BIOLOGICAL EFFECTS OF THE INGESTION OF THE TOXIC PLANT, *CYCAS CIRCINALIS*

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Margaret Elizabeth Campbell
1964

ABSTRACT

BIOLOGICAL EFFECTS OF THE INGESTION OF THE TOXIC PLANT, CYCAS CIRCINALIS

by Margaret Elizabeth Campbell

Weanling albino rats and several species of farm animals were used to study the toxic effects of the consumption of Cycas circinalis. Parts of this plant, particularly the flour prepared from the nut are of value as human food.

Cycad materials were incorporated on a percentage basis into natural grain rations of the experimental animals. Flour prepared from the fresh nut and from the nut washed for the purpose of destroying the toxic properties, were studied. Cycasin, the toxic glucoside isolated from the nut and believed responsible for the toxicity thereof was also used in feeding trials. The outer husks of the nut were fed in a third series of studies.

A total of 485 weanling albino rats were used in 11 short term bioassays. These were designed to study variations in the animals response to the toxicity, the stability of the toxic factor under various conditions and the effects of dietary change on the biological response of the animals. Body weight changes and pathological lesions were used to evaluate the relative toxicity of the cycad materials

used. Data collected show these factors exerted an influence on the biologic response.

Long term trials were conducted in which the response of rats fed diets containing 1 or 1.5% cycad were compared with that of rats fed 200 or 400 ppm cycasin. A trial lasting 23 months was designed to study the nutritional safety of washed cycad flour.

Studies in which pigs, rats and cattle were used demonstrated that the toxic factor in cycad flour passes through the placenta and mammary gland. Additional bioassays showed that dimethylaminoazobenzene, diethylnitrosamine and dimethylnitrosamine behaved similarly to the toxic compound in unwashed cycad.

Attempts were made to identify that fraction of cow's milk that contained the toxic factor of cycad. The results of two trials in which rats were fed milk fractions from a cycad-fed-cow were not conclusive.

The husks of the cycad nut caused a toxic response that appears to be different from that caused by cycad nut flour. Hemoglobin and hematocrit values indicated the early development of hemolysis and vasoconstriction which were not apparent in animals fed cycad flour.

Cattle, horses and pigs were all susceptible to the toxicity of the nut flour. Their responses were similar to those seen in rat feeding trials.

BIOLOGICAL EFFECTS OF THE INGESTION OF THE TOXIC PLANT, *CYCAS CIRCINALIS*

By

Margaret Elizabeth Campbell

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Foods and Nutrition

1964

ACKNOWLEDGMENTS

For his help, guidance and unending kindness, I express my sincere gratitude to my major Professor, Dr. Olaf Mickelsen. His enthusiasm and love of learning have set a constant goal for me.

My sincere thanks go to Dr. Dorothy Arata, an inspiring professor in my course work and Dr. Modesto Yang for his willingness and help in instructing me in the methods and practice of research.

To Michigan State University for my Graduate Assistantship, the National Cancer Institute for funds to carry out this research (Public Health Service Research Grant #CA-07052-01), to Dr. Gert L. Laqueur, Chief, Laboratory Experimental Pathology, NIAMD, NIH, Dr. C. K. Whitehair and Dr. Gerald Mugera of the Veterinary Pathology Department, M.S.U. for their cooperation in the pathological work involved in these studies, and to Dr. J. C. Keresztesy, NIAMD, NIH for preparation of the cycad materials, I express my gratitude.

To My Parents

TABLE OF CONTENTS

		Page
I.	REVIEW OF LITERATURE	1
	Literature Cited	9
II.	STUDIES OF THE EFFECTS OF INGESTION OF *CYCAS CIRCINALIS* BY RATS	12
	Introduction	13
	Experimental	17
	Results	18
	Discussion	46
	Literature Cited	51
III.	ADDITIONAL RESEARCH PROJECTS	54
	Washed Cycad Flour	54
	Introduction	54
	General Procedure	54
	Volitional Activity	55
	Respiratory Infection	55
	Reproductive Response	56
	Tissue Preparation	60
	Unwashed Cycad Flour	61
	Introduction	61
	Stability of Toxic Factor and Variations in Biological Response	61
	Comparison of Toxic Response of Cycasin and Unwashed Cycad Flour	62
	Feeding Pregnant Rats	68
	Feeding Weanling Rats	70
	Cycad Husks	71
	Introduction	71
	Effect of Environmental Temperature	72
	Blood Changes	72

Page

Cooperative Studies with the Veterinary Pathology Department 73

Swine 73
Cow and Calf 77
Horses 80

LIST OF TABLES

Table		Page
1.	Composition of cycad flour and white wheat flour	15
2.	Average weight changes of rats of different strains fed unwashed cycad flour #623-6. Five animals per group, average for 5 days	35
3.	Weight changes (g) of Osborne-Mendel rats from 2 sources fed unwashed cycad flour	36
4.	Effect of age on weight changes (g) of young Osborne-Mendel rats fed unwashed cycad flour #623-6. Ten rats per group, average for 5 days	37
5.	Summarizing table of short term bioassays using unwashed cycad flour	63

LIST OF FIGURES

Figure		Page
1.	Growth depression of rats fed either of two lots of unwashed cycad flour for 5 days . .	20
2.	Calculated percentage cycasin in unwashed cycad flour using weight depression curve of rats fed unwashed cycad flour for 5 days	23
3.	Growth of rats fed cycad flour #628-1 incubated for varying intervals at 37°C (5-day bioassay)	26
4.	Growth of rats fed heat treated, unwashed cycad flour for 5 days	29
5.	Growth of rats fed unwashed cycad flour #623-6 stored at 5°C for varying intervals (5-day bioassay)	31
6.	5-day body weight changes of rats fed unwashed cycad flour in diets varying in casein content	39
7.	Mortality of rats fed 2% unwashed cycad flour for 18 or 25 days and/or 1% thereafter	43
8.	Growth of animals fed washed cycad flour . . .	57
9.	% change in hemoglobin and hematocrit from 0 time 0 time animals fed 24% cycad husk diet or control diet (pair-fed)	74

REVIEW OF LITERATURE

Poisonous plants are found in all parts of the world. The tropical regions are virtual greenhouses for species with toxic properties. Keegan and MacFarlane (1) list 8 families of plants, indigenous to Australia which are known to be toxic when ingested and 181 species found on Guam which are poisonous. These include plants which cause toxic reactions after ingestion and/or on contact. Families of such plants are characterized by distinctive poisonous properties which may sometimes be affected by season, climate, soil conditions and cultivation. Moreover, certain parts of these plants may be highly poisonous whereas others may be strictly harmless.

Mushrooms, cottonseed, soybean, the castor oil bean, several species of the common sweet pea and certain plants of the parsley family are some of the commonly known toxic plants (2). A host of plants contain cyanogenetic glucosides which may, under certain circumstances, cause hydrocyanic acid poisoning. These glucosides have been found in corn, sorghum, rough gram, field beans, black gram, kidney beans sweet potatoes, lettuce, the seeds of some fruits, lima beans and manioc. Only in these latter 2 is the content of glucoside great enough to cause poisoning. Manioc is a

staple food in the diet of many Africans. In America manioc is more commonly known as cassava or tapioca. Preparation of the root starch of manioc involves soaking, fermenting or boiling. Each treatment is considered sufficient to cause complete loss of the toxicity responsible for liver, kidney, skin and neural changes which result when unwashed manioc is eaten (3). Growth inhibition in rats has recently been reported by Kadake and Evans (4) when the raw navy bean was fed as the source of dietary protein.

The genus Senecio of the aster family, contains 1250 species, widely distributed throughout the world and known by a variety of local names most commonly "ragwort." Senecio plants are found in parts of America, Europe, Africa, India, Australia and Japan and have probably been the cause of more livestock losses than all other poisonous plants combined (5). The toxic properties of the pyrrolizidine alkaloids produced in these plants have been the topic of extensive research, particularly in Britain and Jamaica (6, 7, 8, 9). Pathological changes seen in animals that have ingested these compounds resemble closely those seen in animals fed flour prepared from the nut of Cycas circinalis (10).

The first record of the toxicity of Cycas is a note in the diary of Cook dated 1770 in which he reports illness in his men when they consumed C. media at Endeavour River, Australia (1). Since that time numerous reports relate the ingestion of various parts of the cycad plants to illness in both men and animals (11).

Cycads resemble the tree ferns in appearance and represent an evolutionary transition between tree ferns and Gymnosperms (12). They are of medicinal, economic and nutritional importance in the tropics and subtropics where the plant is indigenous. The nut, produced at the top of the female plant is of most value (13). In Guam, juice grated from fresh cycad nuts is a recommended treatment for tropical leg ulcers, snake bites, open cuts and wounds. Medicinal teas are also used (11). Limited use has been made of the various parts of the plant in the manufacture of alcohol, fertilizer, laundry starch and to a lesser extent in construction (13).

A sturdy root structure enables the cycad plant to withstand adverse climatic conditions. For this reason the cycad is regarded as a valued emergency food in areas in which it is indigenous. Flour milled from the starchy endosperm is the form most commonly used. One of the values of the nut is that when thoroughly dried it can be stored for long periods. Hard dry pieces of sliced seeds are kept from season to season. They are ground to a fine powder and used in a variety of cooked dishes including tortillas, and soups (11). Starches are also prepared from the roots and stems of some species. "Arrowroot" has often been used to refer to the starches made from the tuberous roots of certain species. "Sago" appears to be restricted to starches obtained from the stems (13). The proximate composition of the

flour prepared from the nut of Cycas circinalis is similar to white wheat flour (Table 1 of Manuscript). In addition to the flour and starches prepared from the plant, the husks covering the nut are chewed fresh as the nuts are harvested and later in dried form as a confectionary.

The cycad has long been recognized as toxic. It was not until 1941 that Cooper (14) isolated from the seeds of Macrozamia spirilis, a species of cycad native to Australia, a compound that exhibited toxic properties. Later this compound was identified as a glycoside of primerverose and methylazoxymethanol (MAM). A glucoside similar to that isolated by Cooper was obtained from Cycas revoluta Thunb. by Nishida in 1955 (15) and from Cycas circinalis by Riggs in 1956 (16). The aglycone, MAM, $CH_3-\overset{\oplus}{N}=N-CH_2OH$ is common to all toxic cycad glycosides identified and considered the toxic factor of the plant (17). Methylazoxymethanol contains nitrogen in linkages not previously found in a natural product (11). On acid or enzymatic hydrolysis of the glycosidic bond, the aglycone which is unstable decomposes spontaneously to produce nitrogen, methanol and formaldehyde. Alkaline hydrolysis yields nitrogen, formic acid, cyanide and traces of methylamine (18).

Through experience, the Guamanians have learned to rid the nut of its toxicity by soaking it cut in eights, in large vats of water. The soaking procedure varies. The water may be changed often or relatively infrequently.

Guamanians report (19) that animals have died after drinking the wash water from soaking nuts. This suggestion that the toxic compound is stable in the water has not been borne out in our laboratory work (Page 22, accompanying manuscript). The soaking time varies from about 10 days to 3 weeks. The nut pieces are spread to dry in the sun before being stored. The nutritional safety of nuts so prepared is presently being investigated.

The "unwashed" cycad causes mild and severe gastrointestinal symptoms when consumed. Such cases most frequently occur during periods of extreme food shortage and can often be traced to inadequate preparation of the nut. Symptoms include swelling of the stomach, abdominal cramps, vomiting and diarrhea. Headache, dizziness, depression and stupor are often concurrent with the gastrointestinal ailments (11). In 1 case, ingestion of inadequately prepared cycad caused the death of 5 members of 1 family (20).

Nuts and leaves of the plant are eaten by animals as well. In Australia, it is reported that animals graze selectively on cycad. Some even become addicted. The losses of cattle and sheep due to cycad poisoning, especially in Australia, is a serious economic problem (21). One effect on cattle and sheep is severe gastrointestinal disturbances, the other is a partial paralysis. This paralysis in animals is first apparent as a slight staggering gait and crossing of the hind limbs in walking (11). Henderson (22) reports a

similar disturbance in oxen pastured in areas where the genus Zamia grows in the Dominican Republic. Slightly more than 1% of the work oxen in these areas are lost each year because of this paralytic disease.

Numerous feeding trials, though sometimes poorly controlled confirmed the development of the acute gastrointestinal symptoms and the chronic locomotor difficulties (11). The course of the locomotor paralysis in cattle follows a characteristic pattern. It is progressive, irreversible and exaggerated by exercise. Death occurs when the animal can no longer move about to get food and water (11,22).

Nishida (18) reported that mice injected subcutaneously or intravenously with crystalline cycasin, the toxic glycoside isolated from Cycas circinalis, exhibited no toxic symptoms, whereas 5 of 6 mice administered similar amounts by stomach tube were dead in 36 hours. Similar amounts given orally were lethal for the guinea pig. Nishida (18) attributed the toxic manifestations appearing after oral administration of cycasin to the absorption of products released by the action of the β-glucosidases present in the gastrointestinal tract. Laqueur (24) has shown that the acute effects of cycasin intoxication are completely absent in germfree rats indicating that cycasin per se is not injurious and that it is not cleaved to glucose and the aglycone in the absence of bacterial enzymes.

Current research into the toxicity of the cycad development from its implication as the neurotoxin responsible for the high incidence of amyotrophic lateral sclerosis, (ALS) among Guamanians. In Guam, the incidence, prevalence and death due to ALS are approximately 100 times that in the United States and Europe (25).

The disease affects the cells of the medulla and cervical cord. It appears usually between the ages of 40 and 50. Weakness of the hands and arms, difficulty in swallowing and talking, weakness and spasticity of the legs are the first symptoms. In a fully developed case, the muscles of the hands, arms, shoulders, pelvis, legs and tongue become atrophied. The weakness of the legs appears as a spastic gait. The course of the disease is rapidly progressive and always fatal. In most cases death ensues in 2 to 5 years (26).

The increased incidence in ALS on Guam and the Marina Islands prompted investigation into possible causative factors of the disease. The small number of families and extensive intermarriage on Guam suggested that ALS may be an expression of a recessive genetic trait. However no genetic patterns became evident after investigation. Environmental factors were also ruled out as being of primary importance (27).

In a broad nutritional survey (11) aimed at identifying toxic foods which may be related to the development of

ALS, Cycas circinalis was suggested because of its association with paralytic diseases in animals (19). However, experiments with several species of animals failed to show the neurotoxic effect of cycad ingestion (11). Even in trials where cattle developed paralysis after being fed cycad, microscopic examination of the brain and spinal cord did not reveal any neurological basis for the disturbance (28). Laqueur et al (10) report the absence of histological lesions resembling ALS in the brain and spinal cord of rats fed the flour of the Cycas circinalis nut. However, animals fed small amounts of this flour over extended periods of time developed benign and malignant tumors. These neoplasms occurred mainly in the liver and kidneys and occasionally in the lungs and intestine (10). These results leave little doubt that the flour prepared from the fresh nuts of Cycas circinalis is carcinogenic and probably not directly associated with the development of ALS.

Literature Cited

1. Keegan, H. L., MacFarlane, W. V. 1963. Venomous and Poisonous Animals and Noxious Plants of the Pacific Region. The Pergamon Press, MacMillan Company, Copyright 1963.

2. Dubois, K. P. Geiling, E. M. K. 1959. Textbook of Toxicology. Oxford University Press, Copyright 1959.

3. Jones, W. O. 1959. Manioc in Africa. Stanford University Press, Copyright 1959.

4. Kadake, M. L., Evans, R. J. 1963. Growth Inhibition in Rats Fed Raw Navy Beans. Fed. Proc. 22(2):551.

5. Campbell, J. G. 1956. An Investigation of the Hepatotoxic Effects in the Fowl of Ragwort with special reference to the Induction of Liver Tumors with Seneciphylline. Proc. Royal Soc. Edinburgh, LXVI:111.

6. Hill, K. R. 1960. Discussion on Seneciosis in Man and Animals. Proc. Royal Soc. Med., 53:281.

7. Campbell, J. G. 1960. Variations of Liver Function in Immature Fowls and Susceptibility to Seneciphylline. J. Path. Bact., 80:399.

8. Schoental, R., Magee, P. N. 1959. Further Observations on the Sub-acute and Chronic Liver Changes in Rats After a single does of Various Pyrrolizidine (Senecio) Alkaloids. J. Path. Bact., 78:471.

9. Schoental, R. 1960. The Chemical Aspect of Seneciosis. Proc. Royal Soc. Med., 53:284.

10. Laqueur, G. L., Mickelsen, O., Whiting, M. G., Kurland, L. T. 1963. Carcinogenic Properties of Nuts from Cycas Circinalis L. Indigenous to Guam. J. Natl. Cancer Inst., 31:919.

11. Whiting, M. G. 1963. The Toxicity of Cycad. Economic Botany, 17:271.

12. Missouri Botanical Garden Bulletin. 1955. 43:65.

13. Thieret, J. W. 1958. Economic Botany of the Cycads. Economic Botany, 12:3.

14. Cooper, J. M. 1941. Isolation of a Toxic Principle from the Seeds of Macrozamia Spirilis. J. Proc. Royal Soc. New South Wales, 74:450.

15. Nishida K., Kobayashi A., Nagahama, T. 1955. Cycasin, A New Toxic Glycoside of Cycas Revoluta Thunb. Bull. Agr. Chem. Soc. Japan, 19:77.

16. Riggs, N. V. 1956. Glycosylazoxymethane, A Constituent of the Seeds of Cycas Circinalis L. Chem. and Industry, 35:926.

17. Matsumoto, H. S., Strong, F. M. 1963. The Occurrance of Methylazoxymethanol in Cycas Circinalis L. Arch. Biochem., 101:299.

18. Nishida, K., Kobayashi, A., Nagahama, T., Kojima, K., Yamane, M. 1956. Cycasin, A New Toxic Glycoside of Cycas Revoluta Thunb: IV Pharmacological Study of Cycasin (In Japanese) Seikagahu, 28:218.

19. Whiting, M. G. (personal communication) NIH, Bethesda, Md.

20. Hiroyshi, T. 1956. Cycad Poisoning on Island of Miyako. Okinawa Times, Dec. 13.

21. Seddon, H. R., Belschner, H. G., King, R. O. C. 1931. Poisoning of Sheep by the Seeds of Burrawang (Macrozomia Spirilis) Vet. Res. Report #6. June. Dept. Agr., New South Wales, Sydney, Australia.

22. Henderson, J. 1964. (personal communication) Conference--Current Progress in Studies of Cycad Toxicity with particular reference to Neurotoxic Properties. NIH, Oct. 26, 1964.

23. Hall, W. T. K. 1956. A Note on Macrozamia and Xanthorrhoea Poisoning of Cattle, Australia Vet. J., July: 173.

24. Laqueur, G. L. 1964. Carcinogenic Effects of Cycads and Cycasin in Rats. Fed. Proc. in the press.

25. Lessell, S. 1962. (personal communication) NIH, Bethesda, Md.

26. Cecil, R. L., Loeb, R. F. 1955. A Textbook of Medicine. W. B. Saunders Co. Philadelphia. p. 1459.

27. Kurland, L. T. 1962. (personal communication) NIH, Bethesda, Md.

28. Hall, W. T. K. 1957. Toxicity of the Leaves of Macroazamia Spirilis for Cattle. Queensland J. Agr. Sci. 14:41.

STUDIES OF THE EFFECTS OF INGESTION OF *CYCAS CIRCINALIS* BY RATS

M. E. Campbell, O. Mickelsen, M. G. Yang,
G. L. Laqueur[1] and J. C. Keresztesy[2]

Department of Foods and Nutrition
College of Home Economics
Michigan State University
East Lansing, Michigan

This investigation was supported by Public Health Service Research Grant No. CA-07052-01 from the National Cancer Institute

[1]Chief, Laboratory of Experimental Pathology

[2]Chief, Section of Fractionation and Isolation
National Institute of Arthritis and Metabolic Diseases
National Institute of Health

submitted to Journal of Nutrition for publication

Introduction

Cycas circinalis L. is one of 9 species of cycads growing in tropical or subtropical areas. Cycads resemble tree ferns and represent an evolutionary transition between ferns and Gymnosperms or evergreens (1). They are of medicinal, economic and nutritional importance in the areas where they are indigenous (2). Although some species of cycad are widely distributed, Cycas circinalis L. is indigenous to Guam and Oceania.

As early as 1770, field observations suggested that cycads contained toxic substances. Reports of these observations indicated gastrointestinal and paralytic symptoms developing in animals and man after ingesting parts of the plant (3, 4). The first sign of paralysis in animals is a staggering gait with a crossing of the hind limbs when resting. In this position, the animal sways from side to side, hence the term "wobbles" used by the Australians to describe this condition (3). Great losses of cattle and sheep have been reported from regions where cycad grows on grazing lands (5).

Some of the first animal studies, although often poorly controlled, confirmed the development of gastrointestinal symptoms and locomotor disturbances (3). Severe

liver damage was a common pathological finding. In some animals, chronic feeding of cycad resulted in a locomotor disturbance that was progressive, irreversible and was exaggerated by exercise. However, no neurological basis for this paralysis was found (6).

Present interest in Cycas circinalis stemmed from an apparent correlation between its use as a human food and a high incidence on Guam of amyotrophic lateral sclerosis. This disease is 100 times more prevalent on Guam than in the United States or Europe (3). The disease is characterized by neural and muscular degeneration, appears usually between the ages of 40 and 50, progresses rapidly and terminates fatally within 2 to 5 years (7).

The use of cycad by the natives on Guam and other Southwestern Pacific Islands stems from the fact that this plant survives adverse climatic conditions. This makes it one of the few foods available during droughts and following devastating typhoons. The nut meat is the part of the plant that is most frequently used by man. Since its toxicity is well recognized, it is routinely soaked in water prior to use. The nut (approximately 4 cm in diameter) is quartered and the center, or nut meat, soaked in vats of water. The water may be changed frequently during soaking which lasts from a few days to 3 weeks. The nut slices are dried in the sun and then ground into flour. The latter is used for thickening soups, puddings, porridge and in tortillas (3).

The proximate composition of cycad flour is similar to wheat flour (table 1). The 34% not identified is thought to be carbohydrate. However, with the exception of glucose and fructose, the other carbohydrates are present in trace amounts only.

Table 1. Composition of cycad flour and white wheat flour.

	Cycad Flour	White Wheat Flour[b]
	(%)	
Nitrogen	2.0	1.8
Water	6.5	12.0
Fat	0.8	1.0
Ash	2.6	0.4
Carbohydrate (known)	45.0[a]	76.1
Unknown	34.0	

[a]The known carbohydrates as a percentage of cycad flour are: free glucose 1.2 - 2.1, glucose (freed by acid hydrolysis) 39.0, free fructose 0.7 - 0.9, fructose (freed by acid hydrolysis) 1.4, cycasin 2.0 and trace quantities of xylose, primeverose (?), myo-inositol, sequoyitol and pinitol (personal communication from Drs. N. K. Richtmyer and S. S. Chernick, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health). Determination of total carbohydrate by the anthrone method (8) showed cycad flour was 52% carbohydrate.

[b]see reference (9).

In 1941, Cooper isolated from Macrozamia spirilis, a species of cycad indigenous to Australia, a glycoside which

was lethal to animals (10). Cycasin, a glycoside similar to the one isolated by Cooper except for the sugar moiety, was isolated from Cycas revolunta Thunb. by Nishida in 1955 (11) and from Cycas circinalis L. by Riggs in 1956 (12). The aglycone common to these glycosides is methylazoxymethanol $CH_3-\overset{\overset{O}{\uparrow}}{N}=N-CH_2OH$. It is considered to be responsible for the toxicity of the glycosides (13).

Evidence suggests that the aglycone is toxic only after hydrolysis of the glycoside by bacterial β-glucosidases in the intestinal tract. Nishida (14) noted that toxic symptoms appeared only after oral ingestion of the glycoside but not following intravenous or subcutaneous injection. The absence of acute toxicity in germfree rats fed cycasin confirms this hypothesis (15).

To determine whether there is any experimental basis for implicating cycad in the etiology of amyotrophic lateral sclerosis, unwashed cycad was fed to various species of experimental animals. The present paper reports the stability of the toxic factor(s) of cycad and variations in the response of rats of different sexes, ages, strains and colonies. Reported, as well, are the effects of dietary changes and discontinuation of cycad feeding on the toxic response.

A previous report of this work (16) indicated that the chronic ingestion of cycad produced no central nervous system lesions in rats. Instead, neoplastic lesions developed in the liver and kidneys.

Experimental

Cycad Materials

Cycad nuts were shipped to our laboratory immediately after collection from various parts of Guam. Each lot of nuts was coded with the year, month and day of delivery (e.g. 623-6). These materials had not been soaked as described earlier, and will be referred to as unwashed cycad. The latter material was chosen because it was thought to contain a higher concentration of the toxic factor and thus results obtained would be more definitive. The nuts were husked, cut open, thinly sliced by machine and dried under vacuum at 40^{o}C. The dried slices were ground into flour in a Wiley Mill.

Bioassay

Sprague-Dawley and Osborne-Mendel male weanling rats were used except where indicated. Previous to the feeding of experimental rations, all animals were fed a basal grain ration until a weight increase of 5 to 6 grams per day was achieved. The average body weight of the animals at the beginning of the experimental feeding trials was approximately 80 grams. In most studies, 10 animals comprised each experimental group. In some of the preliminary work, 3 or 5 animals were used. All were housed individually in suspended wire cages. Feed and water were given ad libitum.

Natural grain rations were fed as the basal diet.[1] Cycad materials were thoroughly mixed with appropriate proportions of the basal diet and refrigerated. Prior to feeding, all cycad materials were stored at 5°C.

Growth rates and liver lesions were used to indicate the relative toxicity of each new lot of cycad flour. Weights were recorded on the first and fifth day of the assay period. On the fifth day, the animals were etherized and sacrificed. The livers were removed, fixed in 4% acetate-buffered-formalin and later examined for pathological lesions. Some animals were maintained for longer periods.

Results

General

Animals fed the unwashed flour as a percentage of their basal ration gained less than controls fed basal ration only. Growth depression of animals varied depending upon the

---

[1] A modification of the Animal Nutrition Research Council Reference Chick Diet (17), the percent composition of this grain ration was: ground corn 60.7, soybean meal (50% protein) 28.0, alfalfa meal (17% protein) 2.0, fish meal (12.5% protein) 2.5, dried whey (67% lactose) 2.5, limestone (38% Ca) 1.6, dicalcium phosphate (18.5% P, 22-25% Ca) 1.75, iodized salt 0.5. Supplementary minerals, vitamins and antibiotics were added to provide per kilogram of feed: (in mg) Mn 121, Fe 95, Cu 7, Zn 4, I_2 4, Co 2, choline chloride 400, calcium pantothenate 6, riboflavin 3, niacin 33, menadione 2, DL Methionine 500, penicillin 2, streptomycin 8, arsenilic acid 968, (in μg) vitamin B_{12} 7, (in International units) Vitamin A 8010, Vitamin D_2 750, Vitamin E 5 (Alpha Tocopherol Powder, Nutritional Biochemical Corporation, Cleveland, Ohio).

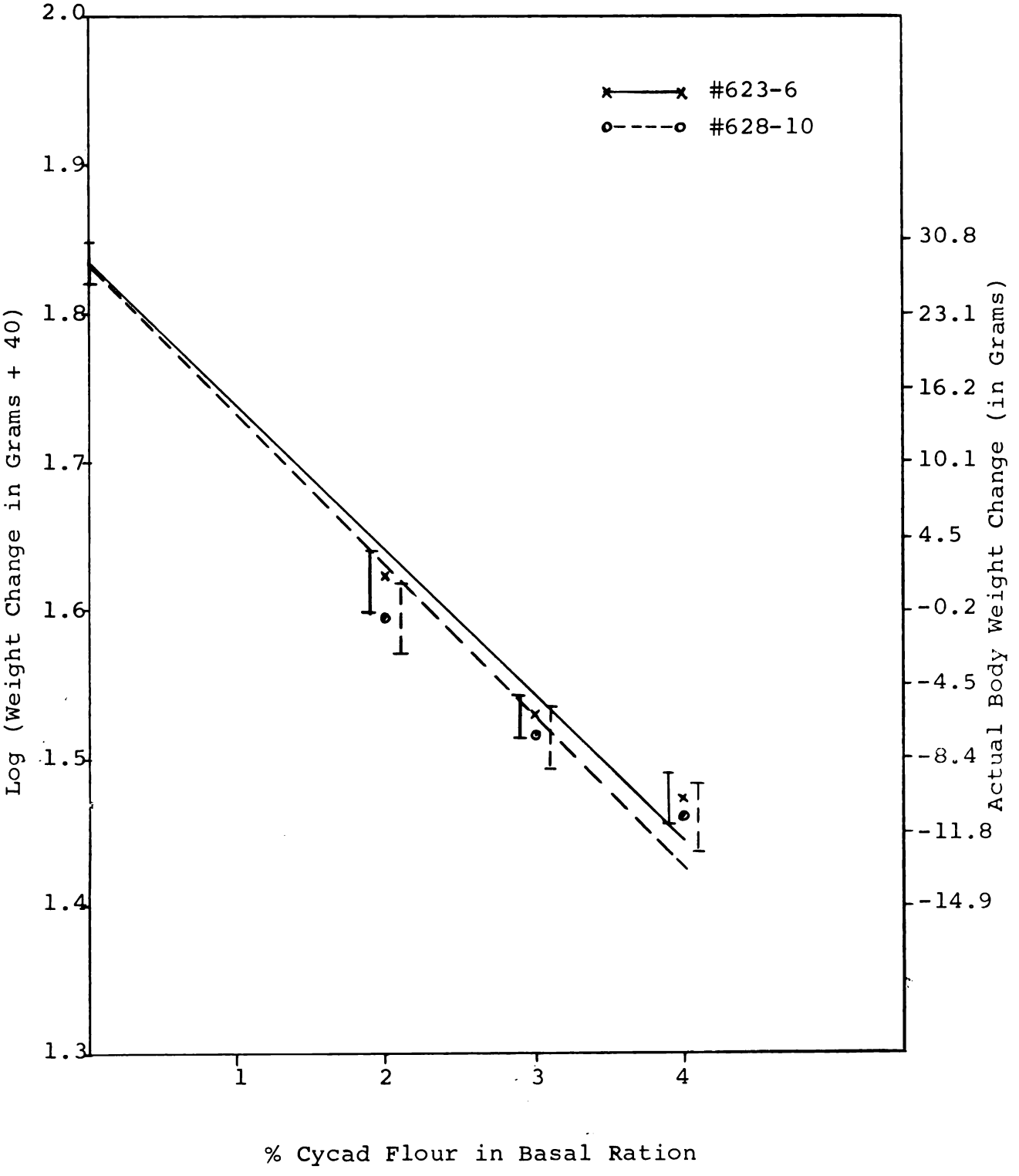
lot of flour used, but, all flours caused a linear response when weight changes were plotted logarithmically against the percentage of unwashed cycad flour included in the ration (figure 1). We have plotted growth depression for all bioassays as the logarithm of the change in weight + 40 (to avoid negative numbers) and the corresponding actual body weight change (in g) on the ordinate. Zero percent cycad indicates the growth response of control rats. Vertical lines are Mantel's standard errors (18). Data were analyzed by analysis of variance (19) and mean differences by Duncan's Multiple Range Test (20).

Decrease and loss of cytoplasmic basophilia from the centrolobular zones and necrosis of individual liver cells in these zones are characteristic of the liver lesions seen in the animals fed experimental rations. With increasing concentrations of cycad flour, there was a corresponding increase in the severity of the liver damage. These lesions are identical with those reported by Laqueur et al (16) as being the early changes characteristic of cycad intoxication.

A considerable amount of the present work has involved the use of cycasin, the major glycoside in the cycad flour. Preliminary studies suggest that most of the toxic symptoms associated with the feeding of unwashed cycad can be attributed to cycasin. Body weight changes for 90 male Sprague-Dawley rats, fed various levels of unwashed cycad flour or cycasin in a 5-day bioassay, suggest that the flour

FIGURE 1

GROWTH DEPRESSION OF RATS FED EITHER OF TWO LOTS OF UNWASHED CYCAD FLOUR FOR 5 DAYS


#623-6
#628-10
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
Log (Weight Change in Grams + 40)
30.8
23.1
16.2
10.1
4.5
-0.2
-4.5
-8.4
-11.8
-14.9
Actual Body Weight Change (in Grams)
1
2
3
4
% Cycad Flour in Basal Ration

contains approximately 2.3% cycasin (dry weight basis) (figure 2). The break in the curve at the 1% cycad level is unexplainable. This was the first assay (in approximately 25) where the curve showed this break. Apart from that, the slope of the main part of this curve parallels that of the previous assays run under comparable conditions.

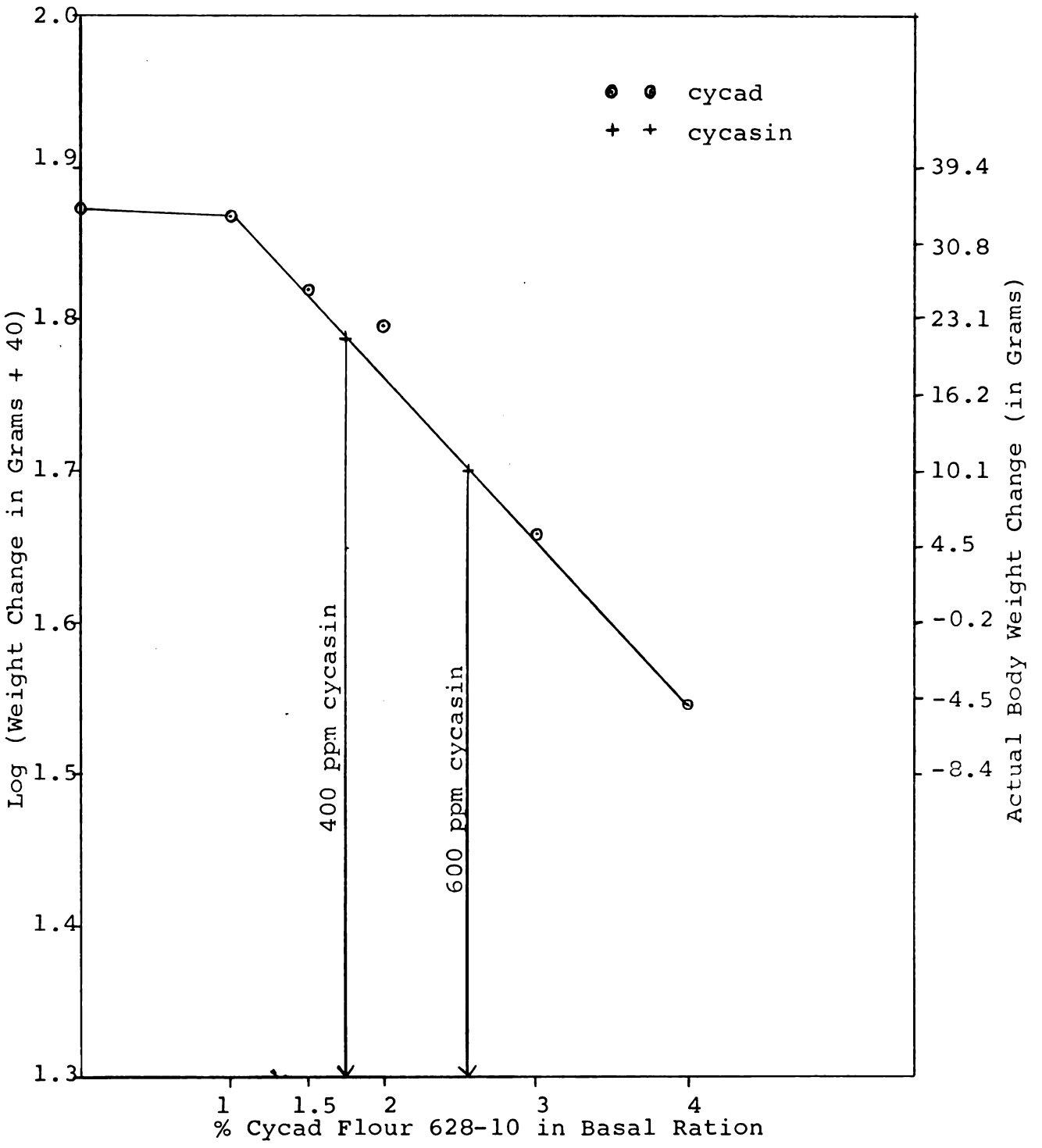
Stability of Toxic Factor

Water Treatment

Reports from Guam suggest the "wash water" from soaking nuts is lethal to animals (21). This would indicate the toxic factor is water soluble, can be leached out of the nut pieces and is stable in solution. To test this hypothesis, 624 g of unwashed cycad nut slices, of known toxicity (see #623-6, figure 1), were soaked in 1.5 liters of tap water at 37°C for 5 days. The solids were removed by filtration, dried at room temperature and ground. The filtrate was concentrated under vacuum to 66 ml. Diets containing either 5% ground solids, an aliquot amount of the concentrated filtrate, or both, were fed to groups of 10 weanling rats. Growth responses for the 5-day assay averaged 31, 32, and 33 g respectively. These increases were similar to gains of control animals, indicating the absence of the toxic factor from both the residue and the filtrate prepared in our laboratories. These data, contrary to reports from Guam,

FIGURE 2

CALCULATED PERCENTAGE CYCASIN IN UNWASHED CYCAD FLOUR USING WEIGHT DEPRESSION CURVE OF RATS FED UNWASHED CYCAD FLOUR FOR 5 DAYS



Calculated % Cycasin in 628-10 Cycad Flour

2.28 for animals fed 400 ppm cycasin

2.35 for animals fed 600 ppm cycasin

suggest a degradation of the toxic compound has occurred during the soaking process.

To study the rate of loss of toxicity, pastes made from unwashed cycad flour and water were incubated at 37°C for varying lengths of time, dried under vacuum at 40°C, and ground into flour. The growth of animals fed a diet containing 15% non-incubated flour was less than that of animals fed diets 15% of which was the incubated flour. The treatment caused a progressive decrease in the toxicity of the pastes with time. Large volumes of gas were generated overnight and all toxic activity was lost some time prior to 90 hours of incubation (figure 3). The gases evolved are believed to be breakdown products (probably nitrogen) associated with the destruction of toxicity. However, not all pastes, made from different lots of unwashed flour, similarly incubated, produced gas. Pastes made from washed (i.e. soaked) cycad flour did not evolve gas with or without the addition of the crystalline glycoside, cycasin.

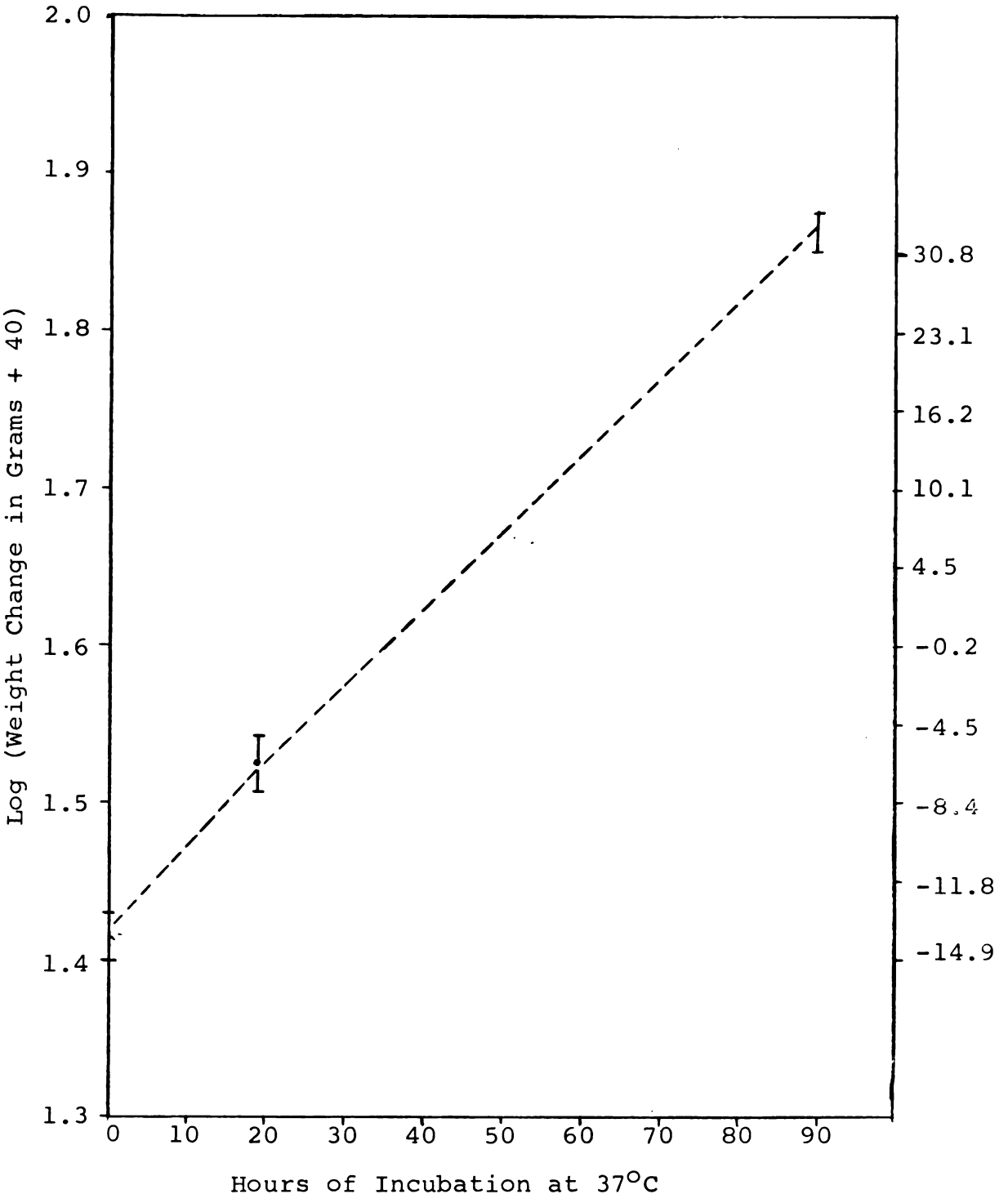
That the toxic component of cycad nuts is destroyed during incubation in the moist state is evident. Experiments are presently underway to test the nutritional safety of the cycad prepared for food by the natives on Guam.

Heat Treatment

Cycad flour is used chiefly as an ingredient in the cooked dishes of the Guamanian people. The effect of heat on the stability of the toxic factor was next investigated.

FIGURE 3

GROWTH OF RATS FED CYCAD FLOUR #628-1
INCUBATED FOR VARYING INTERVALS AT
37°C (5-DAY BIOASSAY)


2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
Log (Weight Change in Grams + 40)
30.8
23.1
16.2
10.1
4.5
-0.2
-4.5
-8.4
-11.8
-14.9
0
10
20
30
40
50
60
70
80
90
Hours of Incubation at 37°C

Toxic unwashed flour (lot #637-3) was cooked at 90°C for 1 hour with 9 volumes of tap water, dried in thin layers at 37°C for 30 hours and ground in a Wiley Mill. An amount of the same flour was heated at 90°C for 1 hour without the addition of water. The growth response of animals fed either of these heat treated flours at levels of 2, 3, or 4% of the ration was in most cases, similar to control animals fed only the basal ration (figure 4). With the exception of the group fed 4% cooked flour (moist heat), the livers from these animals were microscopically normal. This latter group showed loss of some basophilia from the centrolobular areas. These data would indicate the toxic factor in unwashed cycad flour is almost completely destroyed by moist or dry heat at 90°C. This instability of the toxic factor to heat adds a probable margin of safety to the consumption of washed cycad flour when it is used in cooked foods.

Storage

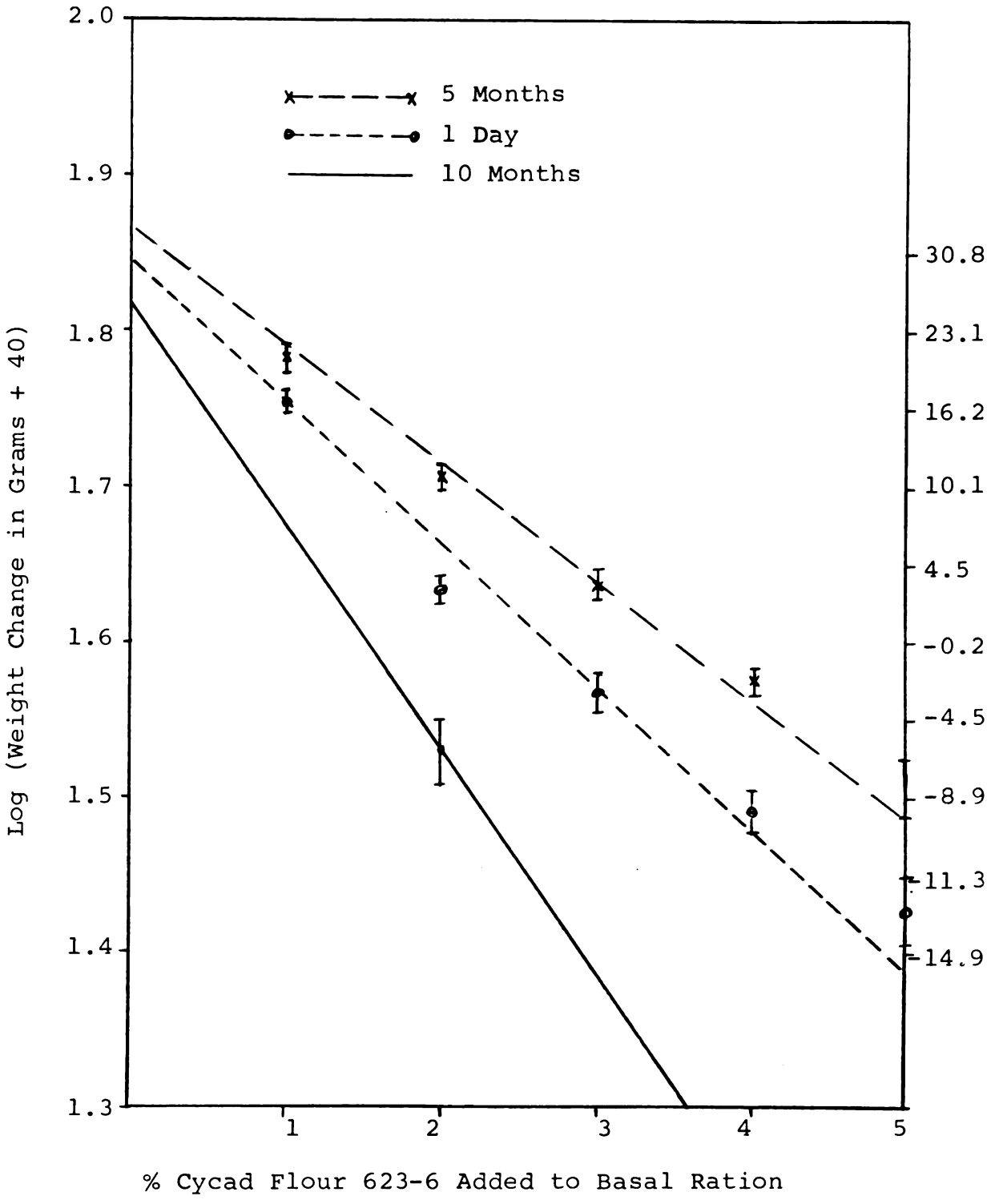
Growth curves from successive animal feeding trials indicated a loss in the toxicity of the cycad flour during storage. Flours stored at -20°C retained more toxicity than flours stored at 5°C. One lot of unwashed flour (#623-6), stored at 5°C, was assayed 4 times over a period of 5 months. Figure 5 represents the data obtained from the first and fourth assays. The curve from the second and third assays

FIGURE 4

GROWTH OF RATS FED HEAT TREATED, UNWASHED CYCAD FLOUR FOR 5 DAYS

FIGURE 5

GROWTH OF RATS FED UNWASHED CYCAD FLOUR #623-6
STORED AT 5°C FOR VARYING INTERVALS
(5-DAY BIOASSAY)


5 Months
1 Day
10 Months
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
30.8
23.1
16.2
10.1
4.5
-0.2
-4.5
-8.9
-11.3
-14.9
1
2
3
4
5
Log (Weight Change in Grams + 40)
% Cycad Flour 623-6 Added to Basal Ration

lies between those shown. A fifth assay at Michigan State University laboratories, after 10 months storage (figure 5), indicated the toxicity of the flour was greater than in the initial assays at NIH. To study this apparent increase in toxicity of 1 lot of flour, the effect of different rations and different strains and age of rats on the bioassay was investigated.

Rations

Various commercial rations were fed as the basal diet. The very poor growth of control rats receiving 1 commercial ration suggested some dietary abnormality. Inquiry revealed that this ration[2] contained a coccidiostat[3] which apparently reduced the food intake and thereby the growth of the rats. Apart from this complication, 3 other grain rations used[4] had no effect on the bioassay.

Strain, Sex and Colony Variations

Body weight losses of Osborne-Mendel rats fed various levels of unwashed cycad flour indicated that this strain was slightly more susceptable to the toxicity than

---

[2]Chick Startena - Ralston Purina Co., St. Louis, Missouri.

[3]3,5-dinitro-o-toluamide, Zoalene - The Dow Chemical Company, Midland, Michigan.

[4]M-1 (see footnote 1), Rockland Mouse/Rat Diet, Tehlad, Inc., Monmouth, Ill., Nutritionally Adequate Natural Grain Pig Ration prepared locally, Michigan State University Pig Grower Ration #1.

were rats of the Sprague-Dawley strain. This was more noticeable in the female than in the male animals (table 2). In addition, male Osborne-Mendel rats obtained from the colony at NIH lost more body weight than did rats of the same strain purchased from a commercial outlet[5] (table 3). These differences however, were not statistically significant ($P > 0.05$).

Age of Animals

Newborn rats appeared to be much more susceptable to cycasin toxicity than weanling animals. In 1 assay, when newborn pups were permitted to suckle a dam fed a natural grain ration containing 400 ppm cycasin, all pups were dead by the fifth day. However, when the same ration was fed to weanling rats, 50% of them survived for more than 7 months. In a third trial weanling animals 25 days old (75g) lost a greater percentage of their body weight than animals 37 days old (130g) when both groups were fed diets containing 2, 3, or 4% unwashed cycad flour (table 4). Although these differences were not statistically important ($P > 0.05$), the 3 trials showed a similar trend, indicating that younger animals are more susceptible to cycad toxicity than are older ones.

---

[5]Osborne-Mendel rats purchased from Camm Research Institute, Wayne, New Jersey. Sprague-Dawley rats purchased from Spartan Research Animals, Inc., Haslett, Michigan.

Table 2. Average weight changes of rats of different strains fed unwashed cycad flour #623-6. Five animals per group, average for 5 days.[a]

Strain		Diet	Male	Female
Osborne-Mendel	Weight gain (g)	Control	29.6	24.2
		2% cycad	11.2	6.4
	Weight decrease (% of Control)		62.2	73.6
Sprague-Dawley	Weight gain (g)	Control	34.6	26.4
		2% cycad	14.2	9.4
	Weight decrease (% of Control)		59.0	64.4

[a]Levels of cycad fed were significantly different at $P < 0.01$; comparisons of sex and strain differences were not significant, $P > 0.05$.

Table 3. Weight changes (g) of Osborne-Mendel rats from 2 sources fed unwashed cycad flour.[a]

% Cycad in Diet	NIH Animals	Camm Research Institute Animals
0	31.1	32.4
1	b	29.5
2	- 0.1	4.0
3	b	- 6.7
4	-16.4	-12.3

[a]2 and 4% cycad were significantly different from each other and from control $P < 0.01$, differences in colonies were not significant, $P > 0.05$.

[b]These levels were not used for NIH Animals.

Table 4. Effect of age on weight changes (g) of young Osborne-Mendel rats fed unwashed cycad flour #623-6. Ten rats per group, average for 5 days.[a]

% Cycad in diet \ Age and weight at start of Expt.	25 Days (75 g)	37 Days (130 g)
0	28.2	32.4
2	2.0	4.0
3	- 6.2	- 6.7
4	-10.3	-12.3

[a]All levels of cycad were significantly different from controls $P < 0.01$, 3 and 4% cycad were different from 2% cycad $P < 0.01$, responses due to age were not significantly different, $P > 0.05$.

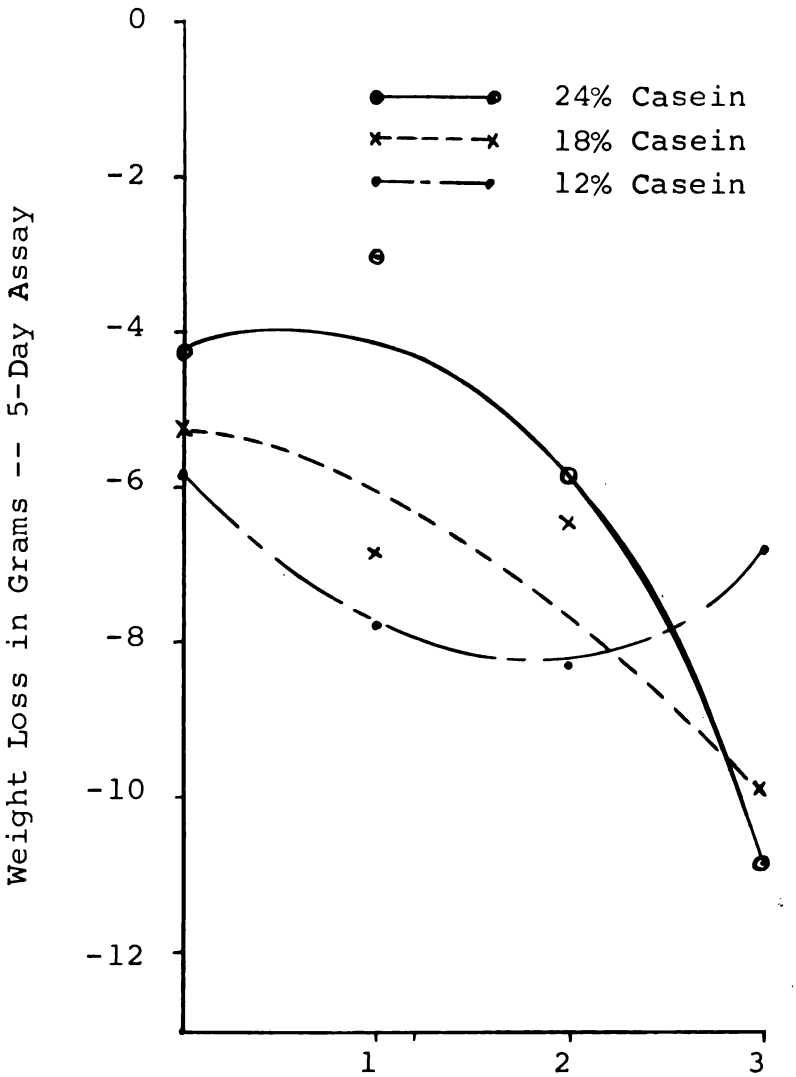
Dietary Effects

Proteins provide the amino acids essential for cell regeneration and they are generally regarded as being potentially able to ameliorate the effects of toxic substances, especially those effecting the liver (22). Since one of the primary effects of cycad ingestion is centrolobular liver necrosis, rations containing different levels of casein were studied for their effect on cycad toxicity. Zero, 1, 2 or 3% unwashed cycad was incorporated into 3 purified rations containing either 12, 18 or 24% casein.[6] These 12 diets were fed to groups of 5 weanling Sprague-Dawley rats for 5 days. All animals were fed the amount of feed eaten by the rats receiving the ration containing 3% cycad flour and 12% casein. Because the food intake was restricted, all animals lost body weight. The degree of weight depression, in the animals fed the various diets, showed the 24% casein diet reduced the toxic response in rats when this diet contained up to 2% unwashed cycad flour ($P < 0.01$). The reverse was true when the dietary levels exceeded 18% casein and 2% cycad ($P < 0.01$). Diets containing 12% casein seemed to prevent an increase in the toxic response when the intake of cycad flour was increased (figure 6). A 2-way analysis of

[6]The source of casein was High Protein casein, General Biochemicals, Chagrin Falls, Ohio. Each diet contained in %: 4, Wesson Salt Mixture, a modification of Osborne-Mendel Salt Mixture, Nutritional Biochemicals Corp., Cleveland, Ohio; 2.2, vitamin supplement, Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corp., 5, corn oil and sucrose to make 100.

FIGURE 6

5-DAY BODY WEIGHT CHANGES OF RATS FED UNWASHED CYCAD FLOUR IN DIETS VARYING IN CASEIN CONTENT



Percent Cycad	Percent Casein		
	12	18	24
0	1.6	1.4	1.4
1	0.8	2.0	1.0
2	1.2	1.2	1.0
3	0.8	1.0	0.6

variance indicated that regardless of the level of protein (casein) in the diets, the weight depression caused by the intake of 3% cycad was significantly different from that caused by 0 or 1% cycad ($P < 0.01$; data broken down according to levels of casein are not shown, but can be, calculated from figure 6).

Methionine, because of its lipotropic activity and sulphydryl group has been considered the effective principle in the beneficial role of high protein diets in modifying experimental hepatic toxicities (22). Preliminary investigation with groups of 3 rats indicated that the addition of 1.0 or 0.5% DL methionine, to a ration containing 2% unwashed cycad flour, exerted a beneficial effect on the growth of the animals but, a similar fortification of a 3% unwashed cycad diet with methionine had a negligible effect. In a later study, using groups of 10 weanling rats, fortification of a 2% unwashed cycad diet with 1% DL methionine[7] exaggerated the growth depression over the 3-week experimental period. Weight gains for animals fed 2% cycad averaged 124 g, those fed the 2% cycad fortified with methionine gained 110 g, and control animals 139 g. The mean weight changes calculated from these data are significantly different from each other ($P < 0.01$). Thus, methionine, added to a ration containing adequate protein, has no beneficial effect on the growth

[7]Nutritional Biochemicals Corp., Cleveland, Ohio.

response of rats receiving cycad and at a level of 1% may actually accentuate the toxicity. No attempt was made to establish if this level of methionine was toxic per se. Our interest was in whether added methionine would reduce the toxic response of animals fed cycad.

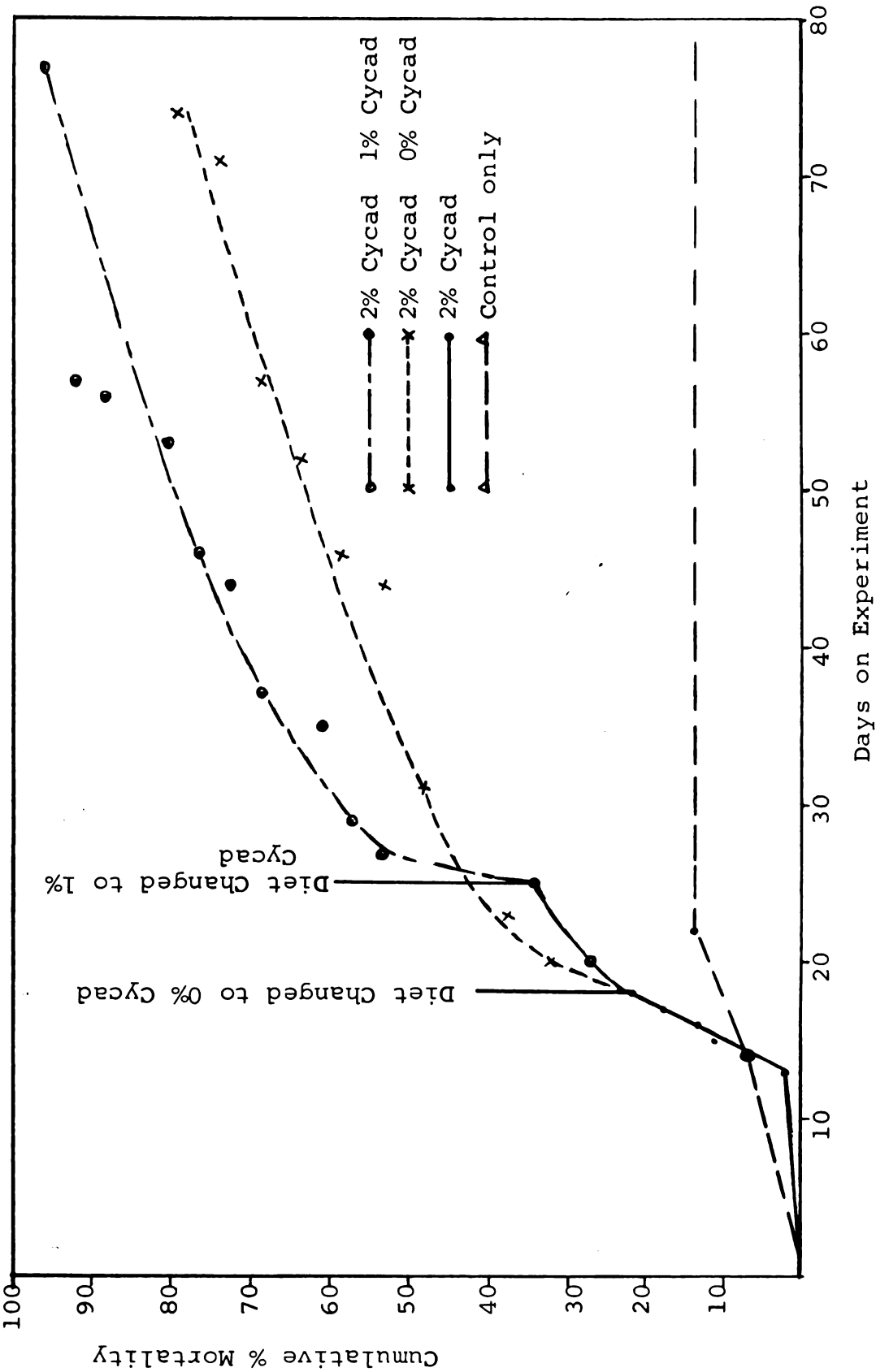
Irreversibility of Toxic Response

Our next investigation was aimed at determining whether the lesions induced by the intake of unwashed cycad were permanent and progressive or reversible. For this purpose 2 groups of male, weanling, Osborne-Mendel rats were fed diets containing 2% unwashed cycad flour. At the end of 18 and 25 days the cycad content of the diet of each group was reduced to 0 (19 animals) or 1% (26 animals) respectively. Dietary changes were made at these times because the animals were rapidly losing body weight. It was reasoned that if this weight response was reversed when the diet was changed, there would be no doubt as to the body's ability to overcome the toxicity. The animals showed a slight weight gain after the dietary change but the mortality of both groups indicated the damage at this stage was permanent. The group fed the 1% cycad ration for the latter part of the experiment had 96% mortality while those fed the cycad-free ration for this period had 79. A third group fed the control ration throughout the study had only 13% mortality (figure 7). These results indicate the irreversibility of the damage initiated

FIGURE 7

MORTALITY OF RATS FED 2% UNWASHED CYCAD FLOUR

FOR 18 OR 25 DAYS AND OR 1% THEREAFTER


Cumulative % Mortality
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
Days on Experiment
2% Cycad 1% Cycad
2% Cycad 0% Cycad
2% Cycad
Control only
Diet Changed to 1% Cycad
Diet Changed to 0% Cycad

in rats fed unwashed cycad flour for as short a time as 18 days.

Absence of Cycad Toxic Factor in Liver

Since the liver plays an important role in the detoxification of harmful substances and is one of the primary targets of cycad toxicity, the possibility that this organ accumulates the toxic substance was investigated. The liver was removed from a heifer that had been fed a calf starter ration containing 2% unwashed cycad flour. After 43 days of feeding, the heifer had gained very little weight and showed symptoms suggestive of neurological disturbances (occasional difficulty in walking and standing). The heifer was sacrificed at this time because it appeared close to death. The liver was freeze-dried and ground to a fine powder. Groups of 10 weanling rats were fed this dried liver or liver from a normal heifer at levels of 2, 5, or 10% of their basal grain ration. Weight gains over a 5-day assay period were similar for all groups. Average gains (in g) for animals fed the liver from the cycad-fed heifer vs. those for the animals fed the liver from a control heifer were respectively 31.8 vs. 32.3 (2%), 32.9 vs. 24.9 (5%), and 28.4 vs. 30.2 (10%). These data suggest that the compound responsible for the toxicity of cycad was not stored in the livers of the animals fed the unwashed cycad.

Discussion

The reliance on the growth bioassay in estimating the toxicity of cycads under various conditions stems from a fairly good correlation between growth rate and the degree of liver lesions (15). Although animal growth followed the food consumption pattern, records indicated the experimental animals consumed near normal amounts of food during the first day but markedly less from the second day onward (unpublished data). Liver changes were recognizable as early as 24 hours after cycad feeding was initiated (15). Food consumption merely reflected this rapidly developing metabolic disturbance.

Bioassays have shown that the toxicity of the cycad nuts is related to their stage of maturity. The green nuts are more toxic than the riper or brown-colored ones. There is evidence that this toxicity is attributable to the glycoside, i.e., cycasin content of the nut (unpublished data). Comparative assays suggest that the flour prepared from the green cycad nuts contains as much as 2 to 4% cycasin. Whether all the toxicity is traceable to the glycoside has been questioned by Matsumoto and Strong (12) who secured evidence for the presence of the free aglycone in Cycas circinalis. The aglycone, methylazoxymethanol, is a water-soluble compound released from cycasin by the action of a β-glucosidase. According to Nishida et al (7), it is the aglycone

which is responsible for the toxicity of the cycad plants. The presence of β-glucosidases in the intestine of the rat and their absence in liver and other tissues has been proposed by Nishida et al (7) as the explanation for the observation that cycasin is toxic only when given orally. Injection of this compound over short periods of time has been reported to produce no symptoms of poisoning (7). The absence of a toxic response in germfree animals fed cycasin and the near total recovery of the ingested compound in the urine and feces of these animals confirm the suggestion that bacteria bring about the cleavage of cycasin (15).

The toxic material in the dry unwashed cycad flour loses approximately half of its toxicity when stored for 6 months at temperatures near -20°C. The cycad flour appears to contain enzymes able to destroy the toxic compound (figure 3). The stability of the cycad material during storage probably depends upon the activity of these enzymes.

The fact that the rate of destruction of the toxic factor, as measured by differences in weight gain shows a logarithemically linear progression with time suggests a first-order reaction (figure 3). Despite the implication that the washing process carried out on Guam involves simply a leaching of the toxic factor, it is likely that an enzymatic or chemical degradation of the toxic factor occurs. Temperatures on the island, ranging from 20 to 32°C (23), would favor enzymatic reactions. In addition, the physical

barrier to water penetration, (these slices are approximately 1 cm thick and show little sign of water uptake) argues against the leaching process as being effective in the removal of the toxicity. The foam reported on the surface of the "washing" vat suggests the compound undergoes some degradation involving the loss of a gas, possibly nitrogen, when the nuts are kept in water. The toxic factor is labile to both moist and dry heat. Our observations suggest that dry heat is slightly more effective in destroying the toxicity than is moist heat.

Protein is generally regarded as being protective against liver injury (22) and high dietary intakes of protein are known to modify the effects of some toxic chemicals (24, 25). Such was the case in our experience when the diets contained less than 2% unwashed cycad flour but at levels above 2% this beneficial effect was not evident. In this case, a 12% casein diet, although well below the range recommended for growing rats (26), exerted a slight beneficial effect on the growth depression resulting from cycad ingestion.

Methionine is beneficial in the treatment of some experimental hepatic injuries especially when there has been protein depletion (22). However, the growth depression of rats increased when 1% DL methionine was added to experimental diets containing cycad. Harper (27) reports a methionine toxicity in rats fed 2.5% DL methionine in both adequate and inadequate protein diets. Our data would indicate a

synergistic action of the cycad toxic factor and added methionine.

Variations in the response of animals of different strains and colonies are not unexpected. Elevenfold differences in resistance to thiourea were reported (28) when rats of the same strain but from different colonies were administered this chemical. Greater sensitivity or resistance in animals of 1 sex for some drugs and toxins undoubtedly results from metabolic and hormonal differences (25). However, in the present study, the toxicity of cycad did not vary significantly in rats of different sexes, strains or colonies.

The rapid turnover of body tissue and cellular components may account for the increased sensitivity of younger animals to the toxicity of cycad. In some drug and chemical intoxications resistance is better in younger animals (28) but these observations are the exception rather than the rule.

The liver cells reacted almost immediately to unwashed cycad flour--changes appear as early as 24 hours after ingestion (15). The cumulative response caused by the ingestion of the flour for 18 days resulted in damage that was irreversible. Feeding trials indicated however, that the toxic compound is not stored in the liver of animals fed cycad. Thus, the question arises whether after the initial liver damage takes place, detoxification of the toxic compound occurs in the liver or at a different site.

All of these observations can be of practical value when considered in terms of the consumption of cycad foods by people. Experiments are underway to study the nutritional safety of these foods as they are eaten by Guamanians. Results of these trials should be considered in relation to the response and stability reported in this paper. Our observations raise a number of questions as to the implications of cycad consumption by humans. A considerable amount of additional information is required before definitive answers can be given as to whether cycad consumption by humans should be discouraged. There is an urgent need to determine how much cycad is consumed by humans in those parts of the world where the plant is indigenous and to obtain some indication as to the age groups who use it. It would also be desirable to carry out an epidemiological study of the incidence of various neoplastic diseases among these same people.

Literature Cited

1. Missouri Botanical Garden Bull. 1955. 43:65.

2. Thieret, J. W. 1958. Economic Botany of the Cycads. Economic Botany, 12:3.

3. Whiting, M. G. 1963. The Toxicity of Cycad. Economic Botany, 17:271.

4. Hiroyski, T. 1956. Cycad Poisoning on Island of Miyako. Okinawa Times, Dec. 13.

5. Seddon, H. R., Belschner, H. G., King, R. O. C. 1931. Poisoning of Sheep by the Seeds of Burrawang (Macrozamia Spirilis). Vet. Res. Report #6, June, Dept. Agr. New South Wales, Sydney, Australia.

6. Hall, W. T. K. 1956. A Note on Macrozamia and Xanthorrhoea Poisoning of Cattle. Australia Vet. J., July :173.

7. Cecil, R. L., Loeb, R. F. 1955. A Textbook of Medicine. W. B. Saunders Co. Philadelphia, p. 1459.

8. Seifter, S., Dayton, S., Novic, B., Muntwyler, E. 1950. The Estimation of Glycogen with the Anthrone Reagent. Arch. Biochem. 25:191.

9. Watt, B. K., Merrill, A. L. 1963. Composition of Foods. Agr. Handbook No. 8. U. S. Dept. Agr. Washington, D. C.

10. Cooper, J. M. 1941. Isolation of A Toxic Principle from the Seeds of Macrozamia spirilis. J. Proc. Royal Soc., New South Wales, 74:450.

11. Nishida, K., Kobayashi, A., Nagahama, T. 1955. Cycasin, A New Toxic Glycoside of Cycas Revoluta Thunb. Bull. Agr. Chem. Soc. Japan, 19:77.

12. Riggs, N. V. 1956. Glycosylazoxymethane, A Constituent of the Seeds of Cycas Circinalis L. Chem. and Industry, 35:926.

13. Matsumoto, H., Strong, F. M. 1963. The Occurrance of of Methylazoxymethanol in Cycas Circinalis L. Arch. Biochem., 101:299.

14. Nishida, K., Kobayashi, A., Nagahama, T., Kojima, K., Yamane, M. 1956. Cycasin, A New Toxic Glycoside of Cycas Revoluta Thunb: IV Pharmacological Study of Cycasin. (in Japanese) Seikagaku, 28:218.

15. Laqueur, G. L. 1964. Carcinogenic Effects of Cycads and Cycasin in Rats. Fed. Proc. in the press.

16. Laqueur, G. L., Mickelsen, O., Whiting, M. G., Kurland, L. T. 1963. Carcinogenic Properties of Nuts from Cycas Circinalis L. Indigenous to Guam. J. Natl. Cancer Inst., 31:919.

17. The ANRC Reference Chick Diet. 1956. Feed Age, May.

18. Mantel, N. 1951. Rapid Estimation of Standard Errors of Means for Small Samples. The American Statistician, Oct. :26.

19. Dixon, W. J., Massey, F. J. 1957. Introduction to Statistical Analysis. McGraw-Hill Book Company, Inc. New York.

20. Duncan, D. B. 1955. Multiple Range and Multiple F Tests. Biometrics, 11:1.

21. Whiting, M. G. 1963. (personal communication) NIH Bethesda, Md.

22. Popper, H., Schaffner, F. 1957. Liver: Structure and Function. McGraw-Hill Book Company, Inc. New York, p. 500.

23. Encyclopedia Britannica, Vol. 10, 1964. Encyclopedia Britannica, Inc. William Benton, Publisher, Chicago.

24. Goldschmidt, S., Vars, H. M., Ravdin, I. S. 1939. The Influence of the Food-stuffs upon the Susceptibility of the Liver to Injury by Chloroform and the Probable Mechanism of Their Action. J. Clin. Invest., 18:277.

25. Gyorgy, P., Seifter, J., Tomarelli, R. M., Goldblatt, H. 1946. Influence of Dietary Factors and Sex on the Toxicity of Carbon Tetrachloride in Rats. J. Exp. Med., 83:449.

26. Nutrient Requirements of Laboratory Animals. National Academy of Sciences--Natl. Res. Council. Publication #990.

27. Harper, A. E. 1958. Balance and Imbalance of Amino Acids. Ann. New York Academy Sci., 69:1025.

28. Farris, E. J., Griffiths, J. O. 1949. The Rat in Laboratory Investigation. J. B. Lippincott Co. Philadelphia, Chapter 13.

ADDITIONAL RESEARCH PROJECTS

Washed Cycad Flour

Introduction

Our interest in the toxicity of cycad centered around its potential harm as a food. The quantitation of the daily consumption of cycad, in normal use is difficult. However, cycads are known to be of considerable importance as emergency foods. We became particularly interested in the cycad prepared for human consumption in areas in which the plant is indigenous. This cycad has been washed, dried and milled (see page 14 of manuscript). Washed nut slices were purchased from local markets in Guam, mailed to us and ground to flour in our laboratories. In all, 14 shipments were received. These were obtained at various times of the year and represent many locales of Guam.

General Procedures

Our experimental work began in October of 1962 when 30 male and 30 female weanling rats of the Osborne-Mendel strain were fed diets containing this washed cycad flour. The animals were divided into 4 groups of 7 or 8 males and females. Three groups received 1.5, 5, or 10% washed cycad

flour in a nutritionally adequate commercial chick starter ration[8] while the fourth group received basal diet only. All animals were housed individually--feed and water were given ad libitum. Weights were recorded at the beginning of the experiment, after 5 days, and thereafter at weekly intervals.

Volitional Activity

Before the end of the first month of feeding, 1 animal of the group receiving 10% washed cycad flour showed incoordination of the hind limbs. Because of the earlier suspected role of cycad in the etiology of amyotrophic lateral sclerosis, the animals' spontaneous activity was studied. The male animals were subjected to a week of training in revolving drum cages. The number of revolutions produced by the activity of each rat was recorded every day for a period of 2 weeks. The average number of revolutions differed for each group, but the differences were not correlated with the level of cycad fed. Female animals were not studied for volitional activity because of a suspected respiratory infection in the laboratory where the activity cages were located.

Respiratory Infections

After approximately 3 months, the animals lost a marked amount of body weight and remained at this reduced

---

[8]Purina Startena.

level for 2 months (figure 8). It was suggested that unseasonably low out-door temperatures affected the temperature in the animal laboratory and this in turn affected the animals. All female animals fed experimental rations lost significantly more weight during this period of suspected respiratory epidemic than females fed control diets ($P < 0.01$). The differences in male animals were not significant, but the recovery of lost weight was slowest for those receiving the 10% washed cycad diet.

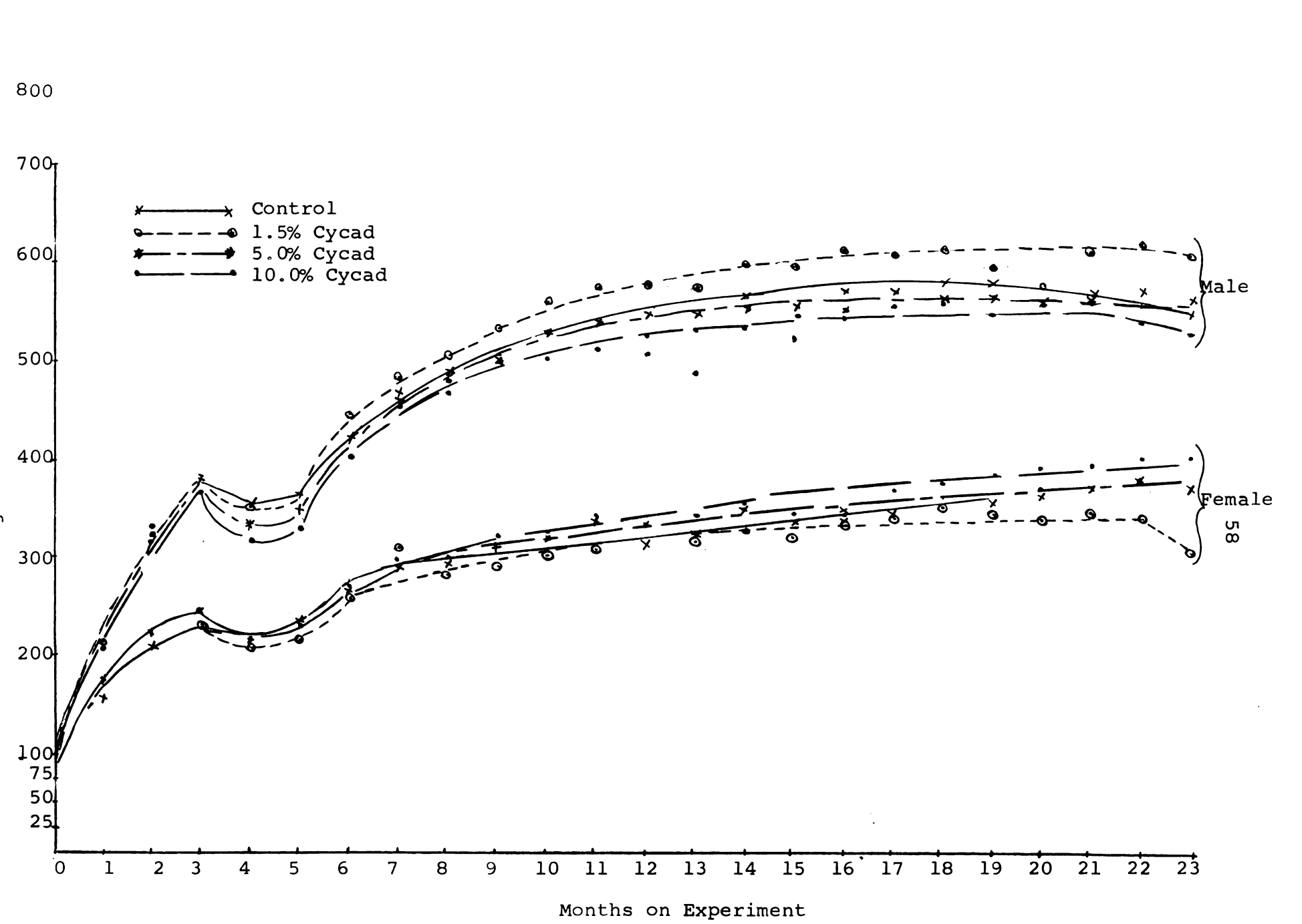
Reproductive Response

Males and females of the same diet group were placed together 4 months after the beginning of the experiment. We thought that the added stress of pregnancy might precipitate a toxic reaction to cycad. The animals did not mate although vaginal smears indicated a normal estrus cycle. To overcome possible infections which may have prevented conception, achromycin was fed at the level of 2 mg/gram of diet for 1 week and 3.5 mg/gram of diet for an additional week. There was no noticeable beneficial effect from the antibiotic therapy. Subsequently, a 5% supplement of ground liver powder, fed for 1 week, or a complete substitution of the grain ration by McCollum's Lactating diet in the control group did not cause any appreciable growth response.

In a separate study, it was observed that animals grew poorly when fed the commercial chick starter ration,

FIGURE 8

GROWTH OF ANIMALS FED WASHED CYCAD FLOUR


800
700
600
500
400
300
200
100
75
50
25
Control
1.5% Cycad
5.0% Cycad
10.0% Cycad
Male
Female
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Months on Experiment

but growth was normal when the rats were fed any one of 3 other nutritionally adequate diets. The commercial chick ration sold during the winter months contained a coccidiostat which affects the food intake of rats and undoubtedly explains the poor growth of our animals. Thereafter, a natural grain ration prepared on campus to our specifications[9] was used instead of the chick starter.

Approximately 2 weeks after the final change in ration, the first litter was born to a control animal; therefore, conception probably occurred shortly after this animal was started on McCollum's Lactating diet, i.e., 4 weeks prior to parturition. Seventy-five to eighty-five percent of the mated pairs had litters, some born as late as 4 months after the males and females were placed together. Because of the dietary complications, the results of the reproductive study were not conclusive.

Mortality of newborn pups was highest in the control group. Since these litters were born earliest, the poor nutritional condition of the mother due to the previous feeding regime could possibly explain the poor survival. Those that did survive to weaning were maintained on the same ration as their parents. Weekly weights were recorded for 3 weeks post weaning. Their growth was normal.

The adult animals were continued on the study for 23 months. Occasional infections were treated with achromycin.

---

[9] see footnote 1.

Male and female animals fed control ration or 10% washed cycad rations were again paired, within the same diet group, 14 months after the beginning of the study. Only a small number of the pairs actually mated. This was probably due to the onset of menopause which is reported to occur in the rat at 450 days of age. Animals born at this time are being kept to 9 months of age. Weights are recorded weekly. The tissues will be prepared for pathological examination at the termination of the study.

Tissue Preparation

Animals that died or appeared moribund were autopsied and the tissue prepared for the pathologist. Autopsy included removal and preparation, after fixation in formalin, of the liver, kidneys, adrenals, spleen, pancreas, complete G.I. tract, urogenital tract, heart, lungs, trachea including thyroid and parathyroid, esophagus, salivary glands, brain, spinal cord and sternum. After macroscopic lesions were recorded, all tissues were sent to the National Institutes of Health (NIH) for microscopic examination. Grossly visible lesions were photographed and arbitrarily scored as to severity. At 23 months all surviving animals were autopsied. The pathologist's findings are not yet completed.

Unwashed Cycad Flour

Introduction

Extensive work at NIH showed that the cycad nut meat not exposed to the washing procedure was acutely toxic to rats when 5% was incorporated into their basal diet. Similar trials at our laboratory caused death in as short a time as 4 days. The NIH group was able to produce in different species of animals by long term feeding trials a very high incidence of liver and kidney neoplasms. Because of these dramatic responses unwashed cycad flour has been used in the majority of our investigations.

Nuts harvested in Guam were mailed to NIH for processing (described on page 17 of manuscript). The nuts were processed under carefully controlled conditions so that their potential toxicity would not be changed. The processed material was refrigerated at 5°C immediately after delivery to our laboratory and was kept at this temperature until the time of feeding.

Stability of Toxic Factor and Variations in Biological Response

The unwashed cycad flour was used in 11 short term bioassays. These studies were designed to determine the relative toxicity of various lots of unwashed cycad flour and the changes in toxicity that may result from storing,

heating or cooking of the cycad flour. The effect of the level of dietary protein or dietary methionine on the biological response was also studied. The majority of the bioassays were of 5 days duration. A total of 485 Sprague-Dawley and Osborne-Mendel weanling rats were used. Table 5 summarizes this aspect of the cycad investigation. Many of these assays are reported in the accompanying manuscript.

Comparison of Toxic Response of Cycasin and Unwashed Cycad Flour

Cycasin (see page 16 of manuscript) is regarded as the main, if not the sole, toxic component of the cycad nut and has been isolated in crystalline form by a group at Kagoshima University in Japan. Some of this crystalline material was made available to us for feeding trials. The primary purpose of these trials was to compare the toxic response caused by chronic ingestion of cycasin and unwashed cycad flour.

Groups of 20 males and 20 female Sprague-Dawley weanling rats were fed either control ration, 1.0 or 1.5% unwashed cycad flour or 400 or 600 ppm cycasin in the natural grain basal diet. In addition, groups of 10 male animals were fed 2, 3, or 4% unwashed cycad flour. All were housed individually. Feed and water were supplied in unlimited quantity. Body weights were recorded on the first and fifth day of the study and weekly until its termination. On the fifth day, those animals fed 2, 3, or 4% unwashed cycad flour

Table 5. Summarizing table of short term bioassays using unwashed cycad flour.

Expt. No.		No. of Rats/ Diet Group	Levels of Cycad (%)	Length of Assay (Days)
1	Purpose: To determine the effect of storage on the toxicity of cycad flour. Conclusion: Body weight changes suggested an increase in toxicity of cycad flour stored at 5°C for 10 months or possibly a difference in the response of rats secured from commercial dealers vs. those from NIH where the initial study was done.	10	0, 2.5, 7.5	5
2	Purpose: To verify results obtained in Expt. 1. Conclusion: Again, body weight changes indicated an increased toxic response.	10	0, 1, 2, 3, 4	5
3	Purpose: To determine if the commercial chick starter in current use was responsible for a period of poor growth. Conclusion: All animals fed this ration with or without cycad grew less than is expected for animals of their age, suggesting dietary complication.	10	0, 2, 4	5

Table 5. Continued

Expt. No.		No. of Rats/ Diet Group	Levels of Cycad (%)	Length of Assay (Days)
4	Purpose: To check the possibility that pig grower ration contained a factor protective against cycad toxicity. Conclusion: Weight changes indicated an almost negligible beneficial response when this basal ration was compared to other basal diets.	10	0, 2, 4	5
5	Purpose: To observe the response to cycad toxicity in rats of the same strain but from a different colony than that where previously obtained. Conclusion: Body weight changes indicated that there was a difference in response. Animals from the NIH colony were more susceptible to the toxicity of cycad than were animals of the same strain from a commercial outlet.	10	0, 2, 4	5
6	Purpose: To study the effect of the dietary protein level on the animal's response to cycad toxicity. Conclusion: The growth depression on a logarithmic scale for animals fed a 2% cycad ration was of the same magnitude whether the diet contained 12 or 24% casein. This suggested	5	0, 2, (12% casein) 0, 2, (24% casein)	5 & 14

Table 5. Continued

Expt. No.			No. of Rats/ Diet Group	Levels of Cycad (%)	Length of Assay (Days)
		that the protein content of the diet was not an influencing factor in the biological response to cycad.			
7	Purpose:	To compare the relative toxicity of 3 lots of unwashed cycad flour.	10	0, 2, 3, 4 (lot a) 2, 3, 4 (lot b) 2, 3, 4 (lot c)	5
	Conclusion:	Body weight changes indicated that the 3 lots of flour were similar in their ability to depress growth.			
8	Purpose:	To establish the relative toxicity of 1 lot of unwashed cycad flour.	10	0, 2, 3, 4	5
	Conclusion:	Body weight changes and pathological changes in the liver indicated this flour was similar in its ability to cause a toxic response as were previously assayed flours.			
9	Purpose:	To study the effect of moist or dry heat on the stability of the toxic factor.	10	0, 2, 3, 4 (dried) 2, 3, 4 (cooked & dried)	5
	Conclusion:	Both moist and dry heat caused nearly a complete destruction of the toxicity. The latter treatment was slightly more effective than the former.			

Table 5. Continued

Expt. No.			No. of Rats/ Diet Group	Levels of Cycad (%)	Length of Assay (Days)
10	Purpose:	To study the effect on the growth response of adding methionine to a diet containing cycad.	10	0, 2, 2 + 1% methionine	21
	Conclusion:	1% DL Methionine added to a diet containing 2% unwashed cycad flour exaggerated the growth depression.			
11	Purpose:	To study the effect of storage temperatures on the stability of the toxic factor in cycad stored for 7 months.	5	0, 2, 3, 4 (stored at -20°C)	5
	Conclusion:	In both cases the growth depression indicated the flour had lost approximately 1/2 of its toxicity. The toxic factor was slightly more stable at -20°C than at 5°C.		2, 3, 4 (stored at 5°C)	

were autopsied. The livers were fixed and prepared for pathological examination. Hemoglobin concentrations determined by the Drabkin method (J. Biol. Chem. 98:719 (1932)) were within the normal range although animals receiving the highest levels of cycad flour (4%) had the highest average values. At the same time, electrophoretic analysis of serum proteins was done in triplicate. Essentially no differences were observed in the 3 groups. The animals that were not autopsied were kept on their respective diets. After 2 weeks, the growth depression of animals fed the cycasin rations indicated the animals would probably not survive for an extended length of time. In order to aid in their recovery, these animals were first fed a control ration for 1 week after which the initial concentrations of cycasin were reduced from 600 and 400 to 400 and 200 ppm respectively. The animals were fed the lower levels of cycasin for 8 months. Finally, all animals were fed control ration for 2 months and then autopsied. Animals that died or appeared moribund during the study were autopsied according to the procedure described earlier.

Workers at NIH observed edema in the connective tissue of the digestive organs and the epicardium of calves fed cycad. Collagen is an important intercellular constituent of connective tissue and is distinguishable from most other proteins by its high content of hydroxyproline. To determine if changes similar to those seen in the calves

occurred in the rats, 3-day urine samples were collected and analyzed for hydroxyproline by the method of Prockop and Udenfriend[3] (Anal. Biochem. 1:228 (1960)). All animals fed experimental rations excreted amounts of hydroxyproline comparable to the controls suggesting that collagen metabolism, as measured by this test, was not influenced by the ingestion of cycad or cycasin.

Feeding Pregnant Rats

One member of our group found, in repeated studies, that when cycad was fed to pregnant rats the newborn pups had lesions characteristic of those caused by the direct ingestion of cycad. Also, when the cycad-containing feeds were ingested by dams after parturition only, the suckling animals developed these lesions even though they did not, at any time, have access to their mothers' rations. This work proved that the toxic factor of cycad was transmitted through the placenta and mammary gland.

Because the aglycone of cycasin is similar in chemical structure to several known carcinogens, it may follow a metabolic pathway common to these carcinogens. Three chemicals were used for comparison with cycasin. They were incorporated into the natural grain diets of pregnant rats at levels slightly higher than cited (J. Path. Bact. L:393), as being toxic to weanling rats. Pathological lesions in

---

[3] determination carried out by M. G. Yang, Ph.D.

pups exposed to 200 ppm dimethylnitrosamine, diethylnitrosamine, 400 ppm cycasin or 1000 ppm dimethylaminoazobenzene indicated that all these chemicals were transmitted across the placenta.

In another study, 25 dams were fed these 4 chemicals post-partum. Two pups from each litter, that was suckling the dams, were killed at weekly intervals. These suckling pups were also affected by the toxicity even though their exposure was through the mother's milk only. Body weights of the pups were recorded at birth and weekly thereafter. Physical development was compared to the expected progress for normal pups of corresponding ages. The mortality was high in those litters wherein their dams were exposed to levels in excess of 100 ppm dimethylnitrosamine, 150 ppm diethylnitrosamine, 500 ppm dimethylaminoazobenzene or 300 ppm cycasin. Dams fed these levels or above, produced young with lesions in several body organs. Those that survived to weaning are being fed a natural grain ration, free of the chemicals, until death or 6 months of age. The growth of those that survived the toxicity is comparable to the controls, except for the animals exposed to 100 ppm dimethylnitrosamine. These weighed significantly less at weaning and throughout life than animals exposed to all other chemicals. It has not yet been ascertained whether the initial damage progresses or whether the body is able to regenerate the damaged tissue when the animals are changed to a control

ration post weaning. These 4 chemicals seem to share a common property in that they are transferred across the mammary gland in a form which is able to produce lesions comparable to those seen when the same compounds are given directly by mouth.

Feeding Weanling Rats

Weanling Sprague-Dawley rats in groups of 8 were fed diets containing 125 ppm dimethylnitrosamine or diethylnitrosamine or 1000 ppm dimethylaminoazobenzene. Their response was compared with controls, and with that obtained in the study already described, wherein the animals were fed cycad or cycasin. One animal from each experimental group was killed on the first, second and fourth week of the study. All other animals were kept for 6 months or until death. Those that died, appeared moribund in the interim, or survived until the sixth month were autopsied. Microscopically visible lesions developed in some animals from all groups fed experimental rations. A more detailed pathologic report is to be included in the doctoral dissertation of the pathologist in our group.

Cycad Husks

Introduction

In Guam, the natives chew the fresh husk of the cycad as the nuts are harvested from the trees. The husks are also dried and later chewed by the men as they work. Preliminary work at NIH indicated that the husks of the cycad were about 1/5 to 1/6 as toxic as the nut meat. Short term bioassays were the basis for this conclusion.

Further investigation into the toxicity of cycad husks began in July 1964 at our laboratory. A trial was started to compare the effects of chronic feeding of cycad husks with those obtained in the unwashed cycad flour feeding trial mentioned previously. Rations containing 6, 9, 12, 18 or 24% husks were prepared. These levels would correspond to the toxic potency of 1, 1.5, 2, 3 or 4% unwashed cycad flour assuming the reported toxic level is correct. Casein was added to make the husk diets isonitrogenous with the control. The husks were considered to contribute only negligible amounts of nitrogen to the diets. This study, originally planned for 1 year was terminated after 9 days because of high mortality in the experimental animals. Those living were anemic in appearance and cold. Gross examination at autopsy suggested gastrointestinal hemorrhage to be the immediate cause of death. In most animals, there was accumulation of fluid in the abdominal and thoracic

cavities. Liver, kidney and heart were prepared for pathological examination. High hematocrit and hemoglobin values determined by comparison with a commercial standard[4] suggested hemoconcentration. Both the blood plasma and the urine were markedly yellow. Positive reactions to Ictotest[5] indicated bilirubin was present in the urine. Blood smears were taken for differential count.

Effect of Environmental Temperature

The lowered body temperatures of the rats fed the husk diets may have caused a peripheral vasoconstriction and the apparent anemia. There were however, no differences in hematocrit and hemoglobin values of 5 animals fed cycad husks at room temperature (77-83°F) as compared to animals fed the same level of husks when the environmental temperature was raised to 88-90°F. Both groups of animals had elevated hematocrit and hemoglobin concentrations when compared to animals fed control rations under both conditions.

Blood Changes

As a follow-up to the previous study, 15 male Sprague-Dawley rats of various ages (1-6 months) were fed

---

[4]Hycel Cyanmethemoglobin Standard, Hycel, Inc.

[5]Ames Laboratories.

the diet containing 24% cycad husks. Blood samples were taken by heart puncture after 0, 1, 2, 5, 7 and 9 days of feeding. Average hemoglobin and hematocrit values were significantly higher on the first day of feeding than at 0 time. These values remained elevated to the end of the experiment. Since there was a possibility that the hematocrit and hemoglobin concentrations observed in this study may have been influenced by a lowered food consumption after the first day (0-5 g/rat/day), another study was initiated. Six rats were fed the 24% husk diet and another 6 pair-fed a control diet. The results of this study confirmed the findings of the previous trial. The hematocrit and hemoglobin concentrations obtained during 4 consecutive days of feeding were markedly elevated for the rats fed the husks but only slightly increased for the pair-fed controls (figure 9). The elevated values of the experimental animals suggested hemoconcentration and/or decreased plasma volume. Attempts to measure blood plasma volumes of these animals were not successful.

Cooperative Studies with the Veterinary Pathology Department

Swine

Piglets

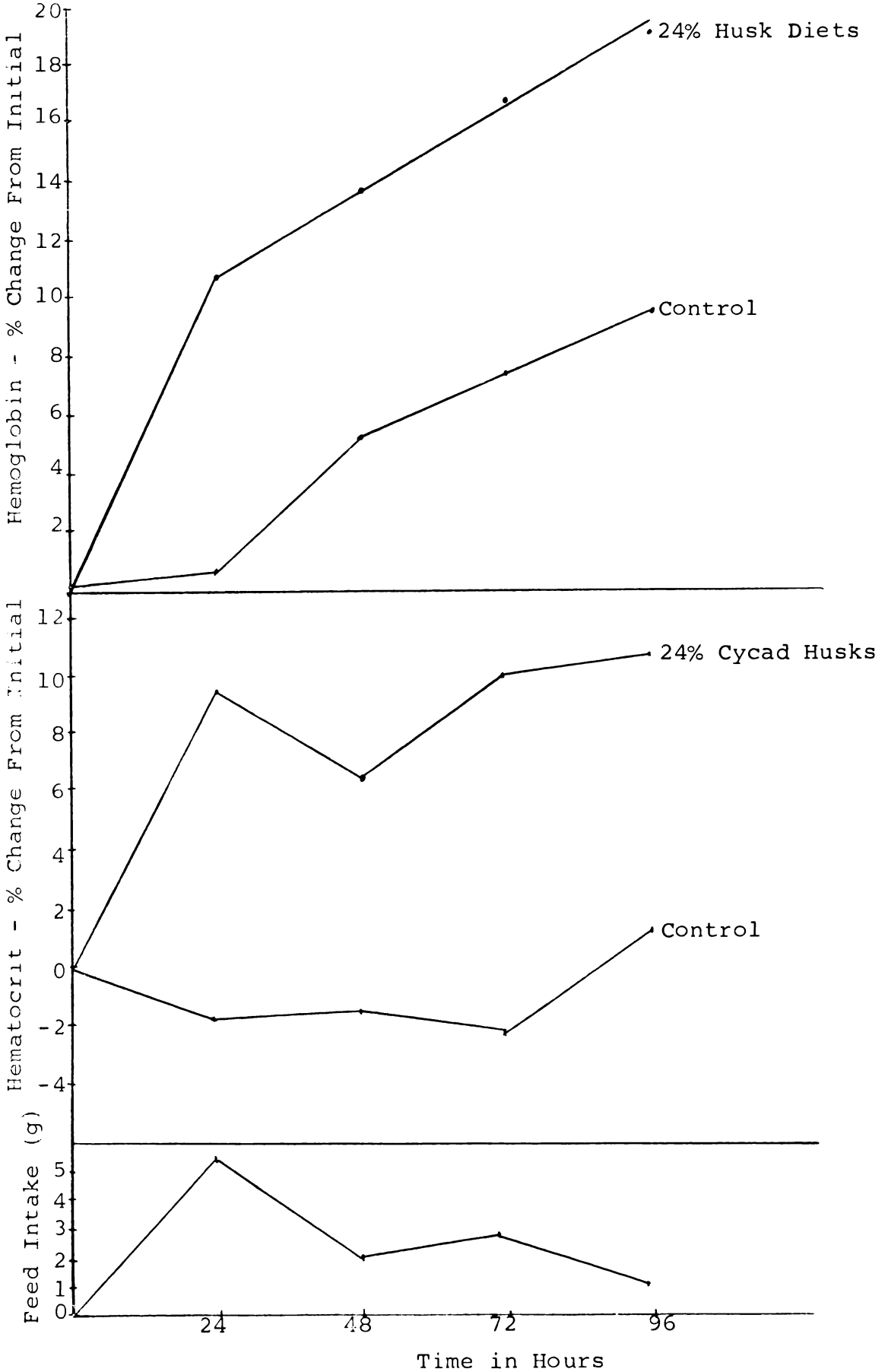
One male and 1 female pig weighing approximately 50 lb each, were fed a pig grower ration containing 2% unwashed

FIGURE 9

% CHANGE IN HEMOGLOBIN AND HEMATOCRIT FROM 0 TIME

ANIMALS FED 24% CYCAD HUSK DIET

OR CONTROL DIET (PAIR-FED)


Hemoglobin - % Change From Initial
20
18
16
14
12
10
8
6
4
2
24% Husk Diets
Control
Hematocrit - % Change From Initial
12
10
8
6
4
2
0
-2
-4
24% Cycad Husks
Control
Feed Intake (g)
5
4
3
2
1
0
24
48
72
96
Time in Hours

cycad flour. Two control animals were fed the grower ration alone. The 2 groups of pigs were housed in separate indoor pens at the Veterinary Pathology barns. Feed and water were provided ad libitum. The experimental animals ate poorly and gained weight slowly. One animal of each group was autopsied after 9 weeks, the remaining after 13 weeks. Both experimental animals were affected by the toxicity of the flour. Characteristic lesions in the liver, kidney, heart, pancreas and G.I. tract were observed. In addition, considerable fluid had accumulated in the abdominal, thoracic and pericardical cavities.

In another trial, 3 pigs, 2 weeks of age were fed various levels of cycad husks. Our purpose was to determine if a species other than the rat was susceptible to the toxicity of husks. Previously we had observed the most dramatic responses in younger animals so pre-weaned shoats were used in the trial. All animals were fed normal cow's milk previous to their receiving experimental rations. Cycad husks were first given in milk and later in a mash of grain, molasses and milk. One control animal received milk or mash without cycad. They ate poorly, developed diarrhea and vomited after the first day on experiment. One experimental animal was dead after 3 days; all including the control animals were dead by the ninth day. Consumption of husks varied from 3 grams for the animal that died on the third day to 15 grams for the one that was autopsied on the ninth

day. Gross pathological examination suggested that the animals died of starvation.

Suckling Shoats

Because the toxicity of cycad was found to pass through the mammary gland of the rat, we became interested in determining whether other species would respond similarly. A sow was fed a grain concentrate containing 2% unwashed cycad flour immediately after farrowing. Her 9 shoats were nursed for 8 weeks. Care was taken so that the young did not have access to the mother's feed. At the end of the suckling period 3 shoats and the sow were autopsied. The remaining 6 shoats were weaned to a control ration. Three of these were autopsied at the eighth and the remainder at the eleventh month. The lesions in the young, autopsied immediately after suckling were more severe than those in the mother. Animals killed after 8 or 11 months had marked or complete regeneration of damaged tissue.

Cow and Calf

In a similar study, a calf nursed while its mother received 0.1 g of cycad/kg of body weight for 10 weeks and 0.2 g/kg for an additional 9 weeks. The cow was fed a 50-50 mixture of oats and corn at the level of 1 lb/100 lb body weight/day. Both animals had free access to pasture grass. Routine analyses of blood taken from the cow and calf at

weekly intervals were carried out in the pathology department. Body weights were recorded weekly. Both animals were sacrificed after 19 weeks. The calf responded as did the suckling shoats; it suffered more severe tissue damage than did the cow.

An attempt was made to locate the fraction of cow's milk which contained the toxic factor resulting from cycad ingestion. A second cow, that had recently freshened, was fed a natural grain concentrate and pasture grass. The grain was supplemented with 0.02 lb of cycad flour/100 lb body weight/ day. Her calf nursed for short periods of time each day during the study. Most of the milk was taken for rat feeding trials. The calf served as a partial control for the transfer of the toxicity and kept milk production at a maximum. It was recognized that the calf might not show very pronounced lesions due to the fact that it was getting milk only sporatically. This is in contrast to the previous experiment where the calf received all of its mother's milk.

Two feeding trials of 8 or 6 weeks duration were carried out. In the first study, 52 young male rats (32 days old) were fed control milk or milk diets made of fractions prepared from the milk obtained from the "treated" cow. Centrifugation and acidification (and neutralization) were used to fractionate the milk. Each fraction was reconstituted with the missing fractions from control milk. "Treated" milk was also heated or stored before being fed.

All diets were supplemented with Fe, Cu, I_2 and Mn and then homogenized. Daily consumption and weekly weights were recorded. One animal from each of the 9 diet groups was autopsied after the second and sixth week. Blood samples for routine analyses were taken by heart puncture. Liver, pancreas, kidney and heart were fixed for pathological examination.

Sixteen day old rats were used in the second trial because of the observation that young animals were more susceptible to the toxicity of cycad. The 3 experimental diets used contained 90% "treated" skim milk or 10 or 20% "treated" cream in control fractions. These contained an estimated 40 and 60% fat (dry wt basis) respectively (AOAC Eighth Edition, 1955). The responses of animals fed these diets were compared to those of animals fed normal milk containing 10 or 20% cream. Daily milk consumption and weekly weights were recorded. Two animals from each of the 5 diet groups were autopsied at 1, 2 and 3 weeks after the beginning of the study. Blood samples and tissues taken then, and at the termination of the study at 6 weeks, were sent to the pathologist for examination. Both studies failed to establish with certainty the fraction in which the toxic factor was located.

The cow and her calf were autopsied after 5 months. Neither animal had pathological lesions at the time of autopsy.

Horses

Four horses (2-3 years old) were used in another experiment. Since neurological distrubances have been reported as a cardinal manifestation of cycad ingestion, it appeared desirable to check this species. Horses were used as the experimental animals because they are reported to be particularly susceptible to neurological disturbances.

Two of the horses were fed unwashed cycad flour as 2% of their daily food intake of oats (1/2 lb/100 lb body weight) for 15 weeks and as 4% for an additional 9 weeks. The other animals received oats without cycad. Routine blood analyses were performed each week. Body weights were recorded at the same time. Serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase were followed by the pathologist. One experimental and 1 control animal were autopsied after 24 weeks. A neuro-pathologist reported that the brain and spinal cord of both animals were normal. Lesions in the liver, pancreas, heart and kidney were characteristic of lesions seen in other species fed cycad. The remaining horses have been fed pasture grass, hay and oats free of cycad for an additional 9 months. The animals appear to be in good health. There are no clinical signs of any neurological disturbances.