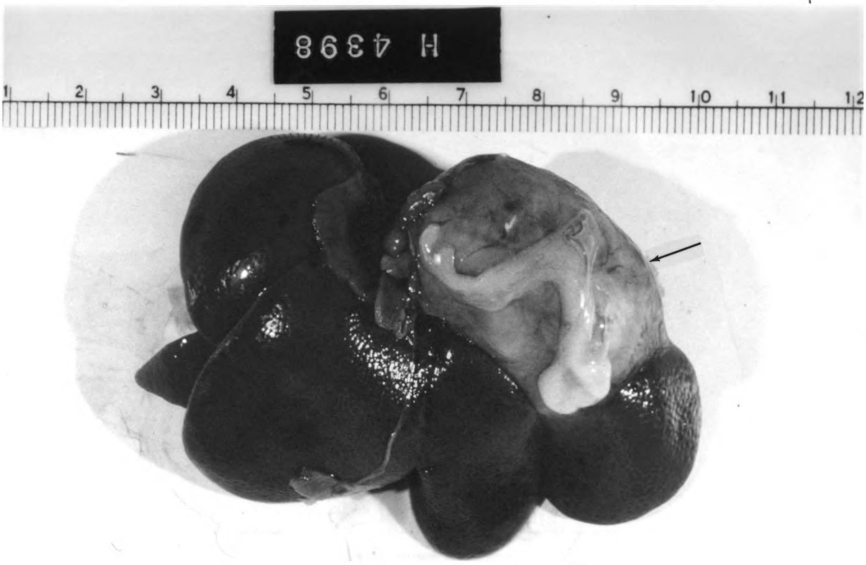


Fig. 31.--The liver of a rat fed 1% cycad flour for 8 months. Arrow shows liver hepatoma.

Fig. 32.--Cross section of a hepatoma on the liver of a rat. Same liver as Fig. 31.

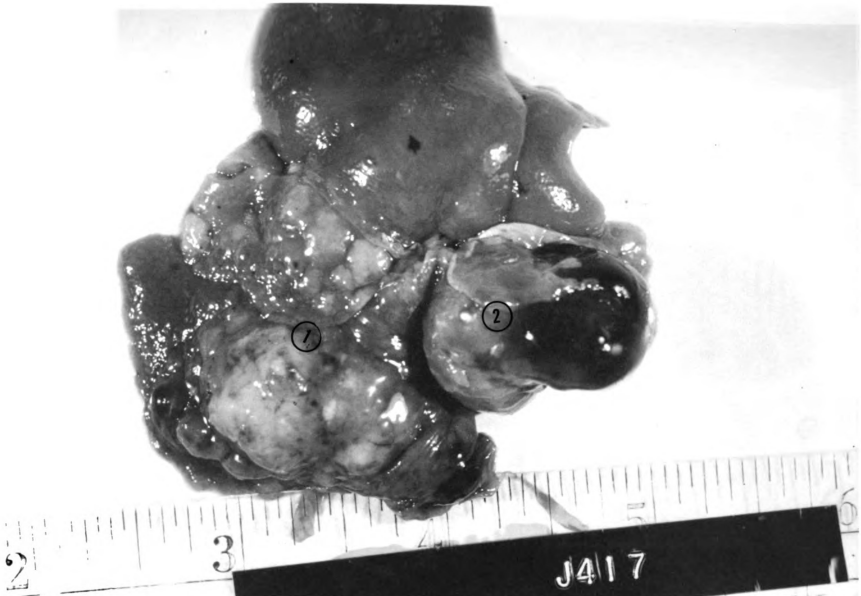


Fig. 33.--Liver of a rat fed 125 p.p.m. of diethylnitrosamine for 150 days. (1) Hepatoma; (2) Hemangioma.

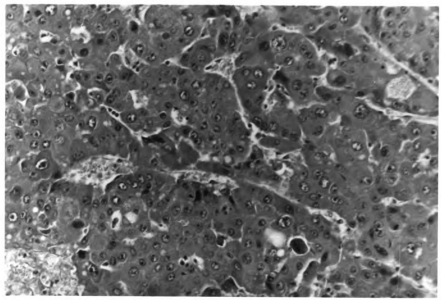


Fig. 34.--Hepatoma. Section taken from the liver in Fig. 32. H & E stain: x187.

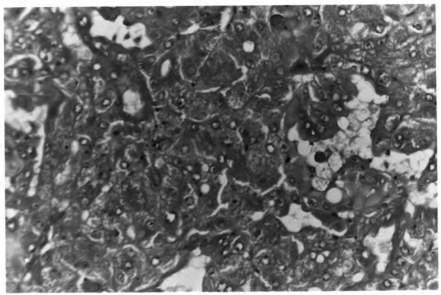


Fig 35.--Hepatoma. Section taken from the liver in Fig. 33 at No. 1. H & E stain: x187.

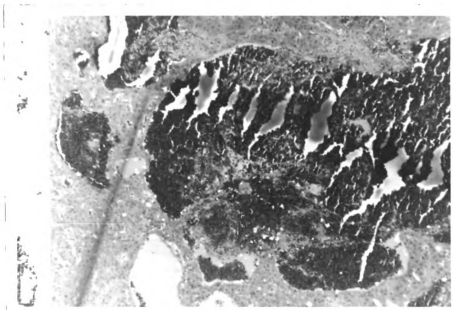


Fig. 36.--Hemangioma. Section taken from a liver of the rat in Fig. 33 at No. 2. H & E stain: x75.

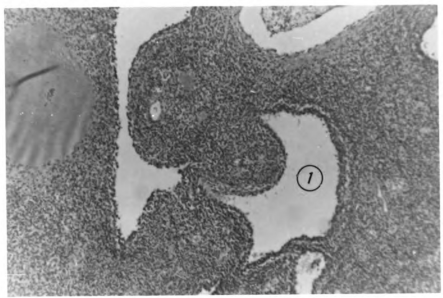


Fig. 37.--Reticulum cell sarcoma in the liver of a rat fed 1% cycad for 300 days. (1) Dilated liver sinusoids. H & E stain: x75.

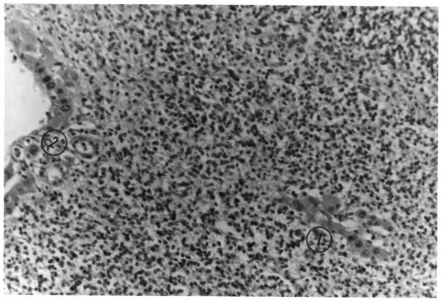


Fig. 38.--Reticulum cell sarcoma in the liver of a rat fed 1% cycad for 300 days. Higher power of Fig. 37. (1) Remains of hepatic cells among the tumorous cells; (2) Remains of portal area of liver lobule. H & E stain: x187.

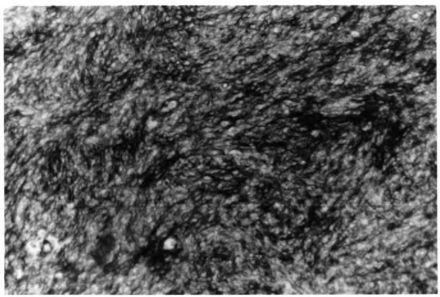


Fig. 39.--Reticulum cell sarcoma. Same section as Fig. 38. Gridley's reticulum stain: x187.

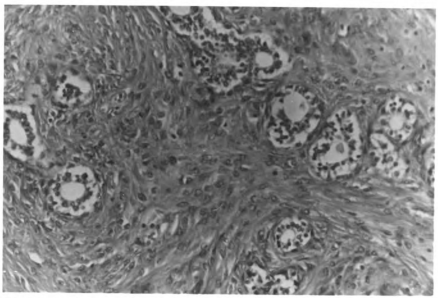


Fig. 40.--Fibroadenoma on abdominal wall. Rat fed 1% cycad for 300 days. Same rat as in Fig. 38. H & E stain: x187.

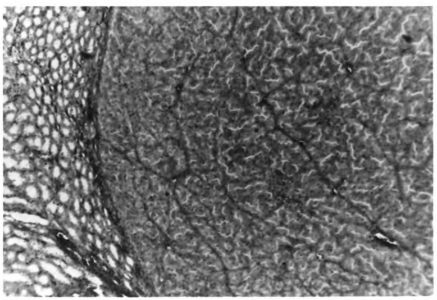


Fig. 41.--Solid renal adenoma in the kidney of a rat fed 2% cycad for 168 days. H & E stain: x75.

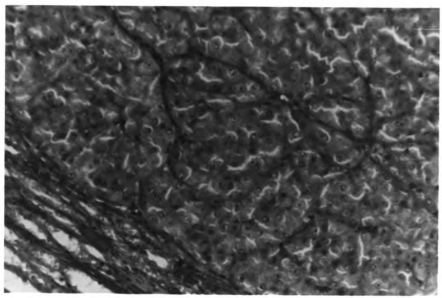


Fig. 42.--Solid adenoma in the kidney of a rat fed 2% cycad for 168 days. Higher power of Fig. 41. H & E stain: x187.

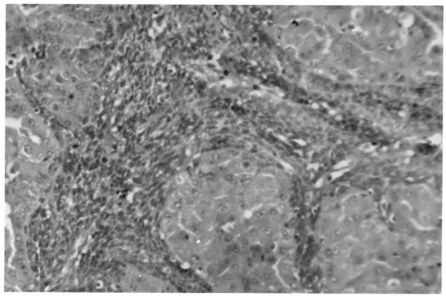


Fig. 44.--Embryonal nephroma in the kidney of a rat fed 2% cycad for 240 days. Section taken at the junction of adenoma and sarcomatous region. H & E stain: x187.

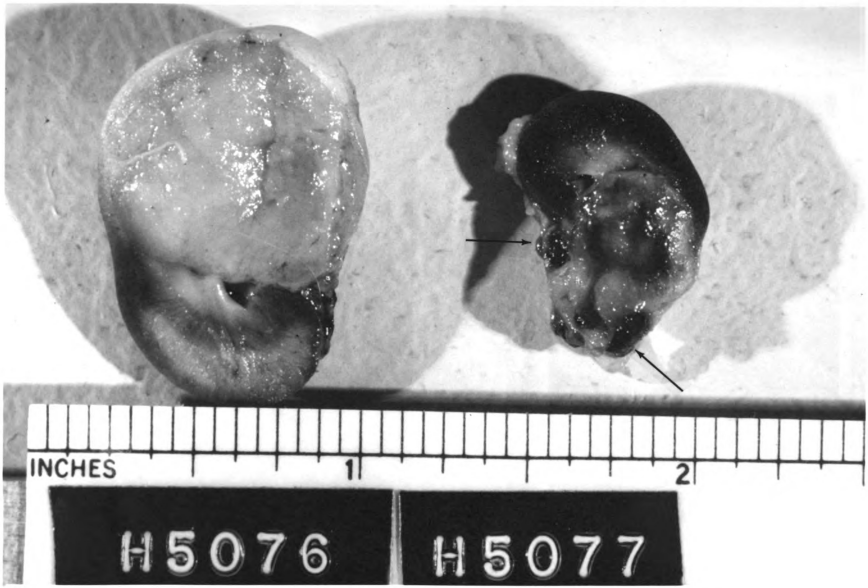


Fig. 43.--Cross sections of kidneys with embryonal nephroma from rats fed 2% cycad for 240 days. Arrows show cysts on the tumorous mass.

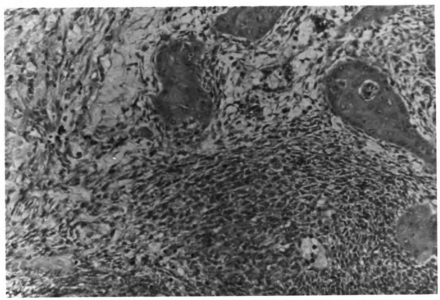


Fig. 45.--Embryonal nephroma in a kidney of a rat fed 2% cycad for 240 days. Note epithelial sheets in a less dense sarcomatous region. H & E stain: x187.

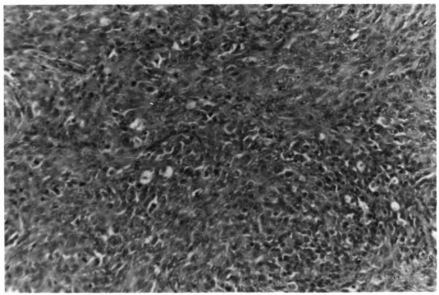


Fig. 46.--Embryonal nephroma of a kidney of a rat fed 2% cycad for 240 days. Anaplastic sarcomatous area. H & E stain: x187.

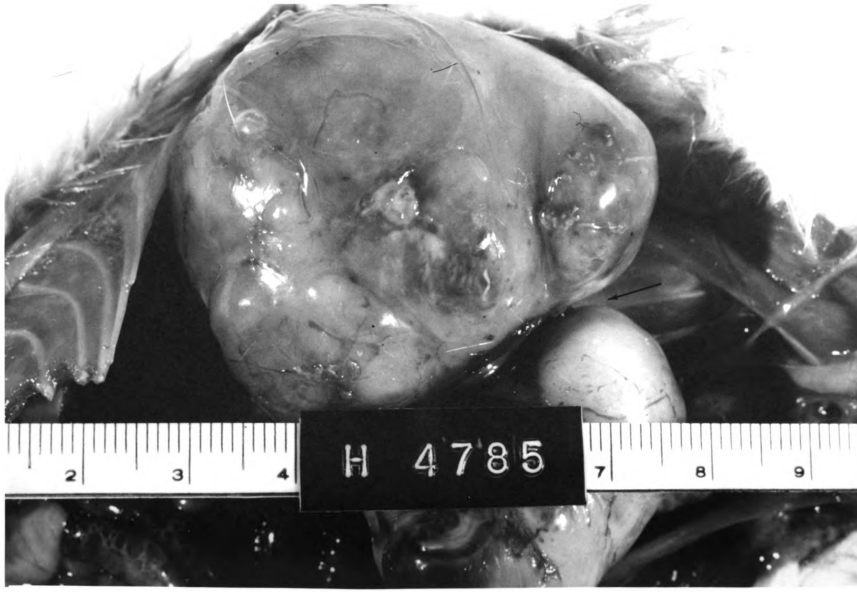


Fig. 47.--Large kidney tumor of a rat fed 2% cycad flour for 210 days. Arrow shows constriction on the tumor mass.

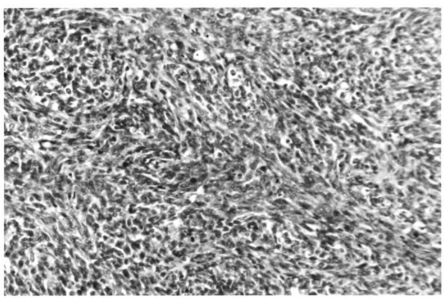


Fig. 48.--Fibrosarcoma in kidney of a rat fed 2% cycad for 210 days. Section taken from kidney shown in Fig. 47. H & E stain: x187.

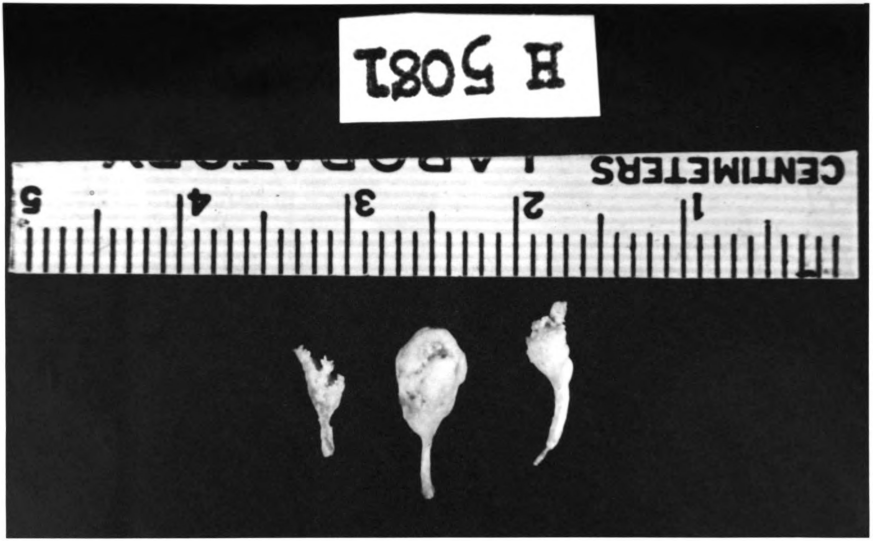


Fig. 49.--Calculi removed from urinary bladder of a rat with a large fibrosarcoma.

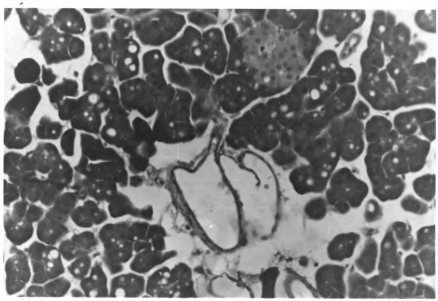


Fig. 50.--Edema of pancreas with vacuolation of cytoplasm of acinar cells in a rat fed 2% cycad for 8 days. H & E stain: x187.

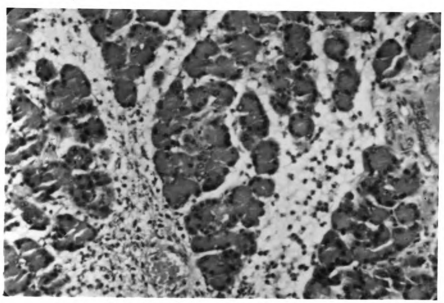


Fig. 51.--Interlobular edema with leukocytic infiltration in the pancreas of a rat fed 2% cycad for 18 days. H & E stain: x187.

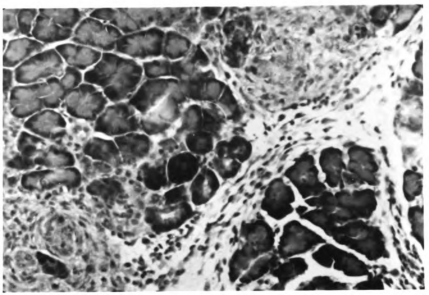


Fig. 52.--Early fibrosis in acinar tissue of the pancreas of a rat fed 2% cycad for 26 days. H & E stain: x187.

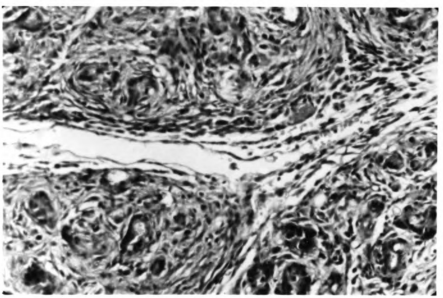


Fig. 53.--Replacement of acinar tissue by fibrous connective tissue in the pancreas of a rat fed 2% cycad for 70 days. H & E stain: x187.

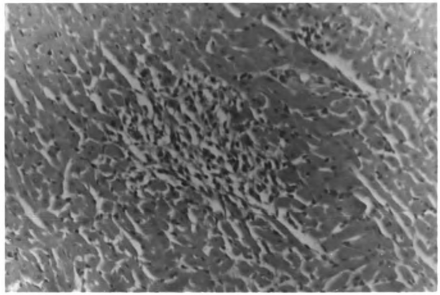


Fig. 54.--Patchy fibrotic myocarditis of a rat fed 2% cycad for 31 days. H & E stain: x187.

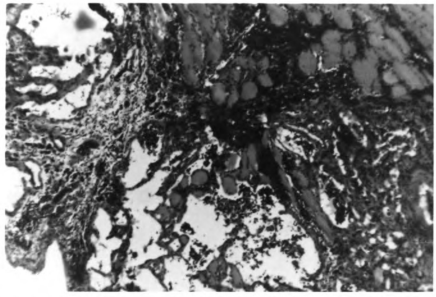


Fig. 55.--Hemangioma on diaphragm of a rat fed 2% cycad for 240 days. Section taken from the center of the lesion. H & E stain: x187.

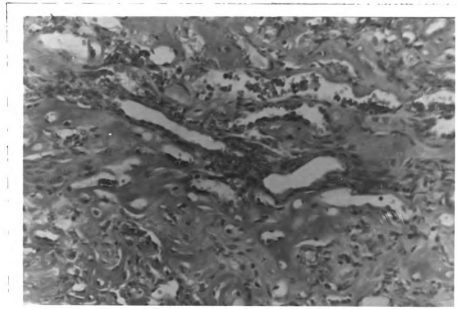


Fig. 56.--Hemangioma on the diaphragm of a rat fed 2% cycad for 240 days. Section taken at the periphery of the lesion, where lesion appeared more fibrotic. Same rat as in Fig. 55. H & E stain: x187.

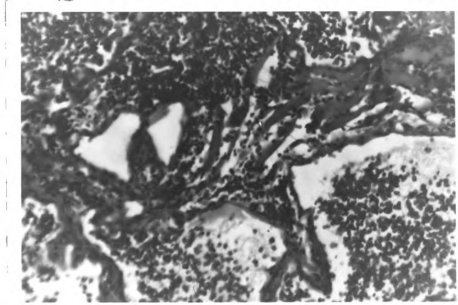


Fig. 57.--Hemangioma in a diaphragm of a rat fed 2% cycad for 240 days. H & E stain: x75.

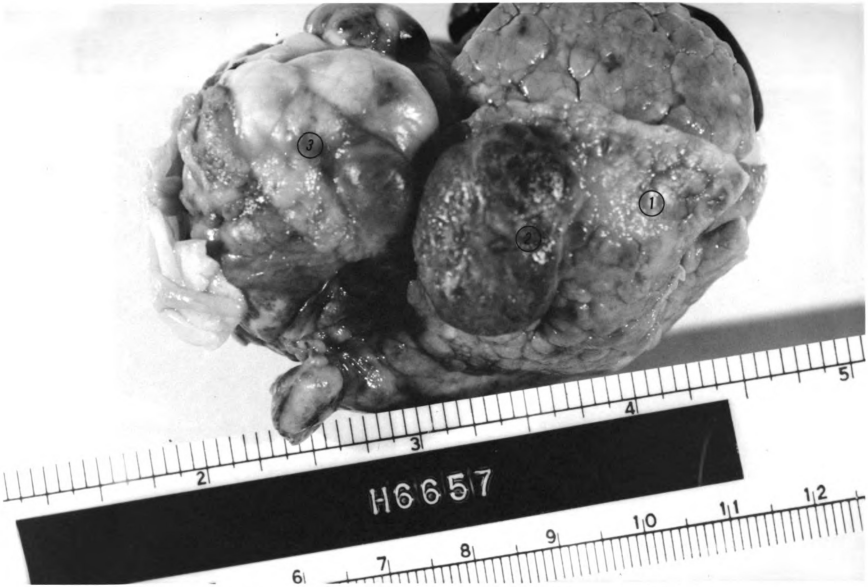


Fig. 58.--Liver of a rat which had been fed 1000 p.p.m. of dimethylaminoazobenzene for 150 days. (1) Hepatoma; (2) Hemangio-endothelioma; (3) Adenocarcinoma.

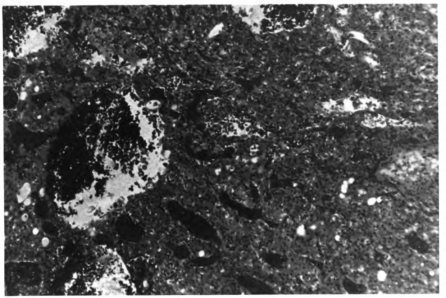


Fig. 59.--Hemangio-endothelioma in the liver of a rat fed 1000 p.p.m. of dimethylaminoazobenzene for 150 days. Section taken from the liver in Fig. 58, at No. 2. H & E stain: x75.

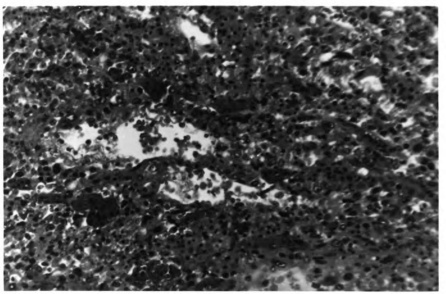


Fig. 60.--Hemangio-endothelioma in the liver of a rat. Higher power of Fig. 59. H & E stain: x187.

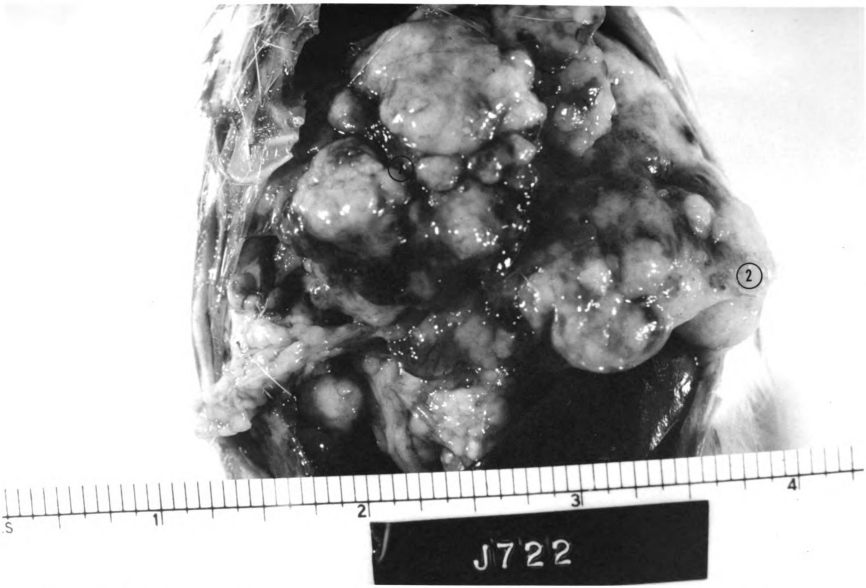


Fig. 61.--Liver of a rat which had been fed 1000 p.p.m. of dimethylaminoazobenzene for 150 days. (1) Adenocarcinoma; (2) Hepatoma.

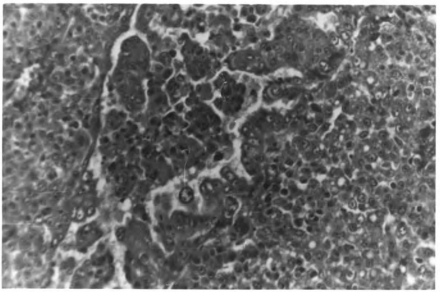


Fig. 62.--Adenocarcinoma in the liver of a rat fed 1000 p.p.m. dimethylaminoazobenzene for 150 days. Section taken from the liver in Fig. 61. H & E stain: x187.

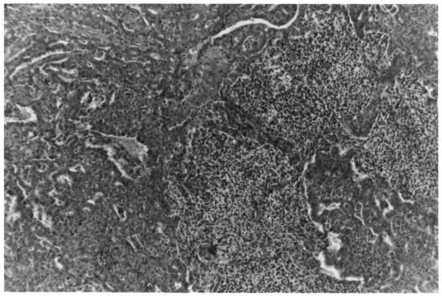


Fig. 63.--Fibrous metaplasia in the adenocarcinoma in the liver of a rat fed 1000 p.p.m. dimethylaminoazobenzene. H & E stain: x75.

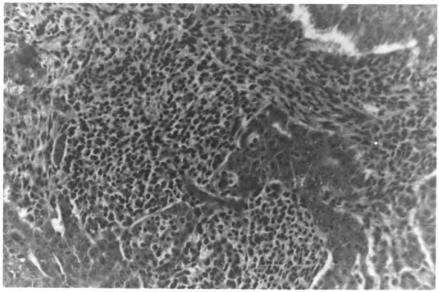


Fig. 64.--Fibrous metaplasia in adenocarcinoma in the liver of a rat. Higher power of Fig. 63. H & E stain: x187.

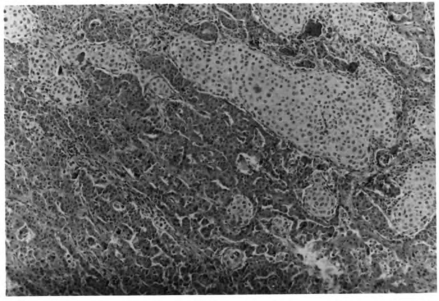


Fig. 65.--Cartilage metaplasia in adenocarcinoma in the liver of a rat fed 1000 p.p.m. dimethylaminoazobenzene for 150 days. H & E stain: x75.

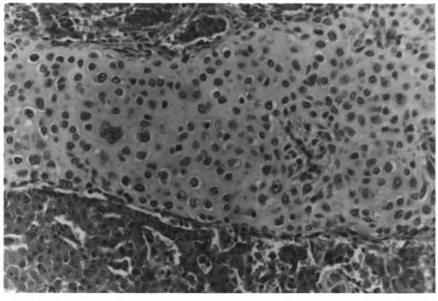


Fig. 66.--Cartilage metaplasia in adenocarcinoma in the liver of a rat fed 1000 p.p.m. dimethylaminoazobenzene for 150 days. Higher power of Fig. 65. H & E stain: x187.

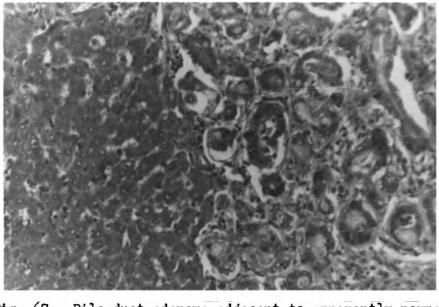


Fig. 67.--Bile duct adenoma adjacent to apparently normal hepatic cells in the liver of a rat fed 1000 p.p.m. dimethylaminoazobenzene for 150 days. H & E stain: x187.

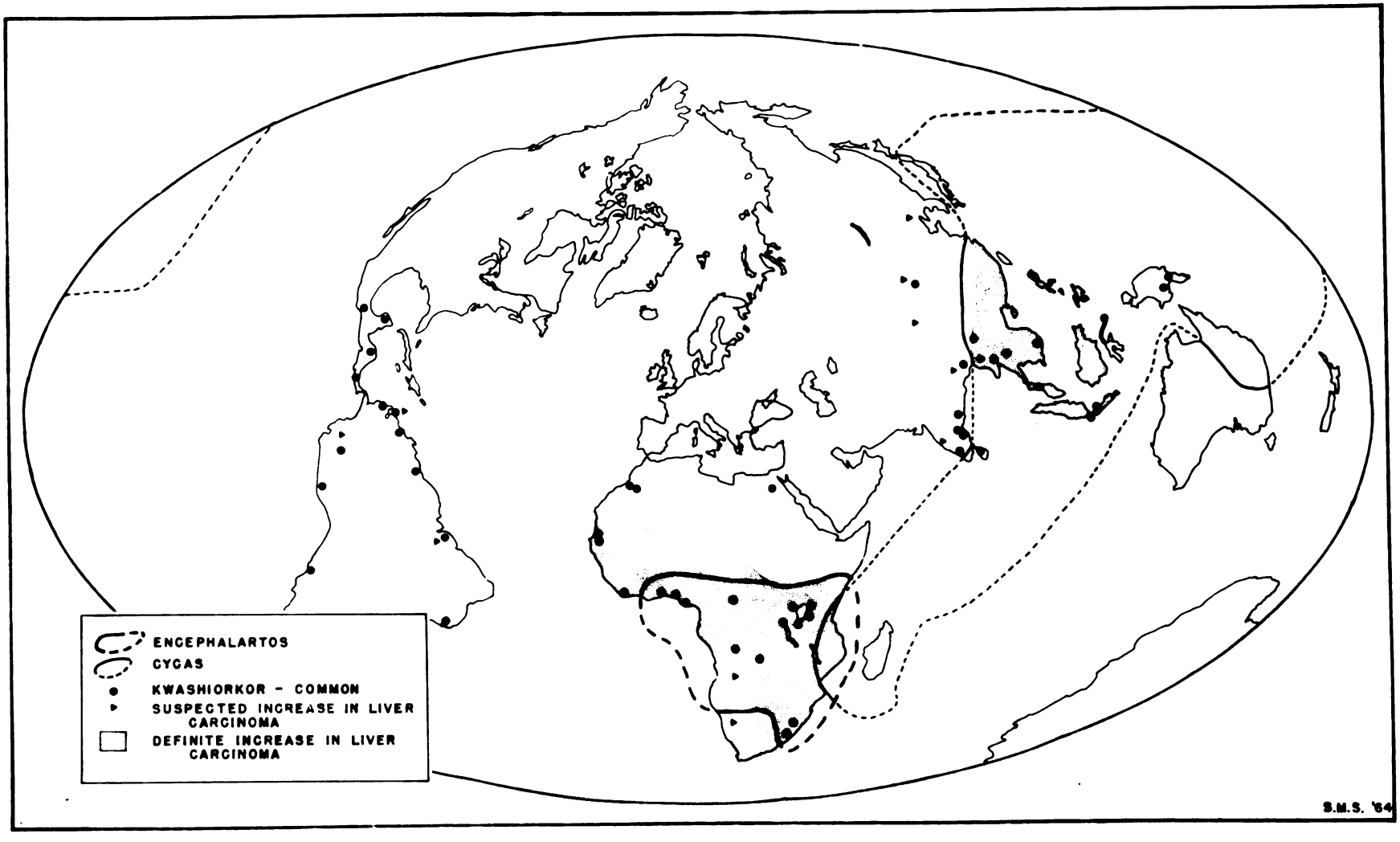


Fig. 68.--World map illustrating the distribution of 2 most used species of cycad; world distribution of kwashiorkor; and the high incidence of the liver carcinoma in the human.

PART III. TRANSMISSION OF CYCAD TOXIC FACTORS AND CHEMICAL CARCINOGENS THROUGH THE MAMMARY GLAND AND PLACENTA

Experiment I. Transmission Through the Mammary Gland and the Placenta

INTRODUCTION

In Experiment I (Part II) it was noted that the young born to the females fed 2% cycad flour died 2 to 4 days after birth, while suckling their mothers. Therefore, additional studies were conducted to determine the cause of death. At parturition the young were destroyed immediately by severing of the spinal cord. The livers were taken and fixed in acetate-buffered 10% formalin. Sections were stained with hematoxylin and eosin and Sudan IV for fat. On histologic examination all sections showed coagulative necrosis of the hepatic cells and occasional fat vacuoles. These lesions were not observed in the young born to mothers fed the basal ration or in young nursing their mothers and fed the basal ration. These observations suggested the transmission of the toxic factor(s) associated with cycad flour and specific carcinogens through the placenta and mammary gland. Two trials were conducted, the first on transfer of factor(s) through the milk and the second through the placenta.

Trial 1. Transmission Through the Mammary Gland

EXPERIMENTAL PROCEDURE

Pregnant females in the latter stages of gestation were used. They were fed the basal ration as previously described until immediately after

parturition. Approximately 2 days before parturition they were placed in an individual cage. After parturition the young were counted and examined for any gross abnormality. The mothers were then fed various levels of cycad flour (.5, 1 and 2%) or selected levels of cycasin, dimethylnitrosamine, diethylnitrosamine and dimethylaminoazobenzene. The number of females fed each ration and the level of the specific substance in each diet are given in TABLE 8.

The diet containing the required amount of the chemical under test was placed in a container designed to prevent spillage. The feed cup was securely fixed on the top of a platform in the cage. The platform was of such a height that even at weaning the young rats could not get to the diet. The mother could only get at the feed by jumping up on the feeder.

Usually, 2 young in each litter were killed at 2, 4, 7, 14 or 21 days of age or when found in a moribund state. The mothers were killed 21 days after parturition. The livers, kidneys, lungs and hearts were fixed in acetate-buffered 10% formalin. Sections were stained with hematoxylin and eosin. Selected tissues were stained with Sudan IV for fat.

RESULTS

TABLE 8 summarizes the litter size, birth weight and other results, including the histological lesions seen at the various ages and the concentration of the chemicals used in the diet. Figures 75 to 82 show the specific histological lesions.

Cycad Flour

Lesions

Liver. The earliest lesion seen in a litter suckling a mother fed 2% cycad flour was a vacuolation of the hepatic cells. This was first observed on the 4th day of suckling. On the 7th day the vacuolation was very marked in the livers from the rats in some litters. The vacuolates varied as fine vacuolates up to the size of hepatic cell nuclei. Young killed at 14 or 21 days had various hepatic cell changes. These varied from fatty infiltration to coagulative necrosis. Significant variations were noted in different litters and also on lobes of the same livers. In some litters, even after 21 days of suckling, no lesions were evident. The litters of the females fed 2% cycad flour usually died between 6 and 18 days of age, with the exception of one litter in which 5 were weaned. These, after weaning, were fed the control diet.

In the pups suckling mothers fed the diet containing 1% cycad flour, the earliest lesion was seen after 7 days. This was fatty infiltration of the hepatic cells. At 14 days, necrosis of hepatic cells was demonstrated in some of the livers. Fourteen young were weaned from the litters of the mother fed 1% cycad flour. These were also fed the control diet at weaning. They were all killed after 6 months or were killed when found in a moribund state.

Cycasin

Lesions

Liver. In the livers of the young suckling 2 mothers fed 400 p.p.m. cycasin, the earliest lesions were seen on the 5th day. In one of these litters all the pups except 1 were dead by the 5th day. Histologically,

there were varying degrees of hemorrhagic necrosis of hepatic lobules. It was very extensive in some animals and only slight in others. Two of the 3 litters from the mothers fed the 600 p.p.m. cycasin and the litter from a mother fed 400 p.p.m. cycasin died between 5 and 21 days of age and histologically there were lesions of the hepatic cells varying from fatty infiltration to coagulative necrosis with hemorrhagic components.

Kidney. There was a slight degeneration of the epithelium of both the proximal and distal convoluted tubules varying from cloudy swelling to fatty infiltration with coagulative necrosis. The lesions were seen only in the young after 14 days of suckling. The other litter from a mother fed 600 p.p.m. cycasin had 11 pups but only 3 survived until weaning. They were then fed the basal ration.

In the livers of pups suckled by mothers fed rations containing 300 p.p.m. cycasin, only fatty infiltration could be demonstrated after 14 days of suckling, and no lesions were noted in the kidney. Most of the litters survived until weaning and were then changed to the control diet.

Dimethylnitrosamine

Lesions

Liver. Pups suckling mothers fed 150 or 200 p.p.m. of dimethylnitrosamine died between 8 and 21 days of age. Those suckling mothers fed 100 p.p.m. lived to weaning. The first histological lesion seen in the liver of these rats was after suckling for 7 days. This consisted of fatty infiltration and centrolobular hemorrhage. The rats killed or dying between 8 and 21 days had histological lesions varying from slight fatty infiltration to extensive necrosis with blood lagoons. The variation here

was very great, in that very extensive lesions could be detected in all lobes of the liver, while others had only slight fatty infiltration.

One outstanding lesion was noted in a rat killed after 8 days of suckling. In this rat the right lateral lobe appeared dark purple in color. It was firm to the touch, while the rest of the lobules had an apparently normal appearance. Histologically, 80% of the right lateral lobe was destroyed and consisted of bloody cysts filled with erythrocytes and necrotic debris. The liver capsule was intact, so that the blood appeared as if inside a pocket formed by the liver capsule. The rest of the hepatic cells surrounding the hemorrhagic area had only vacuolation of the cytoplasm. At the portal areas there was leukocytic infiltration with bile duct proliferation. The other lobes had slight to heavy fatty infiltration without necrosis.

Diethylnitrosamine

Lesions

Liver. The pups suckling the mother fed 200 p.p.m. of diethylnitrosamine all died on the 4th day of suckling. No histological lesions could be demonstrated. The pups from a dam fed 150 p.p.m. diethylnitrosamine had fatty infiltration of the liver after 14 days of suckling. No necrosis was demonstrated, even at 21 days. Those not killed lived to weaning and were then fed the control diet.

Dimethylaminoazobenzene

Lesions

Liver. The litter suckling the mother fed 1000 p.p.m. of dimethylaminoazobenzene died between 8 and 12 days. Those from dams fed 750 p.p.m. dimethylaminoazobenzene died between 6 and 21 days. Those from dams

fed 500 p.p.m. of dimethylaminoazobenzene, when not killed, lived to weaning and were fed the control diet.

After 6 days the rats suckling mothers fed rations containing either 750 or 1000 p.p.m. dimethylaminoazobenzene had livers which had fatty infiltration and cloudy swelling. Between 14 and 21 days fatty infiltration and rarification of the hepatic cell cytoplasm were seen. In a few cases the cytoplasm had eosinophilic staining characteristics but no nuclear changes, indicating necrosis was present. No lesions were seen in pups suckling dams fed 500 p.p.m. of dimethylaminoazobenzene.

Lactating Mothers at 21 Days

At weaning the mother rats fed diets containing various chemicals while suckling their young were killed. Their livers were processed in the same manner as the livers of the young. The histological lesions of the liver were compared with nonlactating rats fed diets containing similar chemicals and killed after 21 days of feeding. The lesions in the livers of lactating dams were mild in some cases, while in others they were similar to those of nonlactating rats of the same age and sex.

Trial 2. Transmission Through the Placenta

EXPERIMENTAL PROCEDURE

To obtain information on the transmission of the toxic factor(s) in cycad flour, 13 mature female rats were used. Some were from Experiment I, Part 2, and some were purchased. The procedure was to place a male with 3 or 4 females and then feed them all the cycad-containing ration. When it was evident the female was pregnant, she was placed in an individual cage and continued on the same ration. After parturition

the female was fed the basal ration. Sixty pups, randomly selected from 13 litters, were used to study the pathologic effects of the cycad flour on the newborn fetus.

To study the transmission of specific carcinogens through the placenta, the results of the histopathologic examination of the young of mothers each fed a specific chemical were used.

RESULTS

The results of feeding the different levels of cycad flour are summarized in the introduction to this experiment. The carcinogen, the level fed and number of young examined are summarized in TABLE 9. None of the substances at the levels fed interfered in conception or caused abortions. Figures 69 through 74 show the specific liver sections. All livers of the pups destroyed immediately after birth from dams fed the various chemicals had necrotic hepatitis. There was a disappearance of the hepatic cell cytoplasm. The cell membranes and walls of the blood vessels were intact. Liver lobules in some areas appeared as a sheet with well demarcated empty spaces outlined by cell membranes. Erythrocytes within the blood vessels were normal, and no hemolysis was detected.

Experiment II. Long Term Effects of Exposing Rats During Suckling to Carcinogens Transmitted Through Their Dam's Milk

INTRODUCTION

This experiment was conducted to see whether the rats that survived after suckling dams fed diets containing various chemicals would develop tumors if kept on a control diet for a long time.

EXPERIMENTAL PROCEDURE

After weaning, the rats that survived suckling mothers fed diets containing various chemicals (Experiment I, Part 3) were fed the control ration for 26 weeks. Their weights were recorded weekly. Those killed or found dead were examined according to standard necropsy procedures. Liver, kidney and pancreas were routinely fixed in 10% acetate-buffered formalin. Sections were stained with hematoxylin and eosin.

RESULTS

TABLE 10 gives the growth rates of the rats exposed to various chemicals during nursing. There was no obvious decline in the normal growth rate in any animals except those which had suckled dams fed dimethylnitrosamine and 600 p.p.m. cycasin. One rat from a dam fed 600 p.p.m. cycasin died in 4 days. One that had nursed a mother fed dimethylnitrosamine died at 8 weeks.

Lesions

Liver. Microscopic lesions were present in 2 pups from dams fed dimethylnitrosamine. In the rat that died 8 weeks postweaning, the liver parenchymal cells had fatty vacuolation and the sinusoids were very congested. In the other rat killed at 26 weeks postweaning, the liver parenchyma had patchy areas of vacuolated cytoplasm.

In the rats that had suckled mothers fed 600 p.p.m. cycasin some lesions were noted. In the rat which died 4 days postweaning, the liver was grossly congested. Microscopically, the hepatic cells were at different stages of degeneration. Fatty infiltration and necrosis of hepatic cells were noted. The sinusoids were congested.

Kidney. In the 2 rats that survived while fed the control diet for 26 weeks, one had bilateral tumorous masses in the kidneys. The right kidney had a whitish mass oval in shape, about 1 cm. in diameter, at the junction of the cortex and medulla. The left kidney had 2 different nodules whitish in color. One extended from the cortex of the kidney to the pelvis, and the other was a small nodule 3 mm. in diameter, situated in the cortex. The other rat had a very small nodule in the right kidney which was only visible during the trimming of the tissue. It was situated in the renal cortex. All the tumors microscopically were similar. They had sarcomatous characteristics. The cellular components were elongated with oval nuclei. Mitotic figures were very common. In general these tumors resembled the sarcomatous areas of the tumors described as embryonal nephroma in weanling rats fed 2% cycad but they did not have adenomatous components. In some areas there were many cysts (Figure 95), as described previously in embryonal nephroma. The tumors were diagnosed as undifferentiated cell sarcoma. The uninvolved part of the kidney had chronic interstitial nephritis with dilated tubules.

Experiment III. Transmission of Toxic Factor(s) in the Milk of Swine and Cattle

INTRODUCTION

This experiment was designed to determine whether cycad toxic factor(s) would be transmitted through the milk of other species besides the rat.

EXPERIMENTAL PROCEDURE

A lactating cow with a 10-day-old calf and a lactating sow with a litter of 9 newborn pigs were used in this experiment.

The cow was fed 0.5 pound of a mixture of oats and ground shelled corn per 100 pounds body weight with 2% unwashed cycad flour incorporated into it. This ration was fed for 76 days. At the end of this time, the level of cycad was increased to 4% and maintained at this level for another 64 days. Blood samples were collected each week from the jugular vein and analyses were carried out as in Experiment I. Both the calf and the cow were weighed weekly. After 149 days of the experiment, both the cow and the calf were killed. Necropsy was done according to standard procedures.

The sow was fed a standard pig ration to which was added 2% unwashed cycad flour. This ration was started 4 days after parturition. After 77 days, while still nursing the sow, 3 pigs (2 males and 1 female) were killed. The sow was killed 21 days later. Necropsy was performed in all animals according to standard procedures. At no time during the experiment were the suckling pigs permitted access to the dam's feed. Of the 6 remaining pigs, 3 males were killed at 220 days and the remaining 3 females were killed at 264 days after each had a litter of normal pigs.

RESULTS

TABLE 11 gives the weekly serum SGOT, SGPT, bilirubin, hemoglobin and packed cell volume values of the cow and the calf. Figures 83 to 92 show the lesions present in the cow and calf. A steady rise of serum SGOT appeared after 7 days on the experiment in both the cow and the calf. This rise in serum SGOT continued for more than 35 days in the

calf and for 63 days in the cow and then started to decrease. After a steady decrease of serum SGOT values there was slight rise of serum SGPT and bilirubin values. Bilirubin values increased steadily until the termination of the experiment. In both the calf and cow, the values were higher at the termination of the experiment than at the beginning.

Lesions - Cow

Liver. This organ had small pale gray depressed lesions on the surface. The lesions varied in size from 1 to 5 mm. in diameter and appeared as necrotic focal lesions (Figure 82).

The grossly depressed areas, histologically, were extensive necrotic foci, where many of the hepatic cells had been turned into a homogeneous eosinophilic debris. Within the necrotic areas was leukocytic infiltration, mainly eosinophils, monocytes and a few neutrophils. These lesions involved more than one lobule, and it was difficult to judge whether they were principally more of the portal or centrolobular lesions. Away from these lesions the portal areas showed chronic proliferation of connective tissue with marked leukocytic infiltration. In some areas bile duct proliferation was quite extensive, while in other areas it was only slight. Around the large bile ducts there was extensive fibrosis, and the epithelial lining of the ducts had necrotic changes. Some of the bile duct columnar epithelium contained intracytoplasmic hyaline droplets. These hyaline bodies were clearly demarcated from the rest of the cytoplasm by hollow spaces. In the rest of the liver lobules, hepatic cells showed different stages of degeneration. The cytoplasm of the hepatic cells appeared vacuolated.

Kidney. No apparent gross lesion was noted in the kidney. Microscopically, some degree of interstitial nephritis with mononucleated cell infiltration was noted.

Peritoneum. There was about 200 ml. of serous fluid in the abdominal cavity.

Pancreas. In the interstitial areas, fat necrosis was seen between lobules of the pancreas. The acinar tissue had vacuolation of the cytoplasm. A few cells had a complete loss of the cytoplasm which had been replaced by vacuoles. The vacuolation only occurred at the periphery of the pancreatic lobules. A few lobules had leukocytic infiltration in the interstitial areas.

Lungs. Several ecchymotic hemorrhages were noted on the surface of the lungs. Patchy areas of consolidation were seen on the apical, cardiac, intermediate and anterior part of the diaphragmatic lobes of the lungs. This involved about 40% of the lung tissue. The consolidated areas were noncrepitating on pressure and were dark brown in color, with patchy purplish-red areas. The consolidated areas were associated with a few emphysematous lobules. On sectioning the involved tissue bulged out. It appeared coarsely lobulated, the interlobular septum being thickened and edematous.

Microscopically the interlobular septa were edematous and had heavy leukocytic infiltration. The leukocytes were mainly eosinophils and lymphocytes. There was peribronchial pneumonia with rings of lymphocytes, eosinophils and mononucleated leukocytes surrounding the terminal bronchioles. The alveoli were distended and filled with cellular

exudate, which stained pink with hematoxylin and eosin. Occasionally, dense fibrinoid material filled the alveoli and some alveoli had fibrin strands within the exudate. Several lung arterioles had thickened walls as a result of intimal proliferation. In a few instances the arterial walls were infiltrated with inflammatory cells.

Spleen. There was both capsular and parenchymal edema in the spleen.

Heart muscle. Heart muscle had vacuolation of muscle bundles. A few bundles showed hyaline degeneration. These lesions were very patchy in distribution. In a few areas there were Anitschkow cells.

Gastrointestinal tract. The serosal surface of the abomasum, omasum and omentum had ecchymotic hemorrhages. In the fundic region of the abomasum the mucosa appeared thickened and rough. There was catarrhal enteritis in the anterior part of the small intestine.

Microscopically, the fundic glands appeared hyperplastic. A few had several layers of epithelial lining. There appeared to be an excess amount of fibrous connective tissue with the lamina propria associated with leukocytic infiltration in the edematous areas. In the duodenum there was mucinous degeneration of the epithelial lining and an increase of fibrous connective tissue in the lamina propria. Brunner's glands showed some atrophy and were being replaced by rings of proliferating connective tissue and infiltrating leukocytes. These lesions within the lamina propria extended into all parts of the intestinal tract. The submucosa was edematous the whole length of the intestine.

Lesions - Calf

The gross pathology of the suckling calf resembled very much that of the dam. The specific lesions in the cow and calf are shown in Figures 83 to 92.

In the liver the necrotic lesions were smaller in the calf than in the dam, as shown in Figures 82 and 83. The heart appeared enlarged and flabby. Microscopically, the lesions were similar to those of the mother. In some instances the lesions were more marked in the calf than in the dam. In the lung of the calf the lesions were more extensive and more chronic. The pink staining exudate was very extensive in every alveolus and it appeared as an eosinophilic membrane. Associated with the exudate was a heavy leukocytic infiltration. Thromboses and periarteritis were more extensive in the lung arterioles of the calf. Interlobular bronchi had mucous degeneration with inflammatory exudate in the bronchiole lumen. The areas between the lining epithelium and Reisseisen's muscle were edematous and extended by leukocytic infiltration. Fibrotic patchy lesions were seen on the heart muscle of the calf which were not seen in the cow.

Lesions - Sow

Liver. Grossly, the liver appeared firm in consistency. Microscopically, there was a slight increase of fibrous connective tissue in the portal area, as demonstrated with Heidenhain's aniline blue stain. In several lobules of the liver centrolobular fibrosis was present.

Kidney. No lesion was detected grossly. Microscopically, nephritis with proliferation of connective tissue was noted. Some renal tubules had indications of hyperplasia characterized by formation of several

layers thick of cellular sheets. These epithelial sheets had very closely packed cells, and their cytoplasm was either clear or basophilic in staining characteristics. Some tubules were dilated and were filled with hyaline casts.

Gastrointestinal tract. Microscopically, the epithelium of both the stomach and intestines had mucinous degeneration. The part of the lamina propria adjacent to the epithelial lining had extensive lymphocytic infiltration associated with a few neutrophils and eosinophils. In the duodenal area the lamina propria appeared hyperplastic with an increase of glands of Lieberkuhn.

Lesions - Pigs

Liver. In general, grossly there were many necrotic foci varying between 3 and 6 mm. in diameter. These were seen on the livers of all the pigs that suckled the mother while fed the cycad diet. The number and size varied from one pig to the other.

Microscopically, the liver had extensive portal fibrosis with large bands of connective tissue dividing the lobules into pseudolobules and extending to the central veins. A few lobules had a well established fibrosis in the central vein areas. The grossly apparent necrotic lesions in histological sections were areas with most of the hepatic cells destroyed and being replaced by proliferating fibroblasts with leukocytic infiltration. Normally, these lesions involved morethan one lobule.

Six of the pigs were kept on natural grain ration after weaning and were maintained on this ration for 220-264 days, at which time they were killed and necropsy performed according to standard procedures.

Grossly, no lesions were seen in any of the pigs. Microscopically, in all the pigs there appeared to be more collaginous fibers than normal in the portal area. In one male pig, portal and centrolobular fibrosis was noted in a few liver lobules. The fibrosis was less in extent than that noted in pigs killed after 77 days.

GENERAL DISCUSSION OF TRANSMISSION EXPERIMENTS

The transmission of these carcinogens, including the toxic factor in cycad flour through placental and mammary membranes, is considered a significant finding. The transmission through the mammary gland was not as definite in the case of dimethylaminoazobenzene and diethylnitrosamine, as determined from histological changes of the livers of the rat pups. However, mortality occurred in the pups at the higher dosage, which hardly affected the lactating animal. With cycasin, cycad flour and dimethylnitrosamine pronounced changes were observed in livers of the pups, but variations were marked between litters, between pups in the same litter, and between different lobules of the same liver.

In recent years considerable interest has been expressed in the presence of insecticides in bovine milk. Marth et al. (1959) have characterized insecticide products in milk and in milk products. Davson et al. (1943) were the first to hypothesize that certain compounds may penetrate membranes in their unionized lipid soluble state. Schanker (1962) summarized the passage of drugs across the membrane in accordance with their lipid solubility. Rasmussen (1958 and 1959), in studies with cows and goats, demonstrated that mammary gland epithelium was permeable to the unionized form of drugs and almost impermeable to the ionized forms. Sisodia et al. (1964) stated that in essence, the

concept of passive diffusion was that the unionized fraction of many compounds crosses the biological membrane in consonance with their lipid and water solubility coefficient. The rate of crossing in the case of mammary glands depends on PK_a and milk pH, according to Sisodia et al. (1964). Rasmussen's studies demonstrated that basic compounds appeared in milk in a higher concentration than in plasma. An increase in the pH of the milk increased the concentration of the drug in the milk.

The passage of drugs across the placenta has been shown to depend to a certain degree upon lipid solubility of the drugs. The compounds which have been detected in fetal blood or tissues shortly after administration to the mother include all the anesthetic gases and vapors, many barbiturates, sulfonamides, salicylates, quinine, methadone and meperidine, all of which have moderate solubility in lipids (Argar et al., 1960, Baker, 1960, and Marx, 1961). Dancis (1958) showed that esteriol and the lipid-soluble metabolites of estradiol readily traverse the placenta in either direction, while the lipid-insoluble glucomides of these compounds hardly cross at all.

All the carcinogens used in these studies are lipid-soluble to a varying degree, and their passage across the placental and mammary barriers may be in the same manner as other lipid-soluble unionized acidic and basic compounds.

Cycad plants are widely distributed in semitropical areas and are widely used as food by man and animals. They are also used as a medicine for both adults and children.

The present studies suggest that both the dietary and medicinal use of cycad and its products may have deleterious effects. This is based on the finding that in all these species of animal studies, the

toxic cycad factors were transmitted to the young both during fetal development and during nursing.

The situation among human beings differs from the present study in that cycad nut is cooked in water prior to consumption. This procedure inactivates the toxic factor and renders the cycad flour innocuous. However, there is a possibility that milk cows in those areas of the world where cycad grows may ingest some parts of the plant. The toxic substance in these plants may be transmitted to the infants fed this milk. Further studies should be carried out to determine the potential danger associated with this possibility.

The present studies suggest that the toxic substances present in cycad may be eliminated through the milk and can also pass through the placental tissue. This suggests that cycad toxic factors may be secreted in the milk when lactating women use cycad either as medicine or food and in cows' milk when the cows are fed on cycads or their products.

TABLE 8. Histological Lesions in Rats Suckling Mothers Fed Cycad or Carcinogenic Chemicals

Chemical Fed	Concentration in Ration	No. in Litter	Avg. Wt. at Birth	Age at Death (days)	No. Killed or Dead	Ave. Wt. at Death	Histological Lesions
Cycad	2%	9	5.4 Gm.	2	2K*		No lesion
				4	2K		No lesion
				6	2K	8.9 Gm.	Necrosis of the liver
				8	4D*	9.2 Gm.	Necrosis of the liver
Cycad	2%	8	5.6 Gm.	2	2		No lesion
				4	2		No lesion
				7	2	11.3 Gm.	Vacuolar degeneration
				9	1D		Post-mortem changes
				14	2K	10.2 Gm.	Necrosis of liver and heart muscle
				16	1D		Necrosis of the liver
Cycad	2%	12	5.2 Gm.	2	2K		No lesion
				4	2K		No lesion
				6	all dead		Post-mortem changes
Cycad	2%	11	5.5 Gm.	2	2K		No lesion
				4	2K		No lesion
				6	3D		Coagulative necrosis of liver
				14	1K	9.7 Gm.	Coagulative necrosis of liver
				18	2D	12.0 Gm.	Necrosis of liver and kidney
Cycad	2%	13	5.2 Gm.	2	2K		No lesion
				4	2K		Fatty infiltration of hepatic cells
				6	2K		Fatty infiltration of hepatic cells
				14	2K	13.27 Gm	Fatty infiltration of hepatic cells
				23	2K	21.0 Gm.	Necrosis of liver and kidney, vacuolar degeneration of pancreas acinar cells

TABLE 8--continued

Chemical Fed	Concentration in Ration	No. in Litter	Avg. Wt. at Birth	Age at Death (days)	No. Killed or Dead	Avg. Wt. at Death	Histological Lesions
				Five weaned and kept on control diet			
Cycad	1%	6	6.4 Gm.	2	2K		No lesion
				4	1K		No lesion
				7	1K	9.0 Gm.	Fatty infiltration
				Two weaned at 21 days and kept for 6 months			
Cycad	1%	11	5.4 Gm.	2	2K		No lesion
				9	2K		Extensive fatty infiltration
				14	2	24.0 Gm.	Fatty infiltration
				21	2	31.0 Gm.	Necrosis of hepatic cells
				Three weaned and kept for 6 months			
Cycad	1%	8	5.45 Gm.	7	2K	13.0 Gm.	Fatty infiltration of liver cells
				14	1K	25.0 Gm.	Fatty infiltration
				21	1K	36.0 Gm.	Necrosis of hepatic cells
				Four weaned and kept for 6 months			
Cycad	1%	11	5.6 Gm.	3	3D		Post-mortem changes
				7	1K	11.2 Gm.	Marked fatty infiltration
				9	1D		Fatty infiltration
				17	1K (moribund)	1910 Gm.	Necrosis of liver

TABLE 8--continued

Chemical Fed	Concentration in Ration	No. in litter	Avg. Wt. at Birth	Age at Death (days)	No. Killed or Dead	Avg. Wt. at Death	Histological Lesions
Cycad	0.5%	8	5.6 Gm.	7	2K	18.0 Gm.	No lesion
				14	2K	37.0 Gm.	No lesion
				21	4	51.25 Gm.	No lesion
				Five weaned and kept for long period			
Cycasin	400 p.p.m.	9	5.8 Gm.	5	8D		Hemorrhagic necrosis of liver
					1K	7.0 Gm.	Hemorrhagic necrosis of liver
Cycasin	400 p.p.m.	8	6.3 Gm.	7	2K		Necrosis of hepatic cells with hemorrhage
				9	1D		Extensive fatty infiltration of hepatic cells, hemorrhages in lungs
				11	1D		Post-mortem changes
				14	2K	15.0 Gm.	Necrosis with hemorrhage of liver
				21	2K	20.25 Gm.	Necrosis of liver, heart muscle and pneumonia
Cycasin	600 p.p.m.	11	6.1 Gm.	7	2K	11.5 Gm.	Necrosis of hepatic cells, vacuolation of heart muscle
				14	2K	13.5 Gm.	Necrosis and fatty infiltration of liver, fatty infiltration of heart muscle
				16	6D		Extensive post-mortem changes
				1 missed			

TABLE 8--continued

Chemical Fed	Concentration in Ration	No. in litter	Avg. Wt. at Birth	Age at Death (days)	No. Killed or Dead	Avg. Wt. at Death	Histological Lesions
Cycasin	600 p.p.m.	9	5.9 Gm.	7	2D		Necrosis with centrolobular hemorrhages of liver
					2K		
				9	4D		
				10	1D		
Cycasin	600 p.p.m.	11	6.3 Gm.	7	2K	14.0 Gm.	Fatty infiltration of liver cells
				14	2K	21.0 Gm.	Slight necrosis of liver cells
				17	3D		Extensive post-mortem changes
				21	1K	21.0 Gm.	Centrolobular necrosis of liver with congestion of lungs
				Three weaned to control diet			
Cycasin	300 p.p.m.	9	5.5 Gm.	7	2K	15.5 Gm.	No lesion
				14	2K	26.0 Gm.	Fatty infiltration of hepatic cells
				21	2K	39.0 Gm.	Fatty infiltration in liver cells and heart muscle
				Three weaned and kept for longer period			
$(CH_3)_2NNO$	200 p.p.m.	10		8	2K		Centrolobular necrosis of liver
				10	3D		Extensive post-mortem changes
				10	5K (in moribund state)		Extensive centrolobular hemorrhagic necrosis of liver

TABLE 8--continued

Chemical Fed	Concentration in Ration	No. in Litter	Avg. Wt. at Birth	Age at Death (days)	No. Killed or Dead	Avg. Wt. at Death	Histological Lesions
$(CH_3)_2NNO$	150 p.p.m.	11	6.1 Gm.	7	2K	8.0 Gm.	Fatty infiltration with centrolobular hemorrhages
				14	2K	11.0 Gm.	Centrolobular hemorrhages
				21	7D		Excessive post-mortem changes
$(CH_3)_2NNO$	200 p.p.m.	13	6.2 Gm.	7	2K	7.5 Gm.	Centrolobular necrosis with hemorrhages
				8	11D		Extensive post-mortem changes
$(CH_3)_2NNO$	100 p.p.m.	10		7	2K		No lesion
				14	2K		Slight vacuolation of hepatic cells
				21	2K		Fatty infiltration of hepatic cells
				Four weaned and kept for longer period			
$(C_2H_5)_2NNO$	200 p.p.m.	9		4	9D		Extensive post-mortem changes
$(C_2H_5)_3NNO$	150 p.p.m.	10		7	2K		Slight vacuolation of hepatic cells
				14	2K		Vacuolation of hepatic cells
				21	2K		Vacuolation of hepatic cells
				Four weaned and kept for longer period			

TABLE 8--continued

Chemical Fed	Concentration in Ration	No. in Litter	Avg. Wt. at Birth	Age at Death (days)	No. Killed or Dead	Avg. Wt. at Death	Histological Lesions
$(C_2H_5)_2NNO$	150 p.p.m.	11		7	2K		No lesion
				14	2K		Slight vacuolation
				21	2K		Vacuolation of hepatic cells
				Three weaned to control diet			
DAB**	1000 p.p.m.	8	6.3 Gm.	7	2K	8.0 Gm.	Fatty infiltration of hepatic cells
				10	2K (in moribund state)	8.75 Gm.	Fatty infiltration of hepatic cells
				11	2K		Fatty infiltration of hepatic cells
				12	2K (in moribund state)		Fatty infiltration of hepatic cells
DAB	750 p.p.m.	11		6	2D		Extensive post-mortem changes
				7	2K		Fatty infiltration of hepatic cells
				14	2K		Fatty infiltration of hepatic cells
				21	1D, 1K		Fatty infiltration
				Three weaned died 23rd day			Extensive post-mortem changes

TABLE 8--continued

Chemical Fed	Concentration in Ration	No. in Litter	Avg. Wt. at Birth	Age at Death (days)	No. Killed or Dead	Avg. Wt. at Death	Histological Lesions
DAB	500 p.p.m.	10		7	2K		No lesion
				14	2K		No lesion
				21	2K		Slight fatty infiltration of hepatic cells
				Four weaned			
Control diet	6.02 Gm.	10		7	2K	19.0 Gm.	Slight fatty vacuoles
				14	2K	29.0 Gm.	No lesion
				21	2K	44.0 Gm.	No lesion
				Four weaned			
Control diet	5.9 Gm.	7		7	2K	22.0 Gm.	Slight fatty vacuoles
				14	2K	34.0 Gm.	No lesion
				21	3K	50.0 Gm.	No lesion

*K=killed; D=dead
**DAB - dimethylaminoazobenzene

TABLE 9. Observations on Pups Born to Female Rats Fed Dimethylnitrosamine, Diethylnitrosamine, Dimethylaminoazobenzene or Cycasin During Gestation.

Diet Group	No. Pups Born	Total Wt. at Birth (Gm.)	No. Necropsied at Birth	Wt. at 2 wk.	Eyes at 2 wk.	Wt. at 3 wk.	Eyes at 3 wk.
Dimethylnitrosamine, 200 p.p.m.	13	65	6	34.1 (17 days)	all open	54.4 (24 days)	open
Diethylnitrosamine, 200 p.p.m.	10	70	5	38.0 (14 days)	eyes open	50.0 (21 days)	open
Dimethylaminoazobenzene, 1000 p.p.m.	2	11	2	---	---	---	---
Cycasin, 400 p.p.m.	13 (2 died)	78 (on basis of 5)	5	36.5 (13 days)	eyes not completely open	48.5 (20 days)	open
Control	9	56	5	37.0	eyes open	54.6 (21 days)	

TABLE 10. Average Weights (Gm.) of Rats That Suckled Dams Fed the Basal Ration and Various Levels of Dimethylnitrosamine, Diethylnitrosamine, Dimethylaminoazobenzene or Cycasin During Lactation and Then Placed on the Basal Ration for 26 Weeks.

Diet Group	Sex	No. Rats	Wt. at Weaning	Weeks of Study														
				1	2	3	4	5	8	10	12	14	16	18	20	22	24	26
Control	M	2	44	67	88	160[1]	206	278	334	374	372	366	390	391	396	406	416	413
	F	2	44	74	100	139	164	197	212	229	236	246	253	263	259	271[2]	271	272
Dimethylnitrosamine 1000 p.p.m.	M	4	23	46	80	120	---	209	239	292[3]	298	330	337	340	340	356	366	365
Diethylnitrosamine 150 p.p.m.	M	1	40	53	91	135	179	271	311	321	347	381	406	404	396	402	386	370
	F	6	33	41	68	94	118	156	181	190	198	214	222	221	228	232	239	222
Diethylaminoazo-benzene, 500 p.p.m.	M	2	40	85	129	171	---	231	300	325	340	364	385	379	402	423	401	416
	F	2	39	70	104	134	---	169	196	211	223	233	231	238	246	248	250	251
Cycasin 300 p.p.m.	F	3	41	74	109	134	152	179	203	219	229	233	242	250	247	251	259	---
Cycasin 600 p.p.m.	F	2	21	36	69	104	127	169	187	192	212	210	205	---	---	---	---	---

TABLE 11. SGOT, SGPT, Bilirubin, Hemoglobin, and Packed Cell Volume Values of the Cow and the Calf

Days	Animal	SGOT*	SGPT*	Bilirubin (in mg./100 ml.)	Hemoglobin (in mg./100 ml.)	PCV %
1	Cow	44	8	0	13.7	35
	Calf	38	8	0	12.7	40
8	Cow	56	6	0	10.2	32
	Calf	54	5	0	10.8	34
15	Cow	56	8	0	8.7	29
	Calf	48	8	0	10.4	34
22	Cow	60	8	0.25	10.0	28
	Calf	56	8	0.5	11.0	34
29	Cow	92	8	0.5	10.5	30
	Calf	84	8	0.5	12.6	37
36	Cow	96	12	0.5	10.8	32
	Calf	104	8	0.5	12.0	34
50	Cow	104	12	0.75	10.8	31
	Calf	92	10	0.5	14.0	39
64	Cow	110	12	0.85	11.0	30
	Calf	90	10	0.75	12.6	35
78	Cow	96	16	1.5	11.0	30
	Calf	76	10	0.95	14.0	35
92	Cow	90	18	1.5	10.5	31
	Calf	54	12	1.25	13.7	37
116	Cow	84	10	1.75	11.0	30
	Calf	66	10	1.5	13.7	38

*In Sigma-Frankel units.

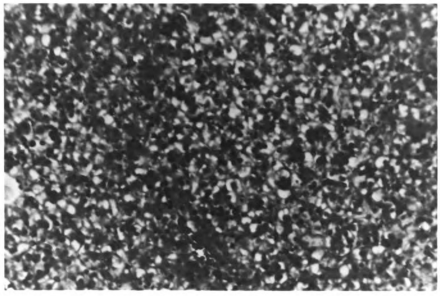


Fig. 69.--Liver of a fetus of a rat born from a dam fed on control diet during gestation period. H & E stain: x187.

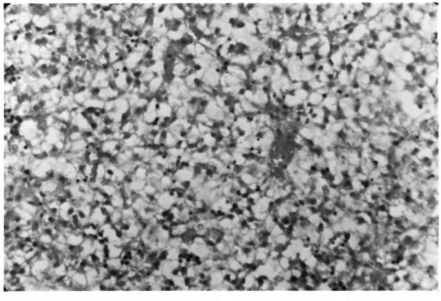


Fig. 70.--Coagulative necrosis of the liver of a rat fetus born from a dam fed 2% cycad during gestation period. Note loss of hepatic cell cytoplasm and nuclei. Fetus killed immediately after birth. H & E stain: x187.

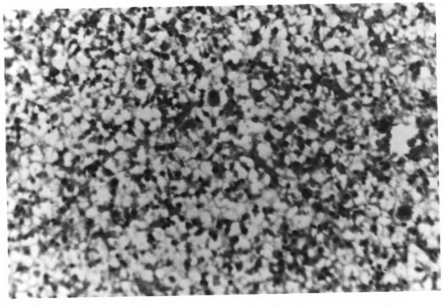


Fig. 71.--Coagulative necrosis of the liver of a fetus of a rat born from a dam fed 400 p.p.m. cycasin during gestation period. Killed immediately after birth. H & E stain: x187.

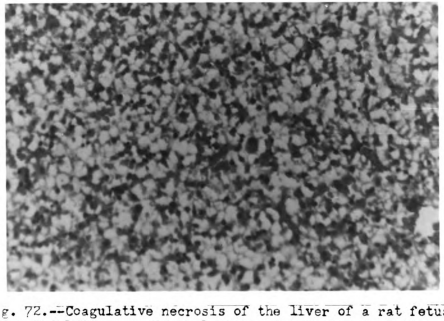


Fig. 72.--Coagulative necrosis of the liver of a rat fetus from a dam fed 150 p.p.m. diethylnitrosamine during gestation period. H & E stain: x187.

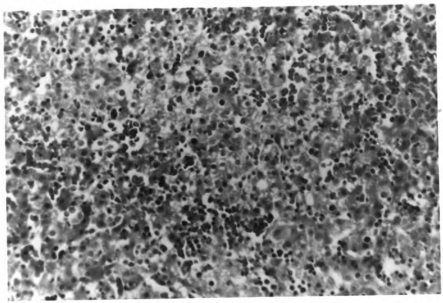


Fig. 73.--Coagulative necrosis of the liver of a rat fetus from a dam fed 150 p.p.m. diethylnitrosamine during gestation period. H & E stain: x187.

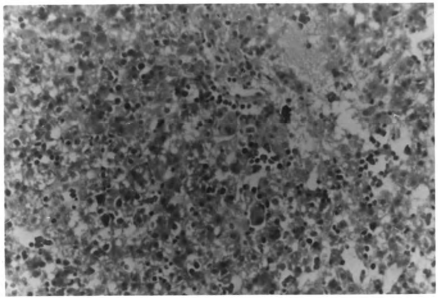


Fig. 74.--Coagulative necrosis of a liver of a rat fetus from a dam fed 750 p.p.m. of dimethylaminoazobenzene during gestation period. H & E stain: x187.

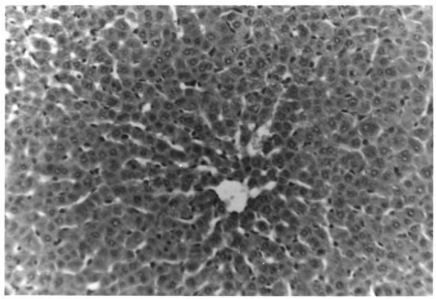


Fig. 75.--Liver of a rat killed after 14 days of suckling mother fed on control diet during lactation. H & E stain: x187.

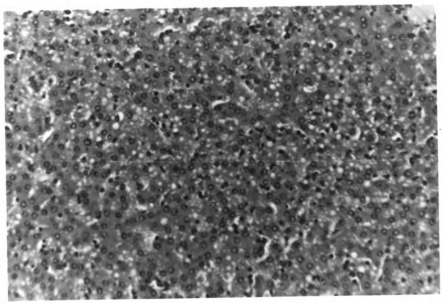


Fig. 76.--Fatty infiltration in a liver of a rat killed after 7 days of suckling mother fed 2% cycad during lactation. H & E stain: x187.

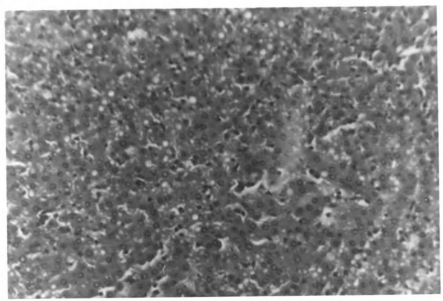


Fig. 77.--Fatty infiltration in the liver of a rat after suckling for 14 days a mother fed 1% cycad during lactation. H & E stain: x187.

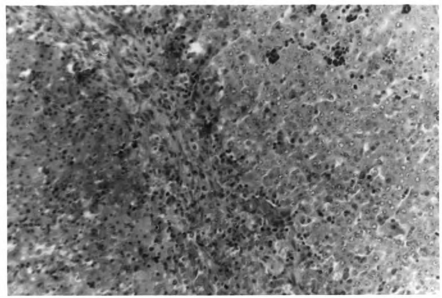


Fig. 78.--Coagulative necrosis of the liver of a rat killed 5 days after suckling a mother fed 600 p.p.m. cycasin during lactation. H & E stain: x187.

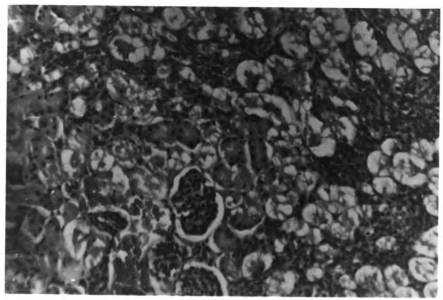


Fig. 79.--Coagulative necrosis of proximal convoluted tubules of kidney of a rat which died 18 days after suckling a mother fed 2% cycad during lactation. H & E stain: x187.

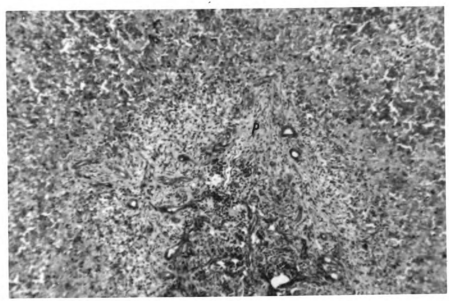


Fig. 80.--Necrosis of the liver of a young rat killed 8 days after suckling a mother fed 150 p.p.m. dimethylnitrosamine. (P) Portal area with proliferating fibroblasts. H & E stain: x75.

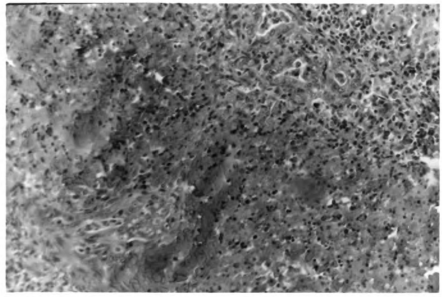


Fig. 81.--Necrosis of the liver of a young rat killed 8 days after suckling a mother fed 150 p.p.m. of dimethylnitrosamine. Higher power of Fig. 80. H & E stain: x187.

Fig. 82.--Liver of a cow that had been fed a diet containing cycad for 140 days. Arrows show focal necrotic lesions.

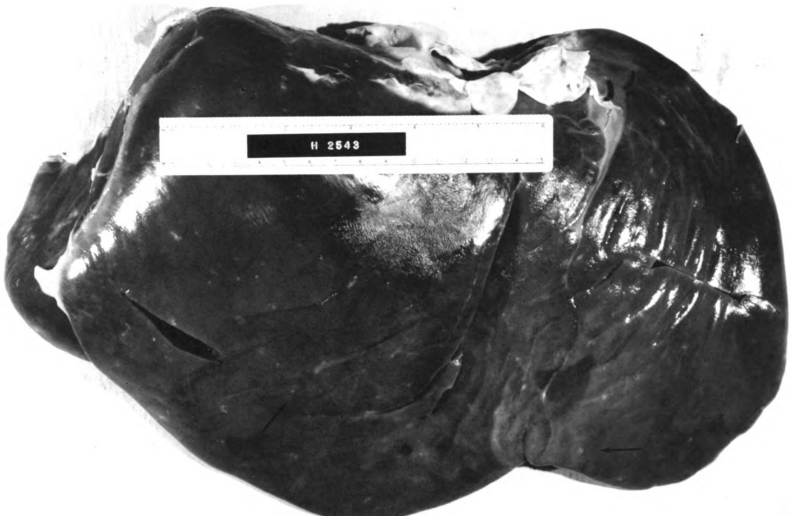


Fig. 83.--Liver of a calf that had been suckling mother while feeding on a diet containing 2% cycad for 140 days. Arrow shows focal necrotic lesions.

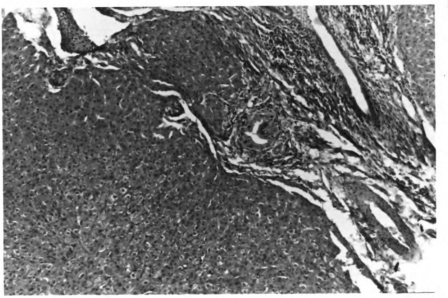


Fig. 84.--Portal fibrosis in the liver of a cow fed cycad for 140 days. H & E stain: x75.

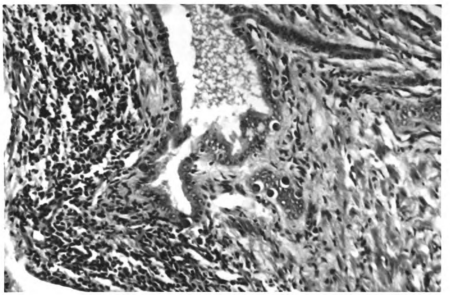


Fig. 85.--Portal fibrosis in the liver of a cow fed cycad for 140 days. Higher power of Fig. 84. H & E stain: x187.

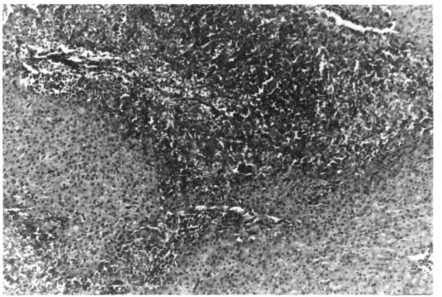


Fig. 86.--Coagulative necrosis in the liver of a cow. Section taken from focal necrotic lesion in the liver of Fig. 83. H & E stain: x750.

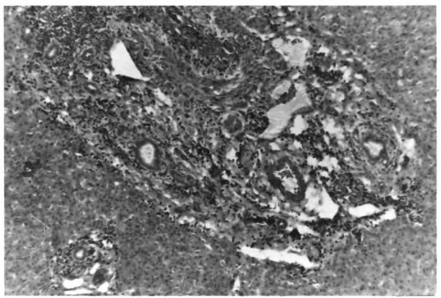


Fig. 87.--Edema and portal fibrosis in the liver of a calf killed after 140 days of suckling a cow fed cycad during the lactation period. H & E stain: x75.

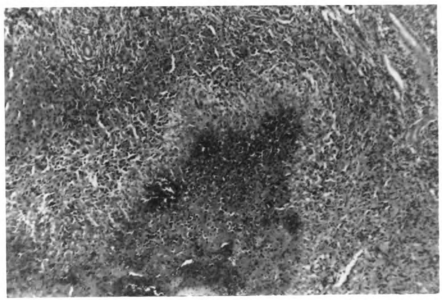


Fig. 88--Caseous necrosis in the liver of the calf. Section taken from focal necrotic lesion on the liver of Fig. 83. H & E stain: x75.

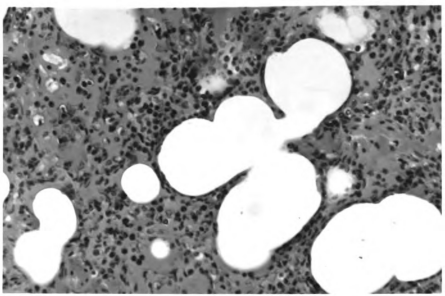


Fig. 89.--Edema and emphysema in the lung of a cow fed cycad for 140 days. H & E stain: x187.

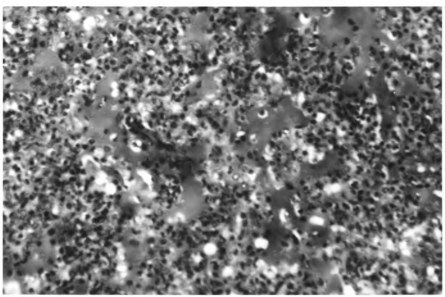


Fig. 90.--Edema with leukocytic infiltration in lung alveoli of the calf killed after 140 days of suckling a cow fed cycad during lactation. H & E stain: x187.

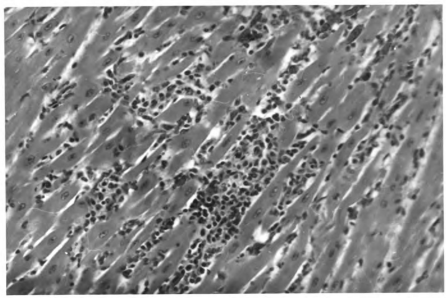


Fig. 91.--Fibroblastic proliferation in the myocardium of a calf killed after 140 days of suckling a cow fed cycad during lactation. H & E stain: x187.

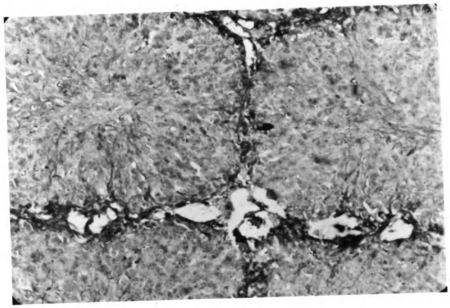


Fig. 92.--Portal and centrolobular fibrosis with occlusion of the central vein in the liver of a sow fed 2% cycad during lactation. Heidenhain's aniline blue stain: x187.

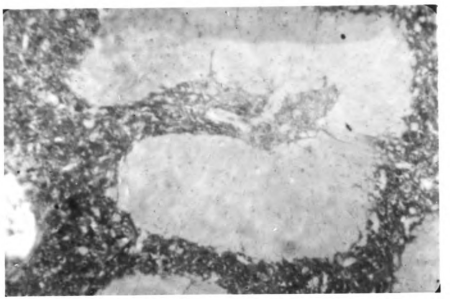


Fig. 93.--Portal and centrolobular fibrosis of the liver of a pig killed after 8 weeks suckling a sow fed 2% cycad during lactation. Heidenhain's aniline blue stain: x137.

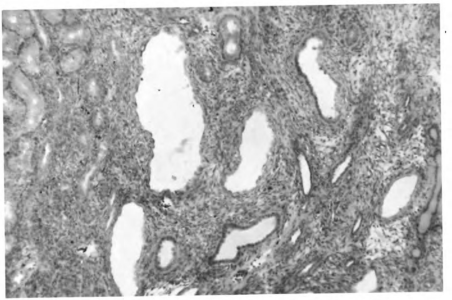


Fig. 94.--Anaplastic undifferentiated cell sarcoma with dilated tubules in a kidney of a rat that suckled a mother fed 600 p.p.m. of cycasin until weaning then fed control diet for 26 weeks. Section taken at the junction of apparently normal kidney tissue and the tumorous mass. H & E stain: x75.

GENERAL SUMMARY

Feeding cycad flour to horses, swine, cattle and rats produced lesions in their livers and kidneys. The lesions consisted primarily of portal and centrolobular fibrosis of the liver and interstitial nephritis. Varying degrees of degeneration and regeneration of hepatic cells were noted in all animals fed cycad. The liver appeared to be more susceptible to the toxic substance than the kidney, and the severity of the lesions was in proportion to the amount fed.

Benign and malignant tumors were produced in rats fed 1% or 2% cycad for 6 to 10 months. The tumors in the liver were hepatocellular carcinomas or reticulum cell sarcomas occurring either simultaneously or independently. The kidney tumors were adenomas, embryonal nephromas or fibrosarcomas. The carcinogenic chemicals cycasin and dimethyl- and diethylnitrosamine produced tissue alterations and tumors similar to those produced by cycad flour. Lesions produced by dimethylaminoazobenzene were different. Therefore, it appears that the cycad toxic factor yields in its metabolic breakdown a carcinogenic compound which may be similar to that resulting from dimethyl- or diethylnitrosamine.

Histopathologic lesions were noted in rats, swine and a calf that suckled dams fed cycad flour. These lesions were similar to those noted in the parents. Lesions were also observed in the fetuses, when pregnant rats were fed cycad. Usually the lesions were more severe in the young and in the fetuses than in the dam. Thus, the toxic factor(s) in cycad are apparently transmitted across the placental and mammary barriers and the young are more susceptible to the toxicosis than their mothers. This appears to be an important discovery of biomedical significance.

This research suggests the need for additional studies on the implications of cycads when used as food or medicine in relation to diseases of man and animals.

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