SOME EFFECTS OF METHYLAZOXYMETHANOL ACETATE ON THE NEUROENDOCRINOLOGICAL AND NEUROLOGICAL SYSTEMS IN THE RAT

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ABSTRACT

SOME EFFECTS OF METHYLAZOXYMETHANOL ACETATE
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By

Yoram Malevski

Rats intoxicated with methylazoxymethanol acetate (MAM-Ac) remained sterile for approximately 10 days post injection. The sterility was associated with alterations in the ovarian, pituitary and hypothalamus metabolism.

Normally cycling Sprague-Dawley rats at metestrus were injected with 10 or 12 µl MAM-Ac or saline. The rats injected with saline were divided into 2 groups: one group was pair fed to the MAM-Ac injected rats and the second was a positive control group fed ad libitum.

As a general response to MAM-Ac intoxication the adrenals underwent hypertrophy associated with hemorrhages. Food intake and body weights were reduced sharply, from about 150 to about 35 g in the first week after injection. At the same time the rats lost approximately 18% of their body weight.

Estrous cycles were significantly prolonged ($p<0.001$) when compared to the control rats. The first cycle after the injection was prolonged from 4.5 days to 9.0-9.8 days

in the MAM-Ac injected rats whereas in the pair fed controls it only increased from 4.5 to 5.4 days. The cycles, however, became normal in length in about 14 days and reproductive performance became normal as evidenced by the number of pups at delivery and at weaning for the treated and control groups.

Ovarian, uterine and pituitary weights expressed as mg per 100 g of body weight were similar in MAM-Ac injected rats and in both control groups when they were categorized according to the stage of the estrous cycle in which the rats were sacrificed. However, when categorized according to the sequence of sacrifice after treatment, it was found that MAM-Ac injected rats had significantly lighter ($p<0.05$) pituitaries (expressed as mg per 100 g of body weight) 4 to 12 days post injection and lighter ovaries and uteruses 1 to 3 days after the injection.

Histological examination of the ovaries and uteruses of the MAM-Ac injected rats revealed that for approximately 8 days after the injections the ovaries were filled primarily with well developed corpora lutea and the uteruses had dense endometriums, narrow lumens and only few glands were present.

MAM-Ac injected rats sacrificed at diestrus had significantly less ($p<0.05$) blood LH and pituitary LH and prolactin than did the controls. Pituitary LH was lower

in the MAM-Ac injected rats sacrificed at proestrus, and estrus than in the controls. One to six days after the injection, MAM-Ac injected rats had significantly lower ($p<0.05$) blood LH than controls and normal levels of blood prolactin. Pituitary LH was lower in MAM-Ac injected rats 1 to 3 days and 8 to 16 days post injection and pituitary prolactin was lower only 4 to 6 days after the injection compared to controls. Prolactin inhibiting factor activity was similar in all groups and luteinizing hormone releasing factor activity was lower in MAM-Ac injected rats 1 to 6 days post injection, only when compared with the positive control.

Pair fed controls, although their food intake was reduced, had normal estrous cycles, normal organ weights and organ histology, and normal blood and pituitary LH and prolactin concentrations. Hypothalamic PIF and LHRF activities were normal as well. Positive controls, fed ad libitum, were found to be normal in all parameters examined.

The blood-brain barrier is not important in the prevention of neurological disorders in weanling and mature rats and mice since implantation with MAM-Ac or cycasin in the third ventricle of the brain by stereotaxic technique, did not cause neurological disorders or gross lesions in the brain.

No brain lesions or neurological disorders were found in rats injected with cycasin or MAM-Ac during their prenatal

life. No tumors were found approximately 6 months after cycasin treatments. However, about 2.5% of the MAM-Ac injected rats had tumors at this time. The surgical procedure used for the injections of MAM-Ac or cycasin directly in to the fetus was stressful, since only 30-41% of the mothers was successful in raising pups and only 8-20% of the pups were weaned.

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To my parents

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INTRODUCTION

Poisonous substances occur in a wide variety of plants, spread all over the world. These substances vary extremely in their chemical and physiological properties. So far many such substances were recognized, among these, the best known are: antienzyme and antivitamin factors, metal binding constituents, hepatotoxins, neurotoxins, hypoglycemic and hypothyroid agents, hemolysis inducing factors, stimulants, depressants and estrogenic factors (Liener, 1969).

Toxic factors are present in many nutritionally important plants. Legumes in particular have the ability to inhibit the proteolytic activity of certain enzymes (Liener and Kakade, 1969). Certain proteins extracted from kidney beans or soybeans, have the property to agglutinate red blood cells (Jaffe, 1969). Thiocyanate and thiourea were found to bear goitrogenic properties. Plants from the genus *Brassica* are especially rich in such substances (Van Ethen, 1969).

Some glucosides are very toxic to man and animals. Cyanogenic glucosides, amygdalin, dhurrin and linamarin, are mainly found in cassava, sweet potato, yam, maize, sugar

cane, peas, beans and the kernels of several fruits (Montgomery, 1969). Different glucosides were isolated from cycads. The active compounds were found to be cycasin and methylazoxymethanol (MAM) which cause cancer, neurological disturbances and other toxic effects (Yang and Mickelsen, 1969). Saponins are glucosides too. They have a bitter taste, cause foaming in aqueous solutions and hemolyze red blood cells. Soybeans, alfalfa and sugar beets are the most important plants where saponins are found (Birk, 1969).

Gossypols which are polyphenolic pigments, were isolated from cotton seeds. Besides their toxic properties they are of economic significance since they are known to cause dark discoloration of egg yolks (Beradi and Goldblatt, 1969). Toxic amino acids were isolated from the seeds of lathyrus species. When introduced to animals, they were found to cause neurological disorders (Sarma and Padmanaban, 1969). Hemolytic anemia following the ingestion of Fava beans or inhalation of its pollen was documented (Mager *et al.*, 1969).

Some toxic factors present in plants, are of mold origin. Toxic alimentary aleukia resulted from eating wheat infested with *Fusarium* species. Cancer of the liver is caused by aflatoxins--a group of mycotoxins produced by *Aspergillus flavus* on peanuts. Rice and corn are infested too by molds in particular *Penicillium* species (Friedman and Shibko, 1969).

The widening gap between world supply of proteins and the growth of population requires development of new protein resources as well as extension and better utilization of the existing resources. Plants, which are an important source of protein are deficient in some essential amino acids and may contain a wide variety of toxic substances. The recognition and understanding of such toxic substances is of most importance for maximum and safe utilization of plant food stuffs. It is necessary that such substances are isolated, identified, that the toxic properties are demonstrated in several species, in both sexes at different ages. It is necessary that the lethal dose is determined using different routes of administration, and methods for qualitative and quantitative determination established.

Cycads which are used as a source of food in some tropic and sub-tropic areas of the world contain toxic glucosides. Toxic effects of cycasin have already been described (Laqueur and Spatz, 1968). However, no work was done on the effects of MAM-Ac on the neuroendocrinological system. For this reason research was started to determine the effects of MAM-Ac on several pituitary hormones associated with reproduction. Furthermore, research was initiated to determine the effects of MAM-Ac or cycasin implanted in the brain of rats or mice or injected directly into the fetus of rats.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Plant Characteristics

During the Mesozoic Era, cycads or plants from the Cycadaceae family were widely distributed on earth. Today only 9 genera and about 100 species survive, mainly in the tropic and subtropic areas of the world (Fosberg, 1964). The genera, *Macrozamia*, *Cycas*, *Zamia* and *Encephalartos* are of particular interest since they have been implicated in toxic effects on human beings (Whiting *et al.*, 1966).

The cycads belong to the gymnosperms (Thieret, 1958). They are palm like trees with a cylindric trunk and a crown of large, stiff, feather-like leaves. In all genera, the stamenate and pistillate organs are borne on different plants. In most genera, the pollen and the ovule bearing organs are borne in strobiles or "cones." The fruit is a naked seed, the fleshy outer portion of which is the outer layer of the seed coat, while the hard inner part is the inner layer of the seed coat (Fosberg, 1964).

The Uses of Cycads

Cycads are mainly used as a source of starch which is derived either from the seeds or from the stems. It is an important source mainly during hard times, and in areas where food supplies as a whole are limited. The kernels

that are rich in starch are made into flour or starch after various treatments to remove the poisonous constituents. The treatment given to the kernels differs in different Islands of the Pacific. In the Andaman Islands *Cycas* seeds are eaten after cooking. In the Key Islands the seeds are roasted, and in Fiji the seeds are boiled and only the softened kernels are eaten. In Guam, *Cycas* kernels are soaked in water for several days with frequent changes in water before they are used. On the Comoro Islands the seeds are eaten after being put through a fermentation process (Thieret, 1958; Whiting, 1963).

The leaves of the Cycads are used for several purposes. Young leaves of several *Cycas* species are cooked and eaten as a vegetable in some parts of the Philippines and Indonesia. Leaves are also used as a raw material for making hats, baskets, mats, fences and brooms. Dried leaves of *Cycas revoluta* are used as a decorative material in many countries. Surface fibers called "palm wool," found at the base of the leaves of several species of *Macrozomia* and *Cycas*, are used for stuffing pillows and mattresses. Fiber from other species is used for making cloth (Thieret, 1958).

Gum is another product of cycads. Most of it is produced from the genus *Cycas*. The gum exudes through wounds in the megasporophylls, stems and leaves. The gum is used to treat malignant ulcers, or as an antidote for snake and insect bites, and as an adhesive agent (Thieret, 1958).

Many diseases are believed to be cured by Cycads. The stem of *Cycas pectinala* is used as a hair wash for diseased hair roots. In Cambodia, the terminal buds of *Cycas circinalis*, crushed in rice water, was used in the dressing of ulcerated wounds, and to treat swollen glands and boils. The juice of young *Cycas* leaves is reputedly a good agent against vomiting blood. The pollen of *Cycas rumphii* is a strong narcotic. *Cycas* seeds, pounded to a paste in coconut oil, are used for sores and various skin complaints (Thieret, 1958).

The Isolation and Identification of the Toxic Substances

The first record of the toxicity of *Cycas* dates back to 1770. Captain Cook reported illness in his men when they consumed *C. media* in Australia (Keegan and MacFarlane, 1963). However, it was not until 1941 that Cooper (1941) obtained a crystalline substance from the seeds of *Macrozamia spiralis*, a species native to Australia, and called it macrozamin. Macrozamin, was later identified as a glucoside of primeverose attached by a β-glucosidic link to its aglycone (Lythgoe and Riggs, 1949). The aglycone itself was identified a few years later (Langley *et al.*, 1951). The same glucoside was found to be present in African cycad as well (Riggs, 1954).

A glucoside closely related to macrozamin was obtained by Nishida *et al.* (1955) from *Cycas revoluta* and by Riggs (1956) from *Cycas circinalis*. Nishida named this

glucoside cycasin, which differs from the macrozamin only in its sugar moiety, which is D-glucose in cycasin.

Once the carcinogenic properties of cycasin became known (Laqueur *et al.*, 1963), a need for quantitative methods for the determination of the glucoside and its aglycone became obvious. Cycasin can be assayed in several ways, including a bioassay method by determining growth rates (Campbell *et al.*, 1966), colorimetrically, by using a chromotropic acid assay (Matsumoto and Strong, 1963), or by a paper chromatographic modification (Dastur and Palekar, 1966). Recently, a direct method for cycasin determination was developed (Wells *et al.*, 1968) using gas liquid chromatography. After a series of studies (Matsumoto and Strong, 1963; Kobayashi and Matsumoto, 1964, 1965; Matsumoto *et al.*, 1965) methylazoxymethanol acetate (MAM-Ac) was synthesized. More progress was achieved when ^{14}C and ^{3}H labelled MAM-Ac was synthesized (Horisberger and Matsumoto, 1968). Methylazoxymethanol is very unstable. Acidic hydrolysis yields nitrogen, methanol and formaldehyde, whereas alkaline hydrolysis yields nitrogen, formic acid, cyanide and traces of methylamine (Nishida *et al.*, 1956). MAM has a characteristic ultraviolet absorption spectrum with a maximum at 215 mμ, which is identical with that of cycasin (Matsumoto and Strong, 1963). Since MAM is destroyed at boiling temperature, it is possible to measure the absorption of a sample at 215 mμ, before and after

boiling. The quantity of MAM present can be calculated from the difference in the reading obtained before and after boiling (Spatz and Laqueur, 1968a). Using chromotropic acid reagent, MAM can be determined colorimetrically or by thin layer chromatography (Spatz and Laqueur, 1968a).

The Conversion of Cycasin to MAM

Macrozamin was toxic to guinea pigs when given orally but not when injected subcutaneously (Lythgoe and Riggs, 1949). Toxic properties of the African cycad *Encephalartos*, where macrozamin is present too, were described (Steyn *et al.*, 1948). Cycasin was toxic for mice and guinea pigs when given enterically, but not when injected intraperitoneally (Nishida *et al.*, 1956).

Because of the similarity in the toxicity of cycasin and macrozamin, it was suggested that the aglycone-methylazoxymethanol (MAM) which is present in both glucosides is causing the toxic effects after being released from the sugar moiety in the digestive system (Nishida *et al.*, 1956). This theory received support from an experiment in which all the cycasin injected intraperitoneally was recovered in the urine of rats (Kobayashi and Matsumoto, 1965). Additional support came from reports indicating that the cells of the small intestine contained glycosidase activity, using 6-Bromo-2-naphthyl as a substrate (Dahlquist, 1965a, 1965b).

When high concentrations of cycasin were given to germ-free rats for a period of 20 days, no toxic effects were observed. On the other hand when the same amounts were given to conventional rats, hepatic centrolobular necrosis and demise resulted within a few days (Laqueur, 1964). Subsequently, the excretion of cycasin from germ-free and conventional rats was measured. It was found that the germ-free rats excreted almost all the ingested cycasin, whereas conventional rats excreted only part, and metabolized the rest (Spatz *et al.*, 1966). Thus, these data strongly suggested that bacterial enzymes in the intestinal tract might be responsible for cycasin hydrolysis. To support this theory, germ-free rats were monocontaminated with bacteria having varying β-glucosidase activities, and then were given cycasin by stomach tube. It was found that the cycasin was hydrolyzed only in the rats that were contaminated with β-glucosidase-containing bacteria. Rats contaminated with the highest β-glucosidase containing bacteria had the most severe liver necrosis, and the least cycasin was excreted in their urine (Spatz *et al.*, 1967b).

A single subcutaneous injection of cycasin to newborn rats was reported to induce kidney tumors (Magee, 1965). This experiment was repeated, and the report was confirmed (Hirono *et al.*, 1968a). The length of the postnatal period in which subcutaneous injection might be toxic to rats was found to be the first 17 days of life (Spatz,

1969). In this study some of the mothers also died of cycasin toxicity, although they were not injected. This suggested that the mothers must have licked the urine off the pups and metabolized the cycasin present in this urine. It has been shown that MAM can pass through milk (Mickelsen and Yang, 1966); later, it was proven that cycasin cycles from the newborn rats to their mother and back to the newborn (Yang *et al.*, 1969).

Further studies showed that the cycling mechanism is not the only possible explanation for cycasin toxicity in one day old rats. A subcutaneous injection of cycasin of one day old germ-free rats, or of artificially fed one-day old baby rats caused the toxic effects typical of cycasin (Spatz, 1969). MAM itself was found in the homogenates of cycasin injected conventional and germ-free rats, thus the possibility of a direct cycasin effect was ruled out (Spatz, 1969). These observations suggested that enzymatic hydrolysis of cycasin occurred in the baby rat. It was not too long later that β-glucosidase activity was found in the crude skin homogenate from both conventional and germ-free baby rats, using cycasin and synthetic β-glucoside as substrates. The greatest enzymatic activity was found during the first few days of postnatal life. It then decreased until the rats were 25 days of age, when it no longer could be demonstrated (Spatz, 1968; Spatz *et al.*, 1968).

Carcinogenicity

The first evidence that crude cycad material is carcinogenic was obtained in studies with rats in 1963 (Laqueur *et al.*, 1963). Since then, tumors were induced by single and repeated doses of cycasin or MAM in rats (Laqueur *et al.*, 1963, 1967; Laqueur and Matsumoto, 1966; Hirono *et al.*, 1968a; Yang *et al.*, 1968; Spatz and Laqueur, 1967), guinea pigs (Spatz, 1964b), mice (O'Gara *et al.*, 1964; Hirono *et al.*, 1969b), hamsters (Spatz, 1969), fish (Stanton, 1966) and monkeys (Kelly and O'Gara, 1965). However, cattle, horses, swine (Mickelsen *et al.*, 1964) and chickens (Newberne, 1965; Sanger *et al.*, 1969), fed unprocessed cycad, showed microscopic liver lesions, but did not develop tumors.

Cycasin is carcinogenic only after passage through the digestive tract, whereas MAM is carcinogenic independent of the route of administration (Laqueur *et al.*, 1967; Laqueur and Matsumoto, 1966). MAM readily passes through the placenta in pregnant hamsters and rats (Spatz and Laqueur, 1968a) and also induces tumors by this route (Spatz and Laqueur, 1967b). MAM induces all the tumors that cycasin induces; but, in addition, when MAM is given by the intraperitoneal route, it induces duodenal tumors as well (Laqueur and Matsumoto, 1966; Laqueur *et al.*, 1967). Regardless of the cycad material used, the sex, and the age of the animal, it takes approximately 6 months for the tumors

to develop (Spatz and Laqueur, 1967c). The rate of tumor induction with MAM is 100%, but is variable with cycasin (Spatz and Laqueur, 1967; Spatz, 1969; Laqueur and Spatz, 1968).

The site of tumor development depends on the duration of the period of cycad feeding. Hepatomas require a longer feeding period than renal tumors. Large intestinal neoplasmas are least dependent on the duration of exposure to cycad (Laqueur, 1965). The age of the animals when cycasin feeding is started determines the frequency of various kinds of kidney tumors. Nephroblastomas, renal sarcomas and the interstitial tumors of the kidney were more common when immature rats were used. However, in mature and young rats, the same frequency of renal adenomas was noticed (Laqueur, 1964; Hirono *et al.*, 1968b). No strain differences to cycad toxicity were described in rats. Similar tumors were induced in Osborne-Mendle, Sprague-Dawley, Fischer and Wistar rats (Gusek *et al.*, 1967).

Tumors induced with crude cycad meal, cycasin, or MAM can be grown in culture (Shimizu, 1969), and are transplantable; but, in some nephroblastomas, the tumors grew more rapidly when the recipient rat and the primary tumor host-rat were of the same sex (Hirono *et al.*, 1968b). More evidence for sex dependence comes from studies in which male Sprague-Dawley rats had a considerably higher incidence of intestinal tumors than did the females of the

same strain under the same conditions (Laqueur, 1965; Hirono *et al.*, 1968a). Some sex differences were described in kidney tumors as well. It was found that only female Fischer rats had, among the interstitial tumors, smooth muscle elements forming leiomyomas of substantial size (Laqueur and Matsumoto, 1966). The cellular composition during tumor induction by cycasin in the rat was determined. In the tumor-bearing rats the DNA and RNA content of the liver was greater than normal. RNA, DNA and nitrogen concentrations were increased in kidney tumors, but not in the uninvolved kidney tissue (Hoch-Ligeti *et al.*, 1968).

Other Biological Effects

Intoxication of rats with cycasin causes liver injuries. With mild injury, loss of cytoplasmic basophilia and glycogen are noticed, whereas diffuse centrolobular hemorrhagic necrosis occurs with severe injury (Laqueur *et al.*, 1963). Examination with an ultramicroscope revealed that, as early as 2 hours after intraperitoneal injections of MAM-Ac, prominent changes occurred. The most prominent lesions were found in the centrolobular zone, while the cells in the periportal fields remained unchanged. The lesions included segregation of nuclear components with depletion of granular material, hypertrophy of the smooth endoplasmic reticulum, decrease in the number of ribosomes, and formation of membrane whorls (Ganote and Rosenthal, 1968).

Intoxication of rats with cycasin causes changes in the normal patterns of protein, lipids and RNA synthesis. After the introduction of cycasin, the levels of liver glucose-6-phosphatase (Spatz, 1964a), liver RNA, total phospholipids (Williams and Laqueur, 1965) and the rate of liver catalase synthesis (Rechcigl, 1964), were decreased. However, blood cholinesterase (Orgell and Laqueur, 1964), hemoglobin and hematocrit (Yang and Mickelsen, 1968) were elevated. Deaggregation of liver polyribosomes occurred after the administration of MAM-Ac intraperitoneally (Shank, 1968), and a reduction in incorporation of ^{14}C leucine to liver proteins of rats fed cycasin was noticed (Shank and Magee, 1967).

Cycasin and MAM were proven to be alkylating agents. In *in vitro* (Matsumoto and Higa, 1966) and in *in vivo* (Shank and Magee, 1967) studies, it was found that liver RNA and DNA were methylated. The purine base guanine was found to be methylated to form 7-methyl guanine (Matsumoto and Higa, 1966; Shank and Magee, 1967).

MAM was shown to be a mutagenic agent. MAM added to the nutrient medium of *Drosophila melanogaster* caused a mark rise in a sex-linked recessive lethal mutation (Teas and Dyson, 1967). MAM caused an increase in the rate of mutation from histidine requiring mutants to histidine independent mutants of *Salmonella typhimurium* (Smith, 1966). When the effect of MAM on insects was studied, it was found that

larvae of *Seirovclia echo* were able to detoxify MAM by converting it back to cycasin. This conversion is done apparently in the gut of the caterpillars by the enzyme β-glucosidase, which was mentioned before as the cleaving enzyme for cycasin (Teas, 1967).

Plants, too, are susceptible to cycasin toxicity. Root-tip cells of onion, which have β-glucosidase activity, showed chromosomal abberations, when exposed to cycasin solution. The same radiomimetic effect was produced by gamma rays (Teas *et al.*, 1965). Compounds like β-mercaptoethylamine and 3-amino 1,2,4, triazole were previously shown to exert a protective effect against x-ray irradiation (Bacq *et al.*, 1953; Feinstein, 1957). When these compounds were administered before the administration of lethal doses of cycasin, the rats were partially protected, they did not die, but they developed tumors later (Hirono and Laqueur, 1967; Hirono *et al.*, 1968a).

MAM has been reported to have teratogenic effects. When MAM was injected intravenously on the eighth day of gestation, it produced a variety of malformations like hydrocephalus, microcephalus and microphthalia in fetuses of hamsters (Spatz *et al.*, 1967a). In later studies, microencephaly was produced in rats which had been exposed to MAM on the 14th to 16th days of gestation. Brain tumors were developed in about 10% of these rats (Spatz and Laqueur, 1968b). Some intellectual deficit was noticed when

a Hebb-Williams maze was used. The rats treated with MAM-Ac during prenatal life had significantly poorer scores than their controls (Haddad *et al.*, 1969). In microencephalic rats, the cerebral hemispheres were diminished in size, the parietal bone was narrowed, the suture lines were soft and open at birth, and the total amount of cortical lipids and proteins were less than in control rats (Spatz, 1969).

Neurotoxicity

A correlation between the consumption of *Cycas circinalis* and high incidence of amyotrophic lateral sclerosis (ALS) on Guam has been suggested. It was found that this disease is 100 times more prevalent in Guam than in the United States and Europe (Kurland and Mulder, 1954; Whiting, 1963). ALS affects the cells of the medulla and cervical cord. It appears usually between the ages of 40 and 50. Weakness of the hands, difficulty in talking and swallowing are the first symptoms. As the disease progresses, the muscles of the hands, shoulders, pelvis, legs and tongue become atrophied. The course of the disease is rapidly progressive and always fatal. Mostly, death occurs within 2-5 years (Cecil and Loeb, 1955).

Cycads were found to be associated with neurological disorders in cattle. The symptoms are a progressive caudal weakness of the hind limbs and paralysis (Britton and Wilson, 1930; Mason and Whiting, 1968). Cattle that have

eaten leaves of *Macrozamia lucida* and *Bowenia serrulata* developed paralysis. When CNS and the spinal cord were examined, it was found that demyelination of the spinal columns occurred. The fasciculus gracilis and the dorsal spinocerebellar tracts were mainly affected (Mason and Whiting, 1966; Hall and McGavin, 1968).

No neurological lesions were found in the brains or spinal cords of over 500 rats fed crude cycad meal (Laqueur *et al.*, 1963). No apparent neurological disorders were noticed when cycasin or MAM-Ac were implanted in the third ventricles of female rats (Malevski *et al.*, 1971). However, a single subcutaneous injection of MAM into newborn mice of the strain C57BL/6 caused hind leg paralysis in 80% of the injected mice (Hirono and Shibuya, 1967). Lesions in the brain of such mice were described (Hirono *et al.*, 1969a).

Neurological disorders can also be induced by a toxic amino acid isolated from *C. circinalis* and other plants (Sarma and Padmanaban, 1969). α-amino-β-methylamino-propionic acid isolated from the seeds of *Cycas circinalis* when injected into chicks resulted in a loss of the ability to extend legs and to stand erect. The birds bent their heads down and tended to "run backwards" (Vega and Bell, 1967). A related compound, α-amino-β-oxalylaminopropionic acid is known to cause lathyrism. This amino acid was not found in cycads (Bell, 1964).

It seems that cycasin and MAM exert several types of mechanisms of action. The similarities between the chemical structure and the biological effects of dimethylnitrosamine (DMN) and cycasin were noted (Laqueur *et al.*, 1963). Both MAM and DMN were found to produce similar subcellular changes when compared at equivalent doses and times (Ganote and Rosenthal, 1968). Both were found to methylate liver RNA and to cause polyribosomal deaggregation under the same experimental conditions (Shank and Magee, 1967; Shank, 1968). It was suggested that both substances may have a common metabolic pathway, forming diazomethane, which is known for its alkylating and carcinogenic properties (Miller, 1964). The pathway by which MAM and DMN are converted to the active form was theorized (Miller and Miller, 1965). Cycasin is deglucosylated to form MAM, which is unstable and releases formaldehyde to form azoxymethane, which in turn loses water to form diazomethane.

This postulated mode of action can explain the carcinogenic and alkylating properties of cycasin. However, no mechanism of action has been suggested, so far, for the neurotoxic, teratogenic, mutagenic and radiomimetic effects of cycasin or MAM.

PART I

THE INFLUENCE OF MAM-Ac ON THE HYPOTHALAMUS-PITUITARY-OVARY AXIS IN THE RAT

PART I

THE INFLUENCE OF MAM-Ac ON THE HYPOTHALAMUS-PITUITARY-OVARY AXIS IN THE RAT

Objective

Pregnant rats fed cycad meal had fewer pups at birth when compared to control rats (Yang, unpublished results). In this experiment all rats were fed *ad libitum* and since cycasin reduces food intake (Campbell *et al.*, 1966) the control rats consumed more than the cycasin fed rats. This made the interpretation of the results difficult since it is not possible to separate the effects of food intake reduction from cycasin consumption.

The only indication available in the literature suggesting alteration in gonadotropin metabolism comes from studies by Spatz and Laqueur (1967c). They found that when crude cycad meal was fed during pregnancy to rats, the mammary glands were less developed, only small amounts of milk escaped from incised mammary gland tissue and little or no milk was found in the stomachs of the young.

For these reasons, we decided to determine the effects of MAM-Ac, which is easily hydrolyzed by blood or tissue esterases to MAM,on the hypothalamus-pituitary-ovary axis.

Review of Literature

The Hypothalamus-Pituitary Axis

No direct neural connections between the hypothalamus and the anterior pituitary are evident. The only connection between them is by the hypophyseal portal blood vessels originating in the stalk and in the median eminence (Popa and Fielding, 1930 a, b).

The first clear postulation that the hypothalamus exerts a control over the anterior pituitary gland was made by Harris (1937) in which he induced ovulation in rabbits by electrical stimulation of the hypothalamus. Hinsey (1937) was the first to postulate that this control was by neuro-humoral substances produced in the hypothalamus and transferred to the anterior pituitary gland by the portal vessels. This postulate received further support when Harris and Jacobson (1952) transplanted pituitary tissue to the median eminence of hypophysectomized rats. After the transplantation the rats showed normal reproductive functions.

Releasing and Inhibiting Factors of the Hypothalamus

The synthesis and release of the anterior pituitary hormones is induced by hypothalamic hormones or releasing factors. The only exception is prolactin which is inhibited by hypothalamic hormone. Direct evidence of the presence of a follicle stimulating hormone-releasing factor (FSH-RF) in rat hypothalamus in an *in vitro* system was reported by

Mittler and Meites (1964) and in an *in vivo* system by Igarashi and McCann (1964). Luteinizing hormone releasing factor (LHRF) was first shown to exist by McCann *et al.* (1960) in an *in vitro* system, and later in an *in vivo* system (McCann, 1962).

Prolactin inhibiting factor (PIF) was first shown to exist in the rat by Meites *et al.* (1961). Other species including sheep, swine and cattle were also shown to contain PIF in the hypothalamus (Talwalker *et al.*, 1963; Schally *et al.*, 1965).

Feedback of Hormones on the Hypothalamo-Pituitary Axis

Ovarian hormones may induce or inhibit the secretion of the gonadotropins from the pituitary. They exert their positive or negative feedback on the hypothalamus and/or the pituitary gland. It has not yet been established whether the hypothalamus or the pituitary is the major site for the feedback. It appears that estrogen and progesterone may inhibit FSH and LH secretion by a direct action on the anterior pituitary (Bogdanove, 1963; Ramirez *et al.*, 1964). However, both steroid hormones induce the secretion of the gonadotropins by a direct action on the hypothalamus (Everett, 1964; McCann *et al.*, 1968; Davidson, 1969, 1970).

Anterior pituitary hormones themselves act to control their own secretion. These hormones act directly on the hypothalamus to inhibit the secretion of the releasing

factors. Several pituitary hormones were found in the median eminence (Guillemin *et al.*, 1962; Schally *et al.*, 1962; Johnson and Nelson, 1966). These hormones reach the median eminence area either via the general circulation or by a reverse blood flow from the pituitary (Torok, 1964).

The first evidence that LH may be controlled in part by itself was presented by Sawyer and Kawakami (1959). Later, when small amounts of LH were placed into the median eminence of rats, a reduction in pituitary and serum LH levels occurred. The decline in LH secretion was associated with irregular vaginal cycling, few corpora lutea and normal follicular growth. These effects were specific to LH implantation (David *et al.*, 1966; Corbin and Cohen, 1966). Recently, Terasawa and Sawyer (1968), reported that LH administration activated the arcuate region, but depressed the preoptic area. These two areas of the hypothalamus are postulated to control LH secretion. Similar types of experiments were conducted to show that FSH too may inhibit its own secretion through an action on the hypothalamus.

The first evidence that prolactin may control its own secretion came from studies by Sgouris and Meites (1953). They found decreased pituitary prolactin following injection of prolactin in rats. These results were confirmed by Sinha and Tucker (1968). Pituitary glands transplanted underneath the kidney capsule of ovariectomized rats secreted mainly prolactin and only small amounts of the other

pituitary hormones (Welsch *et al.*, 1968). Implantation of prolactin into the median eminence of rats resulted in a decrease in synthesis and release of prolactin which was associated with regression of the mammary glands and fewer corpora lutea (Clemens and Meites, 1968).

Prolactin, LH and FSH Levels During the Rat Estrous Cycle

The availability of radioimmunoassays for LH, FSH and prolactin made it possible to measure these hormones during the estrous cycle of rats. The pattern of gonadotropin secretion during the estrous cycle was shown by Gay *et al.* (1970). Serum LH rose at least 50 fold during the afternoon of proestrus but remained low at all other stages of the cycle. LH content of the pituitary was highest on the day of diestrus and it decreased late during proestrus (Monroe *et al.*, 1969). Serum FSH rose only 4-5 fold late in the afternoon of diestrus. It remained low at all other stages of the cycle (Gay *et al.*, 1970). Serum prolactin increased about 10 fold at late proestrus, but it remained high on the morning of estrus (Gay *et al.*, 1970; Amenomori *et al.*, 1970). The anterior pituitaries of estrous rats contain more prolactin than the anterior pituitaries of diestrous rats. Anterior pituitaries of proestrous and estrous rats released more prolactin when incubated *in vivo* and had less PIF when compared to diestrous rats (Sar and Meites, 1967).

The half life for LH, FSH and prolactin on the afternoon of diestrus was measured by the rate of their disappearance from the blood. The half life for LH is 20 min, FSH is 110 min and prolactin is 13 min (Gay *et al.*, 1970).

Prolactin, LH and FSH During Pseudopregnancy in the Rat

It appears that prolactin is the main pituitary hormone responsible for luteal maintenance and progesterone secretion in the rat. Fajer and Barraclough (1967) reported that prolactin and not LH could stimulate progesterone secretion by rat ovaries in an *in vivo* system. Kwa and Verhofstad (1967) reported a rise in serum prolactin levels for the first 3 days of pseudopregnancy and thereafter it declined. Similar results were obtained in pregnant rats (Amenomori *et al.*, 1970). The question of luteal control and maintenance is still unresolved.

Materials and Methods

Two hundred ninety-two, 3-5 month old female Sprague-Dawley rats (Spartan Research Animals, Haslett, Mich.) averaging 250 g were used in these experiments. All rats were fed a grain ration (Campbell *et al.*, 1966) and were allowed free access to water. The rats were housed in a laboratory where the temperature was kept near 24°C throughout the experiments. Lighting was regulated to

provide 12 hours of illumination (8 a.m. to 8 p.m.) and 12 hours of darkness (8 p.m. to 8 a.m.) each day. All rats were individually housed in suspended wire cages except during the time of mating which was done in Trials 1-4. The rats were divided into 8 different trials as indicated in Table 1.

Injections of MAM-Ac or Saline

Prior to the injection of MAM-Ac or saline all rats except those in trials 5 and 8 were examined daily by vaginal lavage to determine the stage of the estrous cycle. The rats were allowed to cycle 2 to 3 times and then only normally cycling rats (4-5 day cycles) at metestrus were injected subcutaneously with MAM-Ac or saline near the left rump area with a micro-syringe. The amounts injected are shown in Table 1.

Feeding Schedule

Feeding cycad material causes a reduction in food intake and as a result a reduction in body weight (Campbell *et al.*, 1966). This is why in all trials but trial 1, one control group was pair fed to the MAM-Ac injected group. The individual rats of the MAM-Ac group were injected 1-5 days ahead of their pair fed controls. This allowed time for measuring the food intake of the MAM-Ac injected rats before the injection of the pair fed mates with saline at metestrus. In addition, in trials 5, 6 and 7 a positive

Table 1. The division of the rats used into 8 trials.

Trial No.	Rats Used As			Amount of MAM-Ac or Saline Injected	Purpose
	Positive Control	Pair fed Control	MAM-Ac Injected		
	(No.)	(No.)	(No.)	(μl/rat)	
1	3[a]	--	11	10	To determine food intake, body weight, length of estrous cycle and number of born and weaned pups.
2	-	10	11	12	Same as in Trial 1.
3	-	13	13	10	To determine food intake, body weight and number of born and weaned pups.
4	-	5	5	12	To determine the number of born and weaned pups.
5	6	6	6	12	To determine the moisture and fat content of the body.
6	55	55	55	12	To determine food intake, body weight, and length of estrous cycle. To determine blood, pituitaries and hypothalami for LH, PRL, LHRF and PIF. Histological examination of ovaries and uteruses.
7	5	6	12	12	Histological examination of adrenals and pituitaries. To obtain the weight of ovaries and uteruses.

Table 1 (Cont'd)

Trial No.	Rats Used As				Purpose
	Positive Control	Pair fed Control	MAM-Ac Injected	Amount of MAM-Ac or Saline Injected	
	(No.)	(No.)	(No.)	(µl)	
8	-	6	9	10 and 12	To determine the weights of the uterus, adrenals and thymuses. Examination of the histological appearance of vaginal smears in spayed rats treated with MAM-Ac.

[a]The rats in the control group were restricted to 60% of the food consumed by the MAM-Ac injected rats.

control fed *ad libitum* was used. The only exception is the control rats in trial 1, their food intake was arbitrarily reduced approximately 40%.

Food intake was measured daily for the MAM-Ac injected rats until the rats consumed pre-injection amounts for at least 3 days, then the rats were fed *ad libitum*. Each pair fed control rat was given only as much food as its experimental mate had consumed during the preceding 24 hours.

Body weights were recorded in trials 1, 2, 3, 6 and 8 from the day of injection up to the day when the rats had regained their initial body weight. Body weight gain was recorded for the positive controls for the same period of time.

Determination of the Stage of the Cycle

Vaginal smears were taken daily between 9 and 11 a.m. as described previously (Evans and Bishop, 1922). The smears which were taken with an eye dropper were put on a glass plate, dried and stained in 1% methylene blue solution for 60 min. The type of cells in the smear was determined using a light microscope. In trials 1, 2 and 3 smear examination was continued until the rats became pregnant and in trials 6 and 8 unitl the rats were sacrificed.

Breeding

Breeding was done in trials 1 through 4. Two weeks after the injection of MAM-Ac or saline, 3 to 4 female rats from the same group were put together with a proven male. Vaginal smears were continued daily until sperms were found. This was considered to be the first day of pregnancy and at this stage the pregnant rats were transferred to maternity cages. The time needed for each rat to become pregnant and the number of the born and weaned pups were recorded.

Fat and Moisture Determination

The rats from trial 5 were used for the determination of fat and moisture. In previous trials it was found that on the 4th day after the injection of MAM-Ac the rats were at their lowest body weight. On the fifth day after injection the rats started to recover, so it was decided to sacrifice the rats on the 4th day after the injection. The rats were overetherized and the contents of their G.I. tract removed and weighed. Moisture and fat were determined as described previously (Mickelsen and Andersen, 1959). The GI.content weight was subtracted from the body weight for the calculations of body fat and moisture.

Histological Studies

Rats from trials 6 and 7 were used for histological studies. Ovaries and uteruses were obtained from the rats of

trial 6 and adrenals and pituitaries from the rats of trial 7. The ovaries and the uteruses were obtained 1 to 6 days, every day and 8 to 16 days, every second day after the injection of MAM-Ac or saline. Adrenals and pituitaries were obtained 4 and 14 days after the injections. All tissues were fixed in 10% buffered formalin. Hematoxylin and eosin stains were used and the examination of the slides was done with a light microscope.

LHRF and PIF Assays

The rats from trial 6 were used for this study. The hypothalami were stored individually in 0.1 N HCl (0.2 ml/ hypothalamus) at -20° until the time of assay. Just prior to the assay the hypothalami were pooled, homogenized, and centrifuged at 12,000 g for 40 min at 4°. The hypothalami from the rats killed 1-6 and 8-16 days after the injection in each group were pooled. The acidic supernatants were placed in protein free medium 199 (Difco Labs, Detroit, Mich.) and the pH was adjusted to 7.4 by adding 0.1 N NaOH. LHRF and PIF were measured by incubating the pituitary halves from normal, mature male rats with pooled hypothalamic extracts. The pituitaries were preincubated for 30 min and the medium was discarded. Fresh medium containing the neutralized acid extract was then added and the incubation was terminated 2 hr later. The pituitary tissue was weighed and the medium stored at -20° until assayed for LH and prolactin (Piacsek and Meites, 1966; Kragt and Meites, 1967).

Radioimmunoassays (RIA)

Blood serum, pituitaries and hypothalamic extracts from the rats of trial 6 were used for RIA. After injections 5 rats randomly selected from each group were sacrificed by decapitation every day from 1 to 6 days and every second day from days 8 to 16. The blood was collected from the neck and the serum separated by centrifugation was kept frozen at -20° until the time of assay. The anterior pituitaries were individually weighed, placed in 0.01 M phosphate buffer in 0.14 M NaCl (pH = 7), homogenized with a cell disruptor and stored at -20° until assayed. The hypothalami were treated as indicated previously.

LH and prolactin from individual serum samples, pituitaries and incubated medium were measured at 2-3 dose levels by RIA for rat prolactin (Niswender *et al.*, 1969), and rat LH (Monroe *et al.*, 1968). Purified rat prolactin and LH were used as references. The purified prolactin preparation was H-10-10-B (23 I.U/mg) obtained from Dr. S. Ellis, National Aeronautics & Space Administration (NASA), Ames Research Center, Moffett, Calif. 94035; and the LH preparation was NIAMD-Rat-LH-RP-1 (Biological potency equal to 0.03 x NIH-LH-S1), obtained from NIH, Rat Pituitary Hormone Program, Bethesda, Maryland.

Spaying and Estrogen Injections

In trial 8, mature rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) and both ovaries were removed. During the recovery period of 2 weeks, vaginal smears were taken. The experimental group was then injected daily with estrogen benzoate (15 µg/kg body weight) in 0.1 ml corn oil. The control group was injected with equivalent volumes of corn oil. Vaginal smears were continued and after 2 weeks all rats were injected with 10 or 12 µl of MAM-Ac. A week later all rats were killed, their uteruses, adrenals and thymuses removed and weighed.

Statistical Analysis

A "t" test (Sokal and Rohlf, 1969) was used to determine the significance of differences between groups. Differences are considered significant only when p is less than 0.05.

Results

Post Injection Food Intake and Body Weights

After the injection of 10 µl MAM-Ac to rats in trial 1 and 3, daily food intake decreased sharply from approximately 20 to 3 g. From the third day on food consumption started to increase until it became normal again a week later (Figure 1). Injection of 12 µl MAM-Ac to rats in trials 2 and 6 caused a decrease in food consumption from a pre-injection intake of 20 g/day to 1-2 g/day by the second

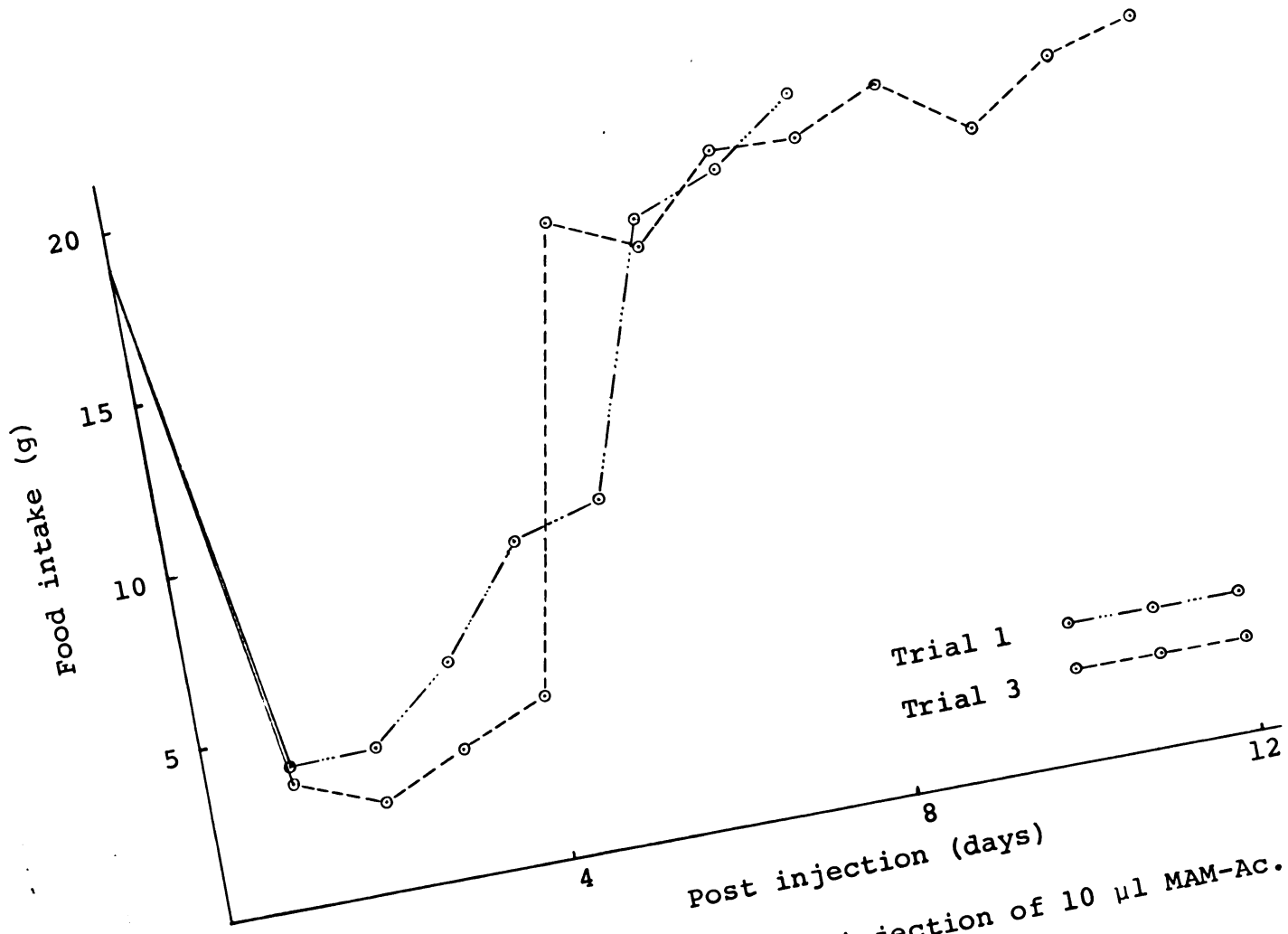

Figure 1. Food consumption after injection of 10 µl MAM-Ac.

day after the injection. Thereafter the intake increased until it returned to normal on the 9th day (Figure 2).

The reduction in food intake was accompanied by a reduction in body weight. The rats injected with 10 µl MAM-Ac lost 12-15% of their body weight by the third day after the injection. Thereafter they gained weight until they restored all loses by the tenth day. Pair fed controls of trial 3 lost identical amounts of body weight as did the MAM-Ac injected rats (Figure 3). Rats injected with 12 µl MAM-Ac lost approximately 18% body weight by the fourth day after the injection, and thereafter gained weight until they restored all the weight lost by the twelfth day. Pair fed controls lost the same amount of body weight, whereas the positive controls continued to gain weight throughout the entire period of the experiment (Figure 4). The positive control rats had significantly ($p<0.05$) more body fat than the MAM-Ac injected rats and the pair fed controls. The same amount of fat was found in the MAM-Ac injected and the pair fed control rats (Table 2).

Adrenal Weights and Histology

Four days after the injection of MAM-Ac the weight of the adrenals increased ($p<0.1$) in comparison with either the pair fed rats or the positive controls. Fourteen days after the injection, the adrenals of the MAM-Ac injected rats remained heavier ($p<0.05$), compared with the pair fed controls (Table 3).

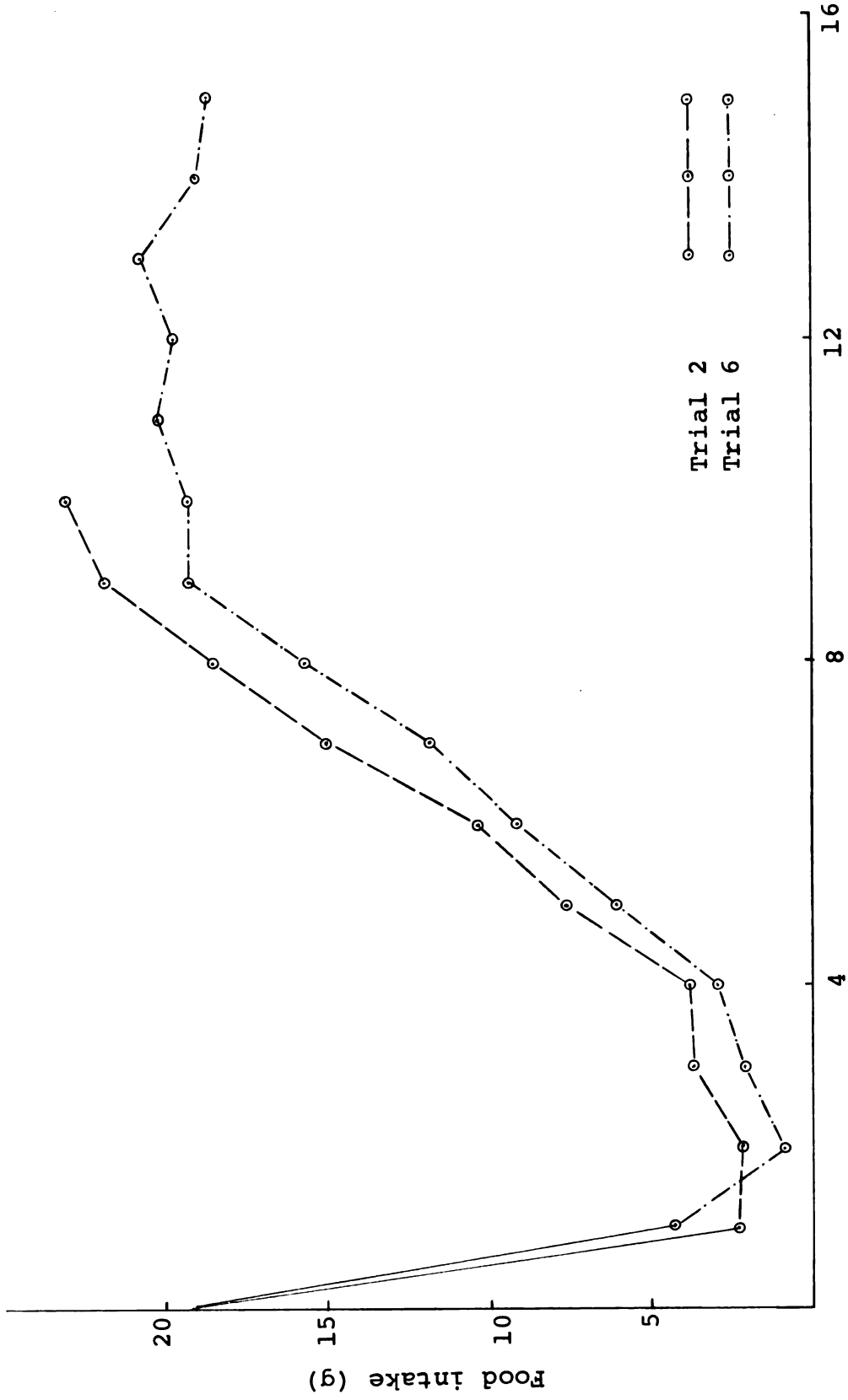

Figure 2. Food consumption after injection of 12 µl MAM-Ac.

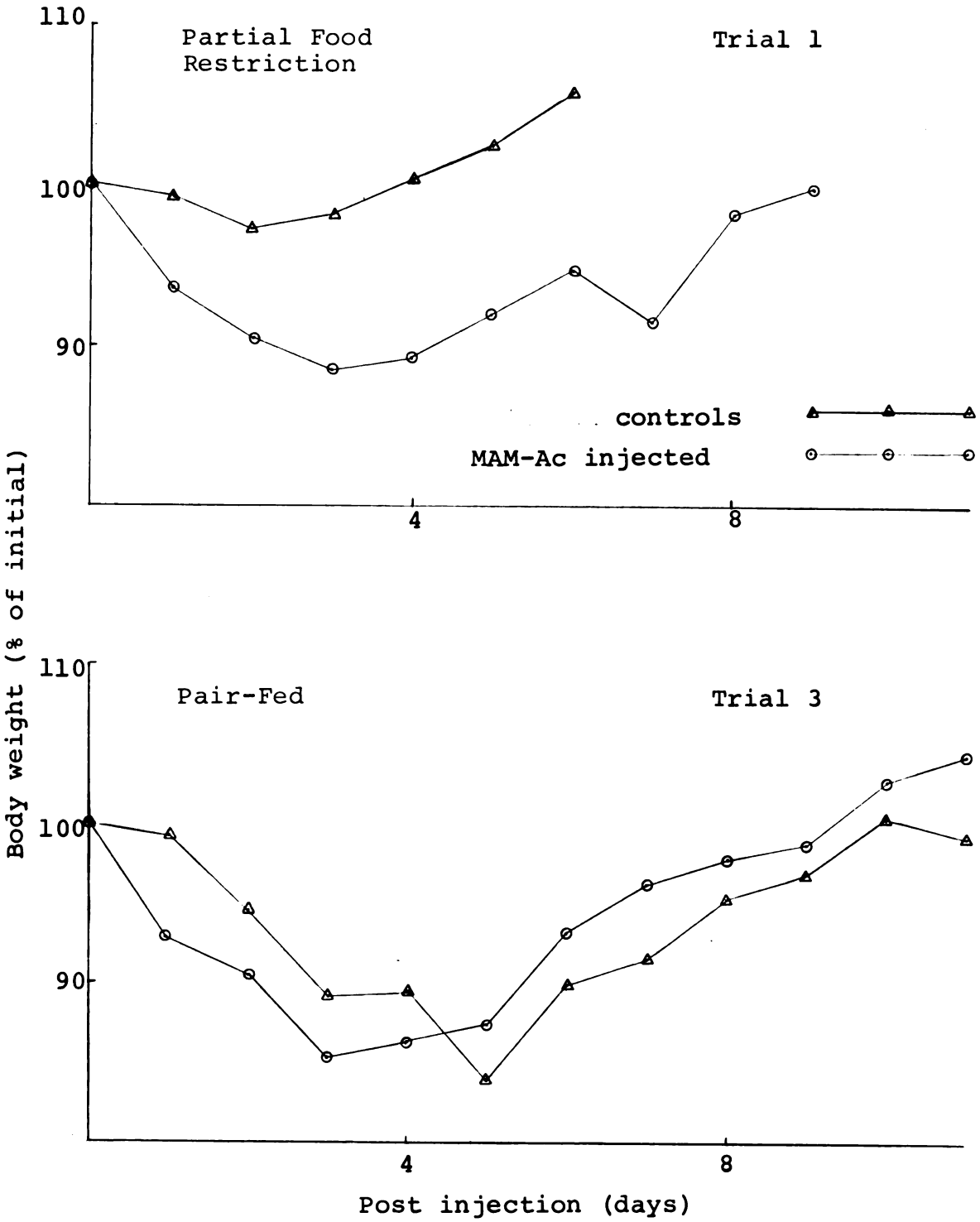

Figure 3. Body weight changes of rats injected with 10 µl MAM-Ac and of their controls.

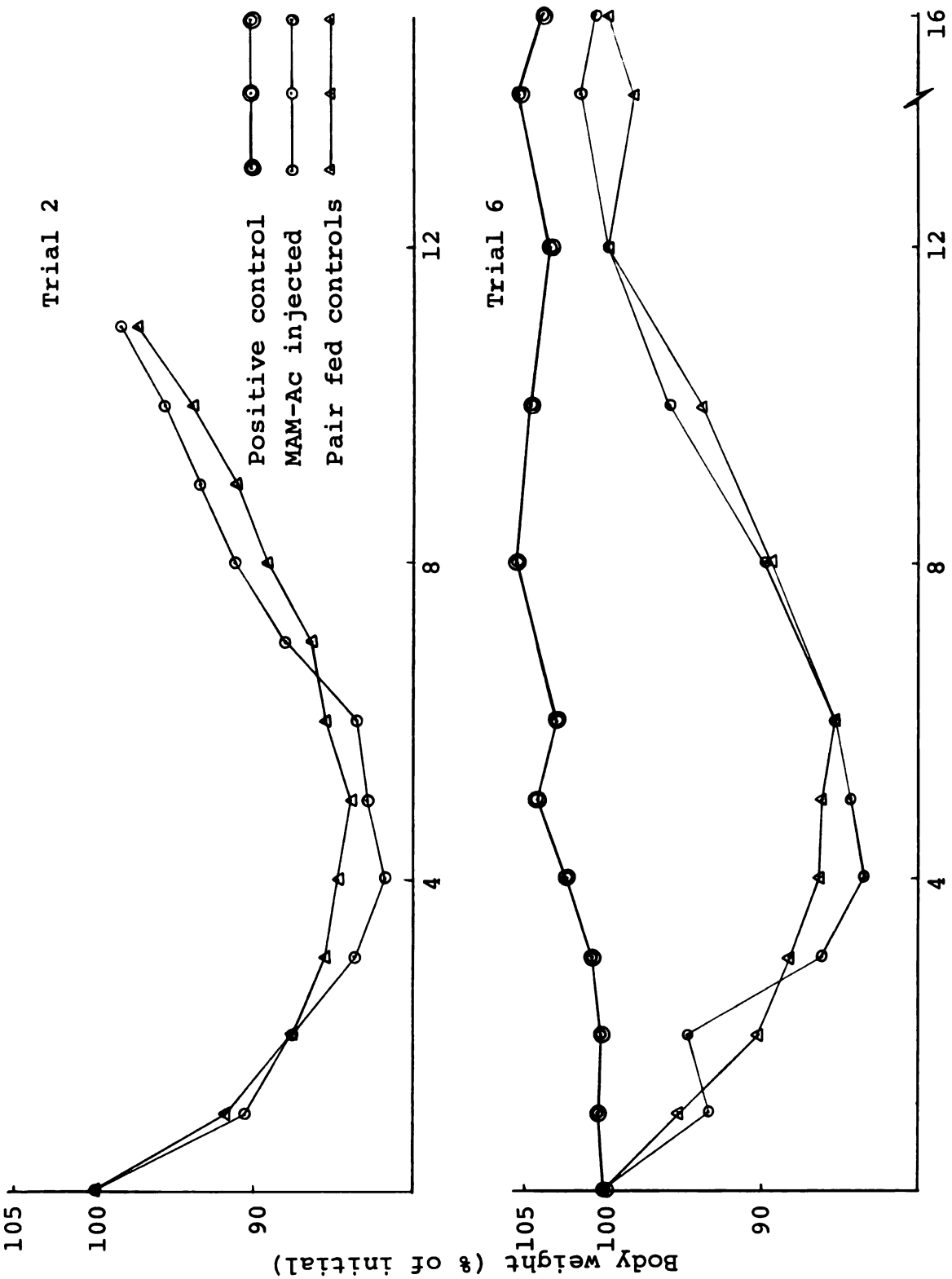

Figure 4. Body weight changes of rats injected with 12 µl MAM-Ac and of their pair fed controls.

Table 2. Moisture and fat content 4 days after the injection of MAM-Ac or saline (Mean ± S.E.)

Group	Rats per Group (No.)	Body wt. at Injection (g)	Body wt. at Sacrifice[a] (g)	Moisture Content (%)	Fat Content (%)	Ash and protein Content[b] (%)
MAM-Ac Injected	6	252.7±7.4	204.0±11.5	63.9±2.0	8.6±0.8[c]	27.5±1.5
Pair-Fed Control	6	251.3±7.6	209.3± 8.2	61.4±1.3	8.7±1.0[c]	29.9±1.0
Positive Control	6	251.6±5.7	242.9± 4.7	60.9±0.3	11.2±0.8	27.9±0.9

[a]Body wt. - G.I. content.

[b]By subtraction of % fat and % moisture from 100.

[c]Significantly different from positive controls ($p<0.05$).

Table 3. Adrenal, ovarian and uterine weights 4 and 14 days after the injection of MAM-Ac or saline (Mean ± S.E.).

Group	Day after Injection (No.)	Rats per Group (No.)	Body wt. at Injection (g)	Body wt. at Sacrifice (g)
MAM-Ac Injected	4	6	240.7±3.1	203.7±5.9
Pair Fed Control	4	3	240.7±3.8	208.0±2.3
Positive Control	4	2	246.0±6.9	255.5±4.5
MAM-Ac Injected	14	5	239.6±6.6	245.2±9.7
Pair-Fed Control	14	3	235.0±4.9	250.7±8.1
Positive Control	14	2	231.5±9.5	260.0±9.0

Table 3 (Cont'd).

Adrenal Wt. (mg/100 g B.W.)	Ovarian Wt. (mg/100 g B.W.)	Uterine Wt. (mg/100 g B.W.)
50.8±4.3[a]	45.6±1.8[b]	123.9± 8.4[c]
39.6±3.8	56.4±3.9	169.5±14.0
32.0±0.0	46.5±5.0	165.8± 9.8
36.2±2.5[b]	36.4±1.7	142.8±12.6
24.0±0.9	40.1±5.2	187.3±31.3
26.0±1.5	41.8±2.2	155.3±16.0

[a]Significantly different from both controls ($p<0.1$).

[b]Significantly different from pair fed controls ($p<0.05$).

[c]Significantly different from both controls ($p<0.05$).

Histological examination revealed that the adrenals of both control groups were free of lesions and the division of the different zones was sharp and clear (Figure 5a, b). However, the adrenals from MAM-Ac injected rats had hemorrhages and the different zones were not divided clearly (Figure 6).

The Effect of MAM-Ac on the Length of Estrous Cycle

Injection of 10 µl MAM-Ac caused prolongation in the length of the estrous cycle from 4.2 to 7.6 days. This prolongation was not statistically significant (Table 4). The injection of 12 µl MAM-Ac caused significant prolongation ($p<0.001$) in the length of the first cycle after the injection mainly by an increase in the diestrus stage. In trial 2, this cycle was prolonged from 4.2 days to 9.0 days in the MAM-Ac injected group and from 4.5 to 5.4 days in the pair fed controls (Table 5). In trial 6 the rats injected with MAM-Ac had a cycle of 9.8 days whereas the pair fed rats and positive controls had cycles of 5.2 and 4.4 days respectively (Table 6).

The Effect of MAM-Ac on the Duration of Time from Mating to Pregnancy and on the Number of Pups

The length of time from the day of mating to the first day of pregnancy was not affected by the injection of MAM-Ac. The number of pups in the uteruses of MAM-Ac injected rats and the controls of trial 1 was found to be the same.

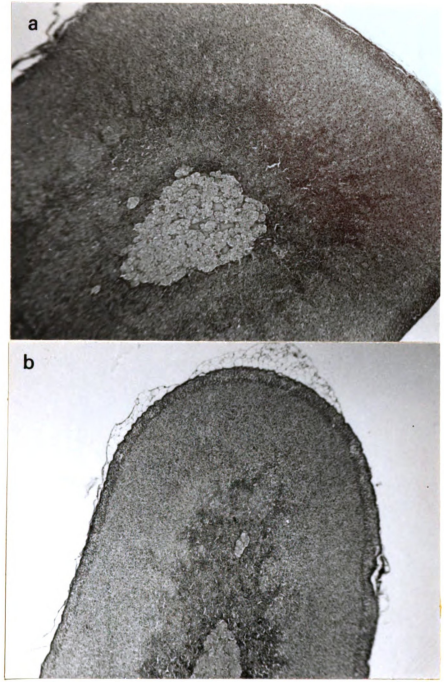

Figure 5. Photomicrographs of adrenal glands from a positive (a) and a pair fed (b) control rats 4 days after the injection of saline. In both, note clear division of zones and the absence of pathological symptoms (hematoxylin and eosin stains x 40).

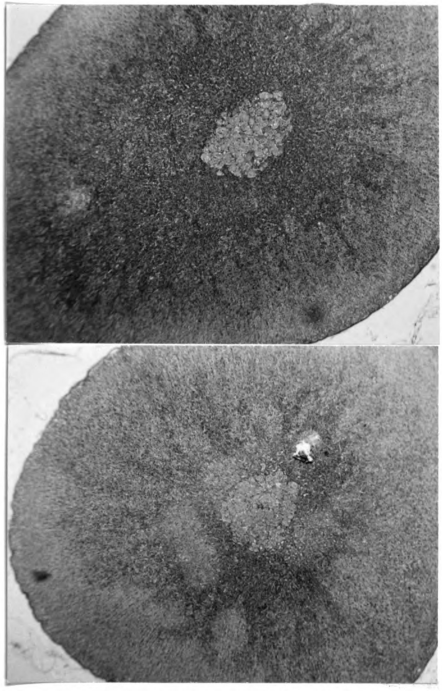

Figure 6. Photomicrograph of adrenal glands from 2 rats 4 days after the injection of 12 µl MAM-Ac. Note unclear division of zones with an extension of the zona fasciculata and presence of hemorrhages (hematoxylin and eosin stain, x 40).

Table 4. Lengths of estrous cycles (in days) before and after injection of 10 µl MAM-Ac or saline (Mean ± S.E.).[a]

Group	Rats per Group (No.)	Pre-injection cycle length			Post-injection cycle length		
		Cycle 1 (days)	Cycle 2 (days)	Cycle 3 (days)	Cycle 1 (days)	Cycle 2 (days)	Cycle 3 (days)
MAM-Ac Injected	10	4.3±0.2	4.4±0.2	4.2±0.1	7.6±1.3	6.9±1.1	4.8±0.3
Positive Control	3	4.0±0.0	4.0±0.0	4.3±0.3	4.3±0.3	4.5±0.5	4.0±0.0

[a]The difference between any two comparisons is not significant.

Table 5. Lengths of estrous cycles (in days) before and after injections of 12 µl MAM-Ac or saline to rats of trial 2 (Mean ± S.E.).

Group	Rats per Group (No.)	Pre-injection cycle length			Post-injection cycle length		
		Cycle 1 (days)	Cycle 2 (days)	Cycle 3 (days)	Cycle 1 (days)	Cycle 2 (days)	Cycle 3 (days)
MAM-Ac Injected	11	4.5±0.2	4.3±0.2	4.2±0.1	9.0±1.0[a]	7.9±1.7	4.4±0.2
Pair Fed Control	10	4.6±0.2	4.5±0.2	4.5±0.2	5.4±0.3	6.5±0.9	4.5±0.2

[a]The cycle is significantly prolonged ($p<0.01$).

Table 6. Lengths of estrous cycles (in days) before and after injections of 12 µl MAM-Ac or saline to rats of Trial 6 (Mean ± S.E.).[a]

Group	Rats per Group (No.)	Pre-injection cycle length			Post-injection cycle length		
		Cycle 1 (days)	Cycle 2 (days)	Cycle 3 (days)	Cycle 1 (days)	Cycle 2 (days)	Cycle 3 (days)
MAM-Ac Injected	46	4.4±0.1	4.2±0.3	4.4±0.2	9.8±0.9[b]	4.5±0.2	- - - -
Pair Fed Control	55	4.3±0.1	4.3±0.1	4.4±0.1	5.2±0.2	6.0±0.4	4.0±0.0
Positive Control	55	4.4±0.1	4.4±0.1	4.7±0.3	4.4±0.1	4.4±0.1	4.6±0.2

[a]Five rats per group were randomly killed at 1, 2, 3, 4, 5, 6, 8, 10, 12, 14 and 16 days after the injections.

[b]The cycle is significantly prolonged ($p<0.001$).

No difference was found between the MAM-Ac injected rats and their controls in trials 2, 3 and 4 in the number born and number of pups weaned (Tables 7-10).

Post Injection Ovarian, Pituitary and Uterine Weights and Histology

Organ weights can be analyzed statistically according to the stage of the cycle in which the rats were sacrificed or to the post injection time of sacrifice. The weights of the ovaries, uteruses and pituitaries per 100 g B.W. of the MAM-Ac injected rats and of both controls were comparable when similar organs were compared on either proestrus or diestrus. MAM-Ac injected rats at metestrus had significantly lighter pituitaries per 100 g body weight and at estrus had significantly lighter ovaries ($p<0.05$). Furthermore, MAM-Ac injected rats at diestrus had significantly lighter ovaries, pituitaries and uteruses when expressed on an absolute basis (Table 11).

It appears that the pituitaries per 100 g B.W. of the MAM-Ac injected rats were lighter ($p<0.05$) than the pituitaries of the pair fed controls, 4 to 12 days after the injection. They were significantly lighter than both controls when expressed on absolute bases. MAM-Ac injected rats had significantly lighter ovaries ($p<0.05$) per 100 g B.W. compared to the positive controls, 1-3 days after the injection. Four to six days after the injection they were

Table 7. Duration of time from mating to pregnancy and number of born and weaned pups in Trial 1 (Mean ± S.E.).

Group	Rats per Group (No.)	Time from mating to pregnancy (days)	Fetuses in Uterus (No.)	Rats delivering (No.)	Pups born (No.)	Pups weaned (No.)
MAM-Ac Injected	4	5.0±1.2	1.0±0.6[a]	-	- - - -	- - - -
Control	2	6.0±4.0	6.0±1.0[a]	-	- - - -	- - - -
MAM-Ac Injected	8	6.9±1.7	- - - -	3[b]	7.3±1.2	3.7±1.8

[a]The pregnant rats were sacrificed between 16 to 18 days of pregnancy to determine the number of fetuses.

[b]5 of the 8 mother rats ate all their pups near delivery.

Table 8. Duration of time from mating to pregnancy and number of born and weaned pups in Trial 2 (Mean ± S.E.).

Group	Rats per Group (No.)	Time from mating to Pregnancy (days)	Rats delivering (No.)	Pups Born (No.)	Pups weaned		
					Total (No.)	Male (No.)	Female (No.)
MAM-Ac Injected	11	8.2±1.8	1[a]	6	6	4	2
Pair Fed Control	10	7.0±1.8	0[b]	0	0	0	0

[a]Out of the 10 that did not deliver: 1 was not pregnant, 2 died during delivery, 4 ate the pups near delivery and 3 had hydrosalpinx.

[b]Out of the 10 that did not deliver: 3 were not pregnant, 2 died during delivery and 5 had hydrosalpinx. The cause of the poor reproductive performance in the controls in this trial is not known.

Table 9. Duration of time from mating to pregnancy and number of born and weaned pups in Trial 3 (Mean ± S.E.).

Group	Rats per Group (No.)	Time from Mating to Pregnancy (days)	Rats Delivering (No.)	Pups born/dam (No.)	Pups weaned		
					Total (No.)	Male (No.)	Female (No.)
MAM-Ac Injected	13	6.0±1.5[a]	9[b]	10.8±0.9[a]	9.1±1.3[a]	4.6±0.7[a]	4.5±0.8[a]
Pair fed Control	13	6.2±1.4	11[c]	11.4±0.4	9.8±1.2	4.9±0.7	4.9±0.7

[a]The difference between the groups is not significant.

[b]Two rats were not pregnant, 2 rats delivered, but ate all the pups.

[c]One rat died during delivery, the second delivered but ate all the pups.

Table 10. Duration of time from mating to pregnancy and number of born and weaned pups in Trial 4 (Mean ± S.E.).

Group	Rats per Group (No.)	Time from Mating to Pregnancy (days)	Rats Delivering (No.)	Pups Born/dam (No.)	Pups weaned		
					Total (No.)	Male (No.)	Female (No.)
MAM-Ac Injected	5	7.5[a] ±1.3	3[b]	10.7[a] ±1.2	10.3[a] ±1.3	6.0[a] ±2.6	4.3[a] ±1.5
Pair-fed Control	5	9.0 ±2.1	4[c]	9.5 ±1.6	8.3 ±1.1	4.8 ±1.3	3.5 ±0.6

[a]The difference between the groups is not significant.

[b]One rat did not get pregnant, the second ate all the pups after delivery.

[c]The rat ate all the pups after delivery.

Table 11. Organ weights after the injection of 12 µl MAM-Ac or saline (Mean ± S.E.).

Group	Stage of Cycle	Rats (No.)	Body Wt. (g)	Anterior Pituitary (mg/100 g B.W.)	Ovarian Wt. (mg/100 g B.W.)	Uterine Wt. (mg/100 g B.W.)
MAM-Ac Injected	Metestrus	3	256.0±4.5	3.50±0.40[a]	41.0±4.3[b]	129.6±20.3[b]
	Diestrus	28	232.7±3.3	4.27±0.10[b]	38.0±2.2[b]	124.9± 5.2[b]
	Proestrus	9	254.4±9.0	4.49±0.40	40.8±4.3	199.3±30.7
	Estrus	6	264.8±6.8	4.62±0.16	33.7±3.4[c]	252.7±74.6
Pair fed Control	Metestrus	13	256.6±5.1	4.79±0.26	40.9±1.6	134.7± 4.0
	Diestrus	16	246.8±3.9	4.67±0.26	41.0±1.9	139.5± 7.5
	Proestrus	14	247.3±5.0	4.14±0.44	39.1±2.6	272.2±32.5
	Estrus	12	242.7±4.5	5.22±0.23	43.0±2.6	197.3±35.2
Positive Control	Metestrus	13	278.6±4.6	4.68±0.13	34.1±2.8	122.4± 6.5
	Diestrus	16	272.4±4.0	4.20±0.21	35.3±2.1	137.8± 8.2
	Proestrus	15	270.1±4.5	4.44±0.17	36.4±2.2	232.3±30.2
	Estrus	11	275.5±4.3	4.73±0.20	41.2±3.2	245.9±33.2

[a]Significantly different from both controls ($p<0.05$).

[b]Significantly different from both controls ($p<0.05$) when expressed on absolute basis.

[c]Significantly different from the pair fed control ($p<0.05$).

significantly lighter when compared with both controls and 14 to 16 days post injection they were lighter when compared with the pair fed control. The uteruses of the MAM-Ac injected rats compared with both controls per 100 g B.W. were significantly lighter ($p<0.05$) 1 to 3 days after the injection. Expressed on an absolute basis the uteruses of the MAM-Ac injected rats were lighter ($p<0.05$) 4 to 6 days after the injections (Table 12).

Histological examination of the ovaries revealed that for a period of approximately 8 days after the injection, MAM-Ac injected rats had ovaries filled primarily with well developed corpora lutea. Pair fed and positive controls had normal ovaries containing both corpora lutea and follicles (Figure 7).

The uteruses of the MAM-Ac injected rats at the same period of time were unstimulated. The endometrium was dense, the lumen was narrow and only a few glands were present. The pair fed and positive controls had normally stimulated uteruses (Figure 8).

Post Injection Prolactin and LH Levels

Pair fed and positive controls sacrificed at the same stage of the estrous cycle had the same concentrations of prolactin and LH in the blood and pituitaries. The only exception was the pair fed rats at diestrus which had significantly more ($p<0.05$) prolactin and LH in their

Table 12. Organ weights after the injection of 12 µl MAM-Ac or saline (Mean ± S.E.).

Group	Post Injection (days)	Rats (No.)	Body Wt. (g)	Anterior Pituitary (mg/100 g B.W.)	Ovarian Wt. (mg/100 g B.W.)	Uterine Wt. (mg/100 g B.W.)
MAM-Ac Injected	1- 3	15	238.1±3.5	4.80±0.20[b, d]	34.8±3.2 [c]	111.0± 7.9 [a]
	4- 6	14	223.2±3.3	4.08±0.10[b, d]	43.5±2.5 [a]	131.2± 7.5 [d]
	8-12	9	259.0±7.5	3.85±0.29[b, d]	36.9±2.7	225.3±36.7
	14-16	8	267.0±4.9	4.29±0.17	32.7±3.0	222.0±55.0
Pair-Fed Control	1- 3	15	246.0±4.0	4.70±0.33	40.9 ±2.1	195.1±18.6
	4- 6	15	235.9±3.1	4.75±0.27	38.0 ±3.4	179.9±30.8
	8-12	15	251.5±4.8	4.69±0.15	39.3 ±1.6	197.8±35.6
	14-16	10	264.8±3.3	5.09±0.37	43.3 ±2.2	164.6±15.2
Positive Control	1- 3	15	272.0±4.6	4.42±0.22	36.0 ±2.1	181.5±35.1
	4- 6	15	273.6±4.4	4.32±0.14	31.0 ±2.0	132.2± 8.7
	8-12	15	275.4±4.4	4.36±0.20	40.7 ±2.3	197.2±28.3
	14-16	10	274.7±4.0	5.02±0.13	38.8 ±3.0	221.8±24.6

[a]Significantly different from both controls ($p<0.05$).

[b]Significantly different from the pair-fed control ($p<0.05$).

[c]Significantly different from the positive control ($p<0.05$).

[d]Significantly different from both controls ($p<0.05$) when expressed on absolute basis.

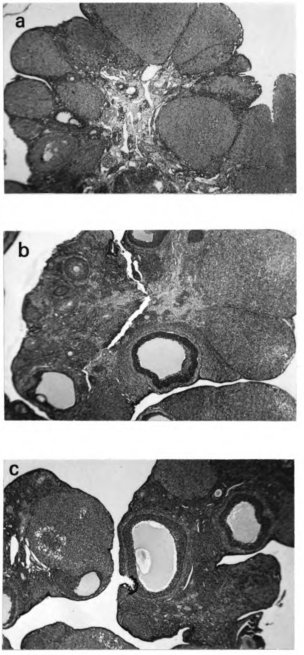

Figure 7. a) Photomicrograph of ovary from a representative rat 3 days after the injection of 12 µl MAM-Ac. Note dominance of well developed corpora lutea.
b) Photomicrograph of ovary from a pair fed control rat.
c) Photomicrograph of ovary from a positive control rat.
In both controls note the presence of both corpora lutea and mature follicles (hematoxylin and eosin stains, x 40).

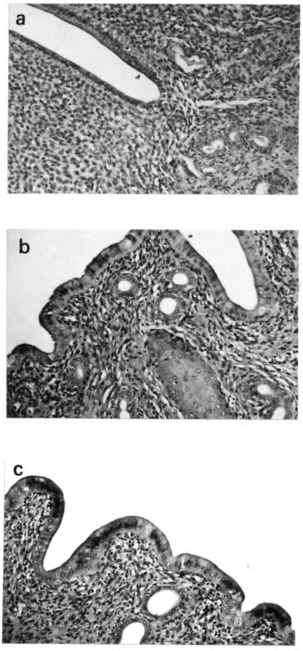

Figure 8. a) Photomicrograph of uterus from a rat 3 days after the injection of 12 µl MAM-Ac. Note the lack of estrogen stimulation.
b) Photomicrograph of uterus from a pair-fed control rat.
c) Photomicrograph of uterus from a positive control rat.
In both controls note the presence of estrogen stimulation (hematoxylin and eosin stains, x 200).

pituitaries. MAM-Ac injected rats had significantly less ($p<0.05$) prolactin in the pituitary during diestrus and significantly less ($p<0.05$) LH during diestrus, proestrus and estrus. Blood LH was significantly lower ($p<0.05$) during metestrus and diestrus (Tables 13-14).

Pair fed and positive controls, 1 to 16 days after the injections, had the same levels of blood and pituitary prolactin except for the period of 8 to 12 days after the injection when pair fed rats had significantly more ($p<0.05$) pituitary prolactin. Pair fed controls had significantly more ($p<0.05$) blood and pituitary LH than the positive controls during the period of 1-3 days after the injection. However, 4 to 6 days post injection they contained more LH in the blood and 14 to 16 days post injection they had significantly more LH in the pituitary. MAM-Ac injected rats had significantly less ($p<0.05$) pituitary prolactin 4 to 6 days post injection and less blood prolactin 14 to 16 days post injection. Pituitary LH was significantly lower ($p<0.05$) 1 to 3 days and 8 to 16 days post injection, while blood LH was lower 1 to 6 and 14 to 16 days after the injection (Tables 15-16).

Post Injection PIF and LHRF Activity

The activity of PIF appeared to be highest in the MAM-Ac injected rats, but statistically it was not significantly different from the activity in both control groups. Both controls had the same PIF activity (Table 17).

Table 13. Blood and pituitary prolactin levels after the injection of 12 µl MAM-Ac or saline (Mean ± S.E.).

Group	Stage of Cycle	Rats (No.)	Prolactin[a] (ng/ml serum)	(µg/mg anterior pituitary)	(µg/anterior pituitary)
MAM-Ac Injected	Metestrus	3	19.6± 4.0	8.6±0.3[b]	67.3±28.9[c]
	Diestrus	28	22.1± 1.6	3.7±0.3[b]	37.4± 3.2[c]
	Proestrus	9	117.9±30.2	4.1±0.6	45.3± 6.5
	Estrus	6	37.8±15.9	9.0±0.9	111.4±13.5
Pair Fed Control	Metestrus	13	24.8± 1.6	6.6±0.8[d]	77.0± 7.9[d]
	Diestrus	16	23.9± 1.4	6.1±0.3[d]	71.4± 6.6[d]
	Proestrus	14	196.9±45.8	3.8±0.5	45.2± 7.0
	Estrus	12	37.0± 6.5	9.4±0.9	123.4±18.3
Positive Control	Metestrus	13	20.5± 1.5	6.0±0.6	71.7± 5.6
	Diestrus	16	19.8± 1.2	4.3±0.2	48.5± 4.9
	Proestrus	15	121.7±33.8	3.3±0.3	38.7± 4.6
	Estrus	11	44.4± 7.5	7.7±0.6	102.8±14.2

[a]Reference preparation H-10-10-B.

[b]Significantly different from pair-fed controls ($p<0.05$).

[c]Significantly different from both controls ($p<0.05$).

[d]Significantly different from positive controls ($p<0.05$).

Table 14. Blood and pituitary LH levels after the injection of 12 µl MAM-Ac or saline (Mean ± S.E.).

Group	Stage of Cycle	Rats (No.)	LH[a] (ng/ml serum)	(µg/mg anterior pituitary)	(µg/anterior pituitary)
MAM-Ac Injected	Metestrus	3	4.0± 0.0[b]	44.6±4.7	517.1±87.8
	Diestrus	28	11.1± 2.5[b]	50.5±3.7[c]	500.4±38.0[c]
	Proestrus	9	479.0±368.0	43.3±4.2[c]	469.4±36.9
	Estrus	6	28.7± 9.9	46.1±6.1	563.4±78.6
Pair-Fed Control	Metestrus	13	50.2± 5.3	49.9±6.8	620.9±96.7
	Diestrus	16	36.3± 5.9	65.7±6.0	750.1±72.4[d]
	Proestrus	14	501.9±238.3	60.4±6.0	733.0±93.7
	Estrus	12	37.9± 2.7	51.3±6.6	642.1±84.9
Positive Control	Metestrus	13	41.0± 6.8	38.6±2.0	504.0±32.4
	Diestrus	16	25.9± 2.2	54.8±5.9	557.2±73.4
	Proestrus	15	84.9± 14.2	52.7±3.2	620.6±43.0
	Estrus	11	43.8± 4.3	51.8±2.3	660.0±29.1

[a]Reference preparation - NIAMD-Rat-LH-RP-1.

[b]Significantly different from both controls ($p<0.05$).

[c]Significantly different from pair-fed controls ($p<0.05$).

[d]Significantly different from positive controls ($p<0.05$).

Table 15. Blood and pituitary prolactin levels after the injection of 12 µl MAM-Ac or saline (Mean ± S.E.).

Group	Post Injection (days)	Rats (No.)	Prolactin[a] (ng/ml serum)	(µg/mg anterior pituitary)	(µg/anterior pituitary)
MAM-Ac Injected	1- 3	15	41.7±16.2	3.3±0.2	38.1± 3.2[b]
	4- 6	14	30.8± 4.9	3.5±0.3[c]	32.0± 3.3[c]
	8-12	9	80.2±32.4[b]	5.9±1.0	58.5±10.4
	14-16	8	22.2± 3.2[b]	8.7±1.0	100.8±18.4
Pair Fed Control	1- 3	15	67.5±27.8	4.6±0.6	65.3±10.2
	4- 6	15	44.9±17.8	6.1±0.6[d]	70.4±10.2
	8-12	15	76.8±27.9	7.2±0.8	83.8±12.3
	14-16	10	106.5±53.8	8.9±1.4	123.7±27.9
Positive Control	1- 3	15	71.1±23.8	4.4±0.7	55.9±12.3
	4- 6	15	22.5± 2.1	4.9±0.3	58.2± 4.9
	8-12	15	56.0±27.4	5.1±0.3	60.8± 5.0
	14-16	10	37.0± 5.6	7.5±0.9	104.7±14.2

[a]Reference preparation H-10-10-B.

[b]Significantly different from pair-fed controls ($p<0.05$).

[c]Significantly different from both controls ($p<0.05$).

[d]Significantly different from positive controls ($p<0.05$).

Table 16. Blood and pituitary LH levels after the injection of 12 µl MAM-Ac or saline (Mean ± S.E.).

Group	Post Injection (days)	Rats (No.)	LH[a] (ng/ml serum)	(µg/mg anterior pituitary)	(µg/anterior pituitary)
MAM-Ac Injected	1- 3	15	8.5± 2.7[b]	49.9±4.6[c]	556.7±55.7[c]
	4- 6	14	22.9± 10.4[c]	49.8±6.8	450.9±48.7
	8-12	9	265.7±211.6[d]	53.0±7.4	505.2±63.4
	14-16	8	25.3± 8.6[d]	47.3±8.0	546.8±99.1
Pair Fed Control	1- 3	15	590.8±369.7[d]	69.0±5.8[d]	808.4±85.5[d]
	4- 6	15	158.6± 97.4[d]	53.0±6.0	592.3±74.9
	8-12	15	50.1± 7.0	68.6±6.5[d]	795.5±74.2[d]
	14-16	10	282.6±207.0	34.2±3.7[d]	462.9±65.5
Positive Control	1- 3	15	35.8± 9.1	51.4±6.1	546.5±80.0
	4- 6	15	30.0± 5.7	45.4±4.1	523.8±34.6
	8-12	15	55.9± 12.2	52.8±5.0	619.7±59.3
	14-16	10	72.1± 10.6	60.0±5.8	824.8±80.2

[a]Reference preparation NIAMD-Rat-LH-RP-1.

[b]Significantly different from both controls ($p<0.05$).

[c]Significantly different from pair-fed controls ($p<0.05$).

[d]Significantly different from positive controls ($p<0.05$).

Table 17. PIF and LHRF in the hypothalami of rats injected with 12 µl MAM-Ac or saline (Mean ± S.E.).

Group	Post Injection (days)	Hypothalami Pooled (No.)	Prolactin Released in Med.[a] (ng/mg anterior pituitary)	LH Released in Med.[b] (µg/mg anterior pituitary)
Pituitary Alone	--	--	438±51	2.37±0.30
MAM-Ac Injected	1- 6	29	198±27	5.10±0.53[c]
	8-16	17	225±19	6.17±0.62
Pair Fed Control	1- 6	30	228±33	5.88±0.74
	8-16	25	283±45	5.23±0.63
Positive Control	1- 6	30	257±37	6.90±0.54
	8-16	25	274±51	5.64±0.30

[a]Reference preparation H-10-10-B.

[b]Reference preparation NIAMD-Rat-LH-RP-1.

[c]Significantly different from the positive control ($p<0.05$).

LHRF was found to be significantly lower ($p<0.05$) in the MAM-Ac injected rats, but only when compared with the positive controls. Both controls had the same LHRF activity (Table 17).

MAM-Ac and Estrogen Interaction in Spayed Rats

Spaying rats caused a constant presence of leukocytes in their vaginal smears. The injection of 0.2 µg estrogen daily caused the disappearance of these cells and the appearance of cornified cells typical of the estrous stage. The injection of MAM-Ac into spayed rats treated with estrogen resulted in the reappearance of leukocytes which lasted 3 days and then started to decrease. The injection of MAM-Ac into rats treated with corn oil without estrogen resulted in the appearance of leukocytes during the entire period.

After the rats were sacrificed, it was found that the injection of 10 or 12 µl MAM-Ac and estrogen resulted in rats with significantly heavier uteruses ($p<0.05$) in comparison to rats injected only with MAM-Ac. Rats injected with MAM-Ac and estrogen also had significantly heavier adrenals and significantly lighter thymuses ($p<0.05$) when compared with rats injected only with MAM-Ac (Table 18).

Table 18. Body and organ weights of spayed rats treated with estrogen and MAM-Ac (Mean ± S.E.).

Treatment	Rats Per Group (No.)	Initial Body Wt. (g)	Body wt. at Injection of MAM-Ac (g)	Body wt. at Sacrifice (g)
Corn oil + 10 µl MAM-Ac	3	225.3±6.3	291.0±9.5	287.7±13.4
Corn oil + 12 µl MAM-Ac	3	236.3±0.3	303.3±4.5	294.0±12.5
Estrogen in Corn oil + 10 µl MAM-Ac	6	229.5±4.3	255.8±4.5	243.0±5.8
Estrogen in Corn oil + 12 µl MAM-Ac	3	224.0±3.8	249.3±3.7	227.3±6.4

Table 18 (Cont'd.)

Uterine Wt. (mg/100 g B.W.)	Adrenal Wt. (mg/100 g B.W.)	Thymus Wt. (mg/100 g B.W.)
38.7± 1.7[a, b]	23.8±1.5[a]	196.6±23.2[b]
30.5± 1.2[c]	17.1±1.2[c]	193.4±11.1[c]
142.2± 9.4	28.4±1.4[d]	139.4± 9.3
147.3±11.5	46.2±5.2	112.0±30.9

[a]Significant difference ($p<0.05$) between corn oil treated rats injected with 10 and 12 µl MAM-Ac.

[b]Significant difference ($p<0.05$) between 10 µl MAM-Ac injected, corn oil and estrogen treated rats.

[c]Significant difference ($p<0.05$) between 12 µl MAM-Ac injected, corn oil and estrogen treated rats.

[d]Significant difference ($p<0.05$) between estrogen treated, 10 and 12 µl MAM-Ac injected rats.

Discussion

It has been well established in rats that starvation or reduction of food intake prolongs the estrous cycle and decreases reproductive performance (Mulinos *et al.*, 1939). This is the reason why positive control and pair fed control groups were used. Conducting the experiments this way enabled the distinction between the effects of reduction in food intake and reduction in food intake plus MAM-Ac injection. Pair fed and MAM-Ac injected rats consuming equal amounts of food were losing the same amount of body weight (Figures 1-4). The similar reduction in body weight was accompanied by the same reduction in body fat (Table 2). The positive control rats had significantly more body fat. The amounts found in this experiment are in good agreement with the amounts found in mature female rats (Schemmel *et al.*, 1969).

Dose response was achieved in the reduction in food intake and body weight when different amounts of MAM-Ac were injected. The higher the level of MAM-Ac injected the more severe was the reduction in food intake and body weight (Figures 1-4).

Yang and Mickelsen (1968) described some of the symptoms appearing after cycad feeding. The rats were pale and had an anemic appearance, very yellow urine, sometimes with blood, and were cold to the touch. The same symptoms appeared when MAM-Ac was injected.

Trauma, infection, intoxication, hemorrhagic shock, cold exposure and other stressful stimuli produced an "alarm reaction." The adrenals became hypertrophic and atrophy of the lymphoid tissues and lymphopenia occurred (Selye, 1936). In these studies, the stress caused hypertrophy of the adrenal and the atrophy of the thymus (Tables 3 and 18). The weights of the adrenals of the pair fed and positive controls were not increased. Signs of general response to stress were noticed too in the histological examination of the adrenals of the MAM-Ac injected rats. In addition to hypertrophy some hemorrhages were noticed. The histological appearance of the adrenals from both controls was normal (Figures 5-6).

MAM-Ac intoxication did not last long. Food intake was restored to normal within a week (Figure 1 and 2) and body weight within 2 weeks after the injection (Figure 3 and 4). Another proof for the short lasting effects of MAM-Ac intoxication comes from the reproductive performance studies (Tables 7-10). Immediately after the injection of MAM-Ac the rats did not become pregnant. However, after the rats had recovered it took the MAM-Ac injected rats and the pair fed controls the same period of time to become pregnant. In addition in both, control and MAM-Ac injected groups,the number of born and weaned pups was the same.

The normal length of an estrous cycle in rats is 4-5 days (Evans and Bishop, 1922). Reduction of food intake is

known to prolong the cycle (Mulinos *et al.*, 1939). In these experiments the reduction in food intake alone was not severe enough to significantly prolong the cycle. The pair fed controls in trials 2 and 6 did not have significantly prolonged cycles after the reduction in food intake (Tables 5 and 6). However, reduction in food intake plus MAM-Ac injection caused a significant prolongation in the first cycle after the injection. Since this cycle was completed within 9.0 to 9.8 days and since the intoxication period was the same, it is expected that the following cycles would be as long as they were in the pair fed controls. The more MAM-Ac that was injected, the more prolonged was the cycle. Rats injected with 10 µl MAM-Ac prolonged their cycles less than rats injected with 12 µl (Tables 4, 5 and 6). The first cycle after injection was prolonged by an increased length of the diestrus stage. Histological examination of the ovaries and uteruses revealed that MAM-Ac injected rats had ovaries and uteruses typical of rats at diestrus. This lasted for approximately 8 days after injection. Except for the predominance of corpora lutea in the ovaries and the lack of estrogen stimulation of the uteruses, no pathological lesions were observed. Pair fed and positive controls had normally stimulated organs throughout the experiment (Figures 7 and 8).

Pituitary, ovarian and uterine weights were statistically the same in both pair fed and positive controls (Tables 11

and 12) and comparable to weights reported by others (Minaguchi *et al.*, 1968; Voogot *et al.*, 1970). Pituitary prolactin and LH were higher in pair fed controls ($p<0.05$) compared to the positive controls only during diestrus. It has been shown that reduction in food intake reduces production and release of the gonadotropins (Leathem, 1966). It was also shown that the maximum content of pituitary prolactin and LH is on the afternoon of diestrus (Monroe *et al.*, 1969). Because of the fact that blood prolactin and LH and the levels of PIF and LHRF were similar in both control groups it does not seem that the pair fed rats were affected by the reduced food intake or that the hormone was discharged to the blood later in the afternoon in the pair fed control rats. The reason for the differences between both controls remains unknown at present. During the period of 1 to 6 days after the injection the same number of rats at the various stages of the cycle was sacrificed in both control groups. This is the reason why the differences in the concentration of LH in the blood seems surprising (Tables 16 and 17). These differences can be easily explained using the findings of Gay *et al.* (1970). They reported that LH is discharged to the blood on the afternoon of diestrus and its half life is 20 min. The rise is very sharp and the increase may be of a magnitude of 50 fold. It is possible that more positive controls were sacrificed before they had discharged the hormones to the

blood, and so pair fed controls had higher levels of hormones in their blood. The large standard error obtained thus supports this hypothesis. During the period of 1 to 3 and 14 to 16 days after the injection, pituitary LH in the pair fed controls was higher than that in the positive controls. This was also true for 8 to 12 days after the injection. As for the higher LH concentrations in the pair fed controls at diestrus, no explanation can be offered for these findings.

Reduction of food intake plus MAM-Ac injection resulted in more rats sacrificed at diestrus than in both control groups. The MAM-Ac injected rats killed at diestrus had significantly lighter ($p<0.05$) pituitaries, ovaries and uteruses when expressed on an absolute basis. Since MAM-Ac injected and pair fed controls had similar body weights after injections and since such reduction in organ weights was not noticed in the pair fed controls, the reduction in organ weights can be attributed to the intoxication by MAM-Ac. The uterus seems to be the first organ that responded to MAM-Ac intoxication. Its decreased weight was noticed one day following the injection and it remained lower than control values for 6 days. Pituitary and ovarian weights decreased starting from the fourth day after injection (Tables 11 and 12).

Blood prolactin levels were similar in all groups 1 to 12 days after the injection and they became lower only during

14 to 16 days after the injection of MAM-Ac (Table 15). It does not seem that this reduction has any physiological significance. Apparently it happened that more rats of the MAM-Ac group were killed before the rise in prolactin late on the afternoon of diestrus. The relatively high levels of prolactin 1 to 6 days post injection compared to the levels of LH at this time are of great importance. The corpora lutea are maintained and supported by prolactin in the rat (MacDonald and Greep, 1968). As indicated before, the rats of the MAM-Ac injected group had ovaries filled primarily with corpora lutea for a period of 8 days. The levels of prolactin needed for the support of corpora lutea have not been established yet, but since prolactin did not decrease as did LH (Tables 15 and 16) it is possible to assume that the amounts present were able to support the corpora lutea. Prolactin levels obtained in rats sacrificed at diestrus were normal, whereas the levels of LH were lower from those sacrificed at diestrus (Monroe *et al.*, 1969; Parlow *et al.*, 1969). Since most of the rats injected with MAM-Ac were sacrificed at diestrus during the first 6 days after the injection, it is expected that the levels of prolactin would be unchanged and the levels of LH would decrease during this period.

In contrast with pair fed controls, MAM-Ac injected rats at diestrus had less prolactin and LH than pair fed controls in their pituitaries. Pituitary LH was found to

be lower too in MAM-Ac injected rats at proestrus and estrus. It is difficult to draw any conclusions regarding the MAM-Ac injected rats at metestrus since only 3 rats were in this stage at time of sacrifice (Tables 13 and 14). The concentrations of prolactin in the pituitary were lower 1 to 3 days after the injection compared with the pair fed control rats and the concentration of LH was lower 4 to 6 days after injection compared with both groups of control. Because of the fact that the pituitaries were lighter after the injection of MAM-Ac, the content of prolactin in the pituitary was lower 1-6 days post injection and the LH content was lower 1 to 3 days and 8 to 16 days post injection compared with at least one of the control groups. Since PIF activity was similar in all groups (Table 17), one would expect equal pituitary and blood levels of prolactin in the 3 groups. However, this is not the case since the pituitary from MAM-Ac groups contained less prolactin during diestrus and 1 to 6 days post injection. This indicates that the pituitary was the organ affected most by the injections of MAM-Ac. LHRF activity was found to be higher in the positive controls than in the MAM-Ac injected rats, 1 to 6 days post injection. As a result one would expect more LH in the pituitaries of the MAM-Ac injected rats. Practically identical levels were found in both groups. Concomitantly, similar LHRF activities were found in MAM-Ac injected and pair fed rats, however more

pituitary LH was found in the pair fed rats. The lower levels of blood LH at this time can be explained on grounds of less LHRF activity. From these results too, it seems that the pituitary is the major gland intoxicated by MAM-Ac.

MAM-Ac was reported to exert radiomimetic effects (Teas *et al.*, 1965). It was found too, that x-ray irradiation of ovaries caused sterility. The ovaries were less responsive to gonadotropins and vaginal smears were continuously cornified for 2 to 14 weeks, then the smears became similar to smears found in spayed rats (Mandl and Zuckerman, 1956). From these experiments it seems that MAM-Ac does not exert the effects described by x-rays. The cells found in vaginal smears after the injection of MAM-Ac were leukocytes which are typical of diestrus and not cornified cells found after the irradiation. Sterility or a reduction in ovarian sensitivity to the gonadotropins causes an increase in the levels of the gonadotropins (Flerko, 1963). In our experiments we found identical blood prolactin in all 3 groups and lower blood LH in the MAM-Ac injected rats than in the controls. The presence of well developed corpora lutea and the absence of mature follicles in the ovaries of MAM-Ac injected rats suggest the influence of low levels of estrogen and FSH and high levels of progesterone.

As a response to MAM-Ac intoxication an increase in the weight of the adrenals and a decrease in the weight of the thymus occurred (Table 18). It has been reported that stresses

may cause an increase in the synthesis and release of progesterone from the adrenals of ovariectomized rats. Stress caused elevation of ACTH levels which in turn increased progesterone secretion (Resko, 1969). In this study it is shown that injection of MAM-Ac to spayed rats receiving estrogen daily caused a change in the histology of the vaginal smears. The typical cells of the spayed rats were replaced with leukocytes which lasted for 3 days and then started to decline. Since the ovaries were removed the only source of progesterone to stimulate the uterus was the adrenals. How much progesterone is secreted from the adrenals after MAM-Ac injections in the normal rat and what is its influence on the ovaries is not known.

It is possible that MAM-Ac acts directly on the brain. The reduction in food intake observed after MAM-Ac injection (Figure 1 and 2) could have been caused by signals from the stimulation of the satiety center in the ventro medial nuclei or the depression of the hunger center in the lateral hypothalamus (Mayer, 1970). The brain was reported to be affected by MAM-Ac in another fashion too. Lesions in the brain of several species were reported after the administration of MAM-Ac to one day old rats or during pregnancy (Spatz *et al.*, 1967c; Hirono *et al.*, 1969a). PIF activity was not affected by MAM-Ac while LHRF activity was lower only when compared with the positive control but not the pair fed control. The concentrations of prolactin and LH

in the pituitary were generally the same for all animals. However, since pituitary weight was decreased in the MAM-Ac injected rats, the total pituitary contents of prolactin and LH were thus less in the MAM-Ac injected rats than in both controls. Although these studies were aimed to examine the mechanism of action of MAM-Ac on the ovary-pituitary-hypothalamus axis, it remains unknown whether MAM-Ac is affecting this axis directly or not. It is still unresolved as to whether the effect is mediated through the ovary, adrenals or brain.

PART II

IMPLANTATION OF CYCASIN AND MAM-Ac IN THE BRAIN THIRD VENTRICLE OF RATS AND MICE

PART II

IMPLANTATION OF CYCASIN AND MAM-Ac IN THE BRAIN THIRD VENTRICLE OF RATS AND MICE

Objective

One day old mice and dogs injected with MAM-Ac developed lesions in the brain (Hirono and Shibuya, 1967; Hirono *et al.*, 1969a). MAM-Ac injections to one day old rats and to mature mice did not result in such lesions. However, rats developed microcephaly and hydrocephaly only when injected with MAM-Ac in prenatal life (Spatz, 1969). Adult humans and cattle developed neurological disorders after the consumption of cycad material (Whiting, 1964; Hall and McGavin, 1968).

This evidence suggested to us that MAM-Ac may have a different potential to enter the central nervous system (CNS) at various stages in life. The purpose of the present experiment was to determine whether the blood-brain barrier prevents the toxicity of cycasin and MAM-Ac in weanling and mature rats and mice.

Review of Literature

The Blood-Brain Barrier

The first observations concerning the blood-brain barrier are attributed to Ehrlich during the years 1885-1887 (Bakay, 1956). In his studies he noted that intravenous injection of Coerulein-S, an aniline dye into experimental animals would stain most tissues of the body readily, while the brain remained uncolored. During the following years such observations were confirmed and the introduction of radioisotopes helped explain the nature of the blood-brain barrier.

The blood-brain barrier is not present early in life, but it develops gradually as the organism develops. It has been shown that in most species the brain undergoes a period of rapid growth earlier than the corresponding period for the rest of the body. The lack of barrier effect seen in the neonatal brain is due to the increased need for substances by the developing brain (Dobbing, 1961). On the other hand some reports suggest that the blood-brain barrier develops early in embryonic development. Bakay (1953), by using P^{32} showed that even during intrauterine life some barrier activity was present. Dobbing (1963) points out that the correct timing in the introduction of various substances is of most importance. The lack of right timing can explain the variation in results when different substances are used at different stages in life.

It is generally accepted that the blood-brain barrier is more permeable to basic dyes while the blood-cerebrospinal fluid barrier is more permeable to acid dyes (Davson and Danielli, 1943; Koenig and Hurbebaus, 1966). Bakay (1953) summarized results of experiments done with radioactive isotopes of ions. It was found that ions of Cl^{38}, I^{135}, K^{42}, S^{35}, Na^{24} and Br^{32} reach a relatively quick equilibrium between the plasma and the tissues except brain. The penetration of such ions to the brain is delayed and only small amounts penetrate. D_2O, on the other hand, was found to penetrate this barrier easily. Rapid exchange exists between plasma and brain amino acids (Lajtha, 1962). However, the entry of radioactively labelled proteins from the blood into the brain is much smaller in amount and slower than its entrance to other organs (Fishman, 1953; Bering, 1955). Some drugs were found to be able to penetrate the barrier. Sulfonamides and penicillin enter the brain from the blood. Streptomycin does not enter (Quastel and Quastel, 1961).

It has been reported that the lipid soluble substances enter the brain faster than water soluble substances (Davson, 1955). Diseases of the central nervous system that are severe enough to alter its structural organization result in an increase in the permeability of the blood-brain barrier (Bakay, 1968). Quadbeck (1968) reported on various drugs affecting the penetration of several ions and dyes.

Materials and Methods

Sixty-two Sprague-Dawley female rats (Spartan Research Animals, Haslett, Mich.) and 33 female C-57BL/6; mice (Jackson Lab., Bar Harbor, Maine) were used in 8 experiments as indicated in Tables 19 and 20. All rats were fed and housed as described in Part I. The mice were housed in the same laboratory as the rats but they were housed three to four mice in one plastic cage. They were allowed free access to Purina mouse chow and water.

Cycasin dissolved in water (547 mg cycasin/ml), liquid MAM-Ac and MAM-Ac mixed with cholesterol were implanted in rats and mice as indicated in Tables 19 and 20. The procedure used was described by Rowland (1966).

Results and Discussion

The third ventricle was chosen as the site of implantation because of its location in the brain and the fact that from this ventricle the implanted material can easily reach other parts of the brain.

Rats implanted with cholesterol pellets with or without 0.2 mg MAM-Ac, and 10-30 mg cycasin in water solution, recovered quickly from the surgery and did not show any signs of MAM toxicity. MAM-Ac implantation resulted in the appearance of the toxic signs typical to MAM-Ac intoxication which were described by Yang and Mickelsen (1968). The more

Table 19. The division of rats into various experiments of cycasin and MAM-Ac implantations.

Experiment	Rats Used (No.)	Age of Rats	Rats implanted with			
			0.2 mg MAM-Ac in Cholesterol pellet (No.)	Cholesterol Pellet (No.)	Cycasin[a] (No.)	MAM-Ac[b] (No.)
R/1	15	2 months	10	5	--	--
R/2	26	2 months	--	-	10	16
R/3	21	21 days	--	-	11	10

[a]The rats in experiment R/2 were implanted with 20-30 mg cycasin and in experiment R/3 with 10-20 mg cycasin.

[b]The rats in experiment R/2 were implanted with 2-10 µl MAM-Ac and in experiment R/3 with 2-4 µl MAM-Ac.

Table 20. The division of mice into various experiments of cycasin and MAM-Ac implantations.

Experiment	Mice Used (no.)	Age of Mice Used (Months)	Mice implanted with			
			0.2 mg MAM-Ac in Cholesterol pellet (No.)	Cholesterol Pellet (No.)	Cycasin[a] (No.)	MAM-Ac[b] (No.)
M/1	6	3-4	3	3	-	-
M/2	10	3-4	-	-	5	5
M/3	5	4-5	-	-	-	5
M/4	5	1	-	-	2	3
M/5	7	1	-	-	-	7

[a]The mice were implanted with 8-12 mg cycasin.

[b]The mice were implanted with 2-4 µl MAM-Ac.

MAM-Ac implanted, the more severe was the toxicity. Although general signs of toxicity appeared, no gross neurological disorders were noticed.

Mice implanted with cholesterol pellets, cholesterol pellets with MAM-Ac, and those implanted with 8 to 12 mg cycasin, recovered quickly from the surgery and did not show any signs of cycasin or MAM toxicity. However, the implantation of MAM-Ac resulted in severe toxicity and sometimes death. Out of the 5 mice implanted with MAM-Ac in experiment M/2, 3 died, 2 of which had trembled slightly before death. In the following experiments, M/3, M/4 and M/5, the same percentage of mice died, but none of them showed trembling or other neurological disorders.

Because of the fact that rats and mice implanted with 0.2 µl MAM-Ac and cycasin did not develop gross neurological disorders following the implantations, it was decided not to implant controls unless neurological disorders were seen following MAM-Ac implantations.

It has been shown in the rat that the enzyme β-glucosidase which cleaves cycasin into MAM is present and active only in the skin for a short period after birth (Spatz, 1968). This is apparently the reason why the rats and the mice were not intoxicated by the cycasin implanted. The rats and the mice were not intoxicated by 0.2 mg MAM-Ac. This amount is apparently too small to cause any damage since even 2 µl MAM-Ac implanted had little effect (Figure 9).

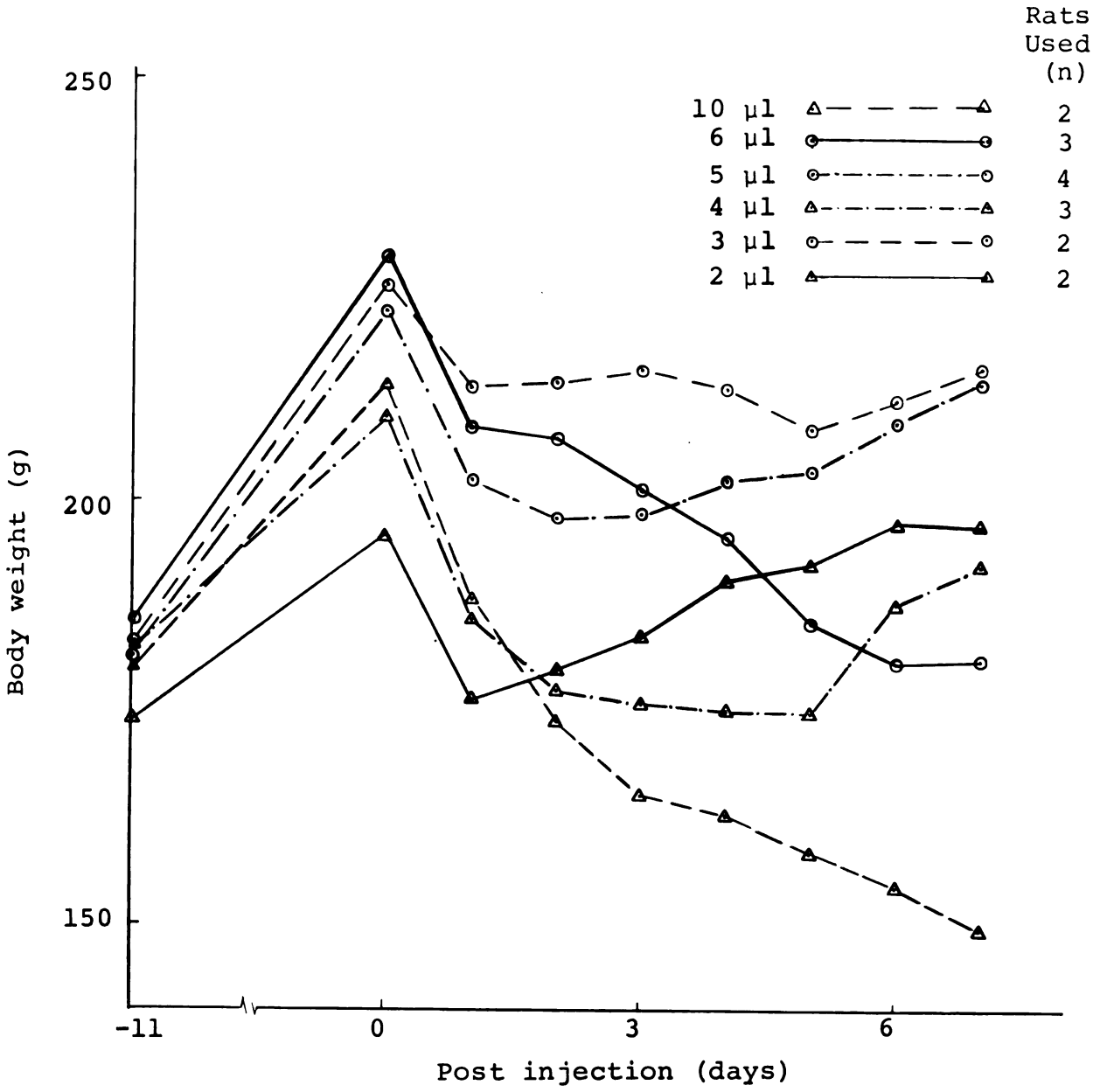

Figure 9. Body weight changes after implantation of 2-10 µl MAM-Ac to rats.

The more MAM-Ac implanted, the more severe the toxicity and body weight loses recorded (Figure 9).

Only 2 mice out of 62 rats and 33 mice implanted, came down with neurological disorders. Such disorders could not have been reproduced although attempts were made. Thus, the only conclusion must be that the implantation of MAM-Ac and cycasin to the third ventricle of rats and mice, mature and young,did not cause any gross neurological disorders.

One of the most important uses of stereotaxic techniques is in experiments where the effects on the brain of substances that can not pass the blood-brain barrier, are determined (Rowland, 1966). So far it is not known how much of the MAM-Ac injected to experimental animals subcutaneously reaches the brain. It is still unknown how effective the blood-brain barrier is in preventing MAM from entering the brain in the different species. These studies show that the presence or absence of MAM in the brain is not the determining factor for the development of neurological disorders in mature rats and mice. It has been reported that only during a limited period of time it is possible to induce neurological disorders and lesions in rats, mice, hamsters and dogs by injecting MAM-Ac (Spatz, 1969; Yang, 1971; Hirono and Shibuya, 1967). Thus, it appears that the most important factor in the etiology of neurological disorders is the stage of brain development at the time of its exposure to MAM. However, this explanation does not

seem to fit with findings in human and cattle studies. It has been reported that adult humans and cattle may develop neurological disorders after consuming cycad material (Whiting, 1964; Hall and McGavin, 1968). Thus, it appears that in human subjects and in cattle the blood-brain barrier may have an important role in preventing greater MAM toxicity to the neurological system.

PART III

PHYSIOLOGICAL AGE AND THE SENSITIVITY OF RATS TO CYCASIN TOXICITY

PART III

PHYSIOLOGICAL AGE AND THE SENSITIVITY OF RATS TO CYCASIN TOXICITY

Objective

One day old mice injected with MAM-Ac developed lesions in their central nervous system (CNS) (Hirono and Shibuya, 1967). Rats at the same age did not develop such disorders. The fact that mice are born after 19 days of gestation whereas rats after 21-23 days, suggested to us that a possible reason for the differences in the reaction to MAM-Ac injection was due to differences in the developmental stage of the CNS at time of exposure to the toxin. Therefore, we decided to examine whether rats at the same stage of CNS development as mice would show the same neurotoxic symptoms subsequent to MAM-Ac or cycasin injection.

Review of Literature

Three major stages of development can be distinguished from the time of fertilization to birth. Embryogenesis is the first stage lasting for 7.5 days in mice and 9.5 days in rats. This stage is followed by organogenesis which lasts up to the 16th day of gestation in the mouse and 18.5 in the rat. Next is the developmental stage of the fetus which lastsuntil the day of delivery. Parturition occurs on the

19th day of gestation in the mouse and during 21-23 days in the rat (Christie, 1964; Edwards, 1966; Rugh, 1968).

Embryogenesis starts with the fertilization of the egg and lasts until the mesoderm and the neural plate are developed. During this stage cell division, implantation and heart appearance are the major events. Organogenesis begins with the appearance of the neural plate. During this stage all tissues and organs start their differentiation. As a general rule the mouse reaches each developmental stage 1-2 days before the rat. The major developmental events during this stage are the differentiation of nerves, brain and sense organs from the ectoderm; digestive system, lungs and glands from the endoderm; and bones, blood vessles and gonads from the mesoderm. A detailed description of the development of mouse and rat embryo can be found in Hicks (1968); Christie (1964); Edwards (1964); and Rugh (1968). The final three days or so of gestation are the fetus stage which is characterized more by growth than by differentiation. This stage ends with parturition.

Most differentiation is in fact completed by the 16th day in the mouse and by day 18.5 in rat. Certain organs such as cerebellum do not mature until after birth. It has been reported that in rats the brain reaches its maximum weight per unit body weight only 10 days after birth. Similar results were obtained in studies with mice (Davison and Dobbing, 1966; Rugh, 1968).

By exposing the embryo to appropriate compounds at the right time one can induce a variety of abnormalities. According to Rice (1969) introduction of such compounds during embryogenesis causes lethal damage to the embryo. Teratogenesis results when such compounds are introduced in early organogenesis and carcinogenesis results when appropriate compounds are introduced during late organogenesis and during the fetus stage.

The effects of diazo dyes on the embryo were studied rather extensively in the past. A dye, trypan blue, was reported to cause cardiovascular malformations in the rat when introduced on the 7th, 8th or 9th day of gestation. Abnormalities of the genito-urinary system were reported when the dye was introduced during the 13th to the 17th day of gestation. During days 16 and 17 of gestation stenosis and occlusion were reported and during days 18 to 20 of gestation the appearance of hydrocephalus was noticed (Beck and Lloyd, 1967). Similar studies were conducted to study the effects of X irradiation on the embryo. A dose of 200 r on the 9th day of gestation kills the embryo whereas 100 r causes cardiac malformation: Abnormalities of the forebrain, spinal cord and cerebral hemispheres are obtained after irradiation during the period of days 13 to 19 of gestation. The cerebellum on the other hand differentiates first on the 16th day of gestation and malformation in it appears if irradiated on the 18th day of gestation and up to one week

after birth (Hicks and D'Amato, 1967). MAM was reported to have similar effects on the brain when introduced during days 14 to 16 of gestation (Spatz, 1969).

Many different drugs and various treatments were studied with relation to malformations in embryos. Cardiovascular malformations in various mammals were reported after the following treatments: administration of trypan blue, thalidomide and streptonigrin; X irradiation, hypoxia and hypercapina; and deficiency in folic acid or pantothenic acid or riboflavin (Takas and Warkany, 1967). Teratogenic effects were reported to be produced by X irradiation, administration of diazo dyes or nitroso and azoxy compounds. Such treatments as hyperthermia causes the same effects (Beck and Lloyd, 1967; Hicks and D'Amato, 1967; Spatz and Laqueur, 1967; Edwards, 1968; Rice, 1969).

Materials and Methods

Fifty-nine pregnant Sprague-Dawley rats were used on days 13 through 20 of gestation. To obtain these timed pregnant rats, 3-4 females were placed with a proven male and vaginal smears taken daily to determine pregnancy. The appearance of sperm in the smear was designated as the first day of gestation. At this time the pregnant rats were transferred to maternity cages. All rats were fed *ad libitum* and were housed in an animal laboratory under the conditions described in Part I (Materials and Methods).

Operational Procedure

The rats were anesthetized with ether and the hairs shaved off the abdomen. A midline abdominal incision was made and the double horned uterus exteriorized. Each pup was injected under the skin with MAM-Ac, saline or cycasin using a micro-syringe. The number of injected fetuses was recorded and the uterus returned to its normal location. The muscles and the skin were sutured separately with silk thread. The rats were allowed to recover and were returned to their cages for continuation of gestation and delivery.

Treatments

The pups were injected in utero with 0.8-2.1 mg cycasin or 3-1 µl MAM-Ac or 1 µl saline, as indicated in Tables 21-23. The number of born and weaned pups was recorded. The weaned pups were allowed to grow for 6 months and then all of them were sacrificed and examined for presence of tumors and malformation. The rats which died during the 6 months were examined for cause of death.

Results and Discussion

After the injection of saline to fetus in utero, only 24% of the injected fetuses were weaned and only 30% of the mothers that underwent laparatomy were able to wean pups. When cycasin was injected, 41% of the mothers that underwent laparatomy weaned pups and about 20% of the injected pups

Table 21. Summary data on rats undergoing laparatomy, injected with cycasin.

Stage of gestation (day)	Cycasin injected (mg/fetus)	Rats used (No.)	Fetuses injected (No.)	Rats that weaned pups (No.)	Rats that died during delivery (No.)	Rats that consumed their pups after delivery (No.)	Pups weaned (No.)
13	0.8	2	20	0	0	2	0
14	0.8	3	37	0	0	3	0
	1.0	1	11	1	0	0	1
	1.3	3	31	0	0	3	0
15	0.8	2	25	2	0	0	6
	1.3	3	25	1	0	2	4
16	0.8	2	23	1	0	1	1
	1.3	4	41	1	1	2	3
18	0.8	3	39	3	0	0	31
	1.3	1	6	1	0	0	3
19	1.3	3	30	2	0	1	19
	1.5	1	9	1	0	0	4
	1.7	2	20	0	1	1	0
20	2.1	2	22	1	1	0	1
21	2.1	2	23	0	0	2	0
Total		34	317	14	3	17	73

Table 22. Summary data on rats undergoing laparatomy, injected with MAM-Ac.

Stage of Gestation (day)	Amt. of MAM-Ac Injected (µl/fetus)	Rats Used (No.)	Fetuses Injected (No.)	Rats that			Pups weaned (No.)
				Weaned pups (No.)	Died during delivery (No.)	Consumed their pups after delivery (No.)	
14	0.3	1	11	0	0	1	0
	0.5	3	27	1	1	1	3
15	0.3	2	24	0	0	2	0
	0.5	2	26	0	1	1	0
16	0.3	2	23	1	0	1	3
	0.5	3	34	2	1	0	5
17	0.5	1	12	1	0	0	2
19	1.0	1	10	0	1	0	0
Total		15	167	5	4	6	13

Table 23. Summary data on rats undergoing laparatomy, injected with saline.

Stage of Gestation (day)	Rats Used (No.)	Fetuses Injected (No.)	Rats that			Pups Weaned (No.)
			Weaned Pups (No.)	Died during Delivery (No.)	Consumed their pups After delivery (No.)	
14	2	22	1	0	1	3
15	2	26	0	0	2	0
16	2	21	1	1	0	3
17	1	8	0	1	0	0
18	1	14	0	0	1	0
19	1	12	1	0	0	9
20	1	8	0	1	0	0
Total	10	111	3	3	4	15

were weaned. In MAM-Ac injections only 8% of the injected pups were weaned compared to 33% of the mothers that underwent laparatomy being able to wean their pups (Tables 21-23).

About 20% of the weaned rats of all treatments died within the first 6 months of their life. Post mortem examination revealed that the lungs were lesioned rather severely. During the 6 months of the experiment no impairment in coordination or other neurological functions were noticed. When the rats were sacrificed at 6 months of age no gross lesions were found in the brain. However, in 4 out of 167 rats injected in utero with MAM-Ac tumors in the liver or kidneys were found. All rats injected in utero with cycasin or saline were free from tumors.

It has been reported that feeding cycad meal to rats at various periods of gestation caused tumors amounting to 18.5% of the weaned pups. In the same experiment, it was found that 42% of the pups exposed to cycasin in their prenatal life died soon after birth (Spatz and Laqueur, 1967). Injections of MAM-Ac to pregnant rats during days 14 to 16 of gestation resulted in brain lesions and brain tumors in 10% of the pups. However, no information on the percentage of the rats that died soon after birth is available (Spatz, 1969). Because of the fact that same percentage of mothers in each group was able to wean pups, it seems that the stressful surgical procedure was the reason

why some of the rats in each group died during delivery or were consumed shortly after birth. The differences in the percentage of survival among the groups can be attributed to the type of compound used; cycasin and saline were much less toxic than MAM-Ac (Tables 21-23). The lower percentage of weaned rats found in this experiment compared with the one reported by Spatz and Laqueur was apparently due to the stressful surgical procedure used in this study.

In this study cycasin was found to be much less toxic than MAM-Ac although equivalent amounts of the substances in terms of MAM were given. It has been reported that cycasin in order to be toxic, must be hydrolyzed first. It has been found that the enzyme β-glucosidase which is responsible for this reaction, is present in the subcutis of pups in their prenatal life (Spatz, 1968). The reason why cycasin was less toxic may be due to a slow hydrolysis rate. It is possible that the injected cycasin was removed via the mother circulation and excreted in to her urine before it was hydrolyzed into MAM in sufficient amounts to cause toxic effects. As mentioned earlier, no brain lesions were found and only about 2.5% of the rats exposed to MAM-Ac in utero developed tumors later in life. It has been reported that introduction of cycasin during gestation resulted in tumors found in 18.5% of the pups later in life. Introduction of MAM-Ac resulted in 10% of the pups having brain tumors (Spatz and Laqueur, 1967; Spatz, 1969). Because of the fact

that in this study many pups were consumed after birth and many others died, it is possible that those rats which would be tumor bearing were consumed selectively by their mothers. It is possible too that the potential tumor bearing rats died earlier because of lung lesions or other reasons and thus the tumors did not have time to develop.

An attempt was made to inject rats in utero with MAM-Ac or cycasin at the same developmental stage as mice are at birth. Mice reach each developmental stage 1-2 days before the rat, thus rats were injected at 19-20 days of gestation. The neurological disorders seen in one day old mice injected with MAM-Ac were not seen in rats at the same developmental stage. Thus, the differences between the response of rats and mice to MAM-Ac and cycasin cannot be attributed to differences in development at the time of injection. It is more likely that what determines the response to MAM treatment is the species of the animal itself.

SUMMARY AND CONCLUSIONS

A total of 292 female Sprague-Dawley rats were used in 8 trials to study the effects of MAM-Ac on the neuro-endocrinological system associated with reproduction. Normally cycling rats at metestrus were injected subcutaneously with 10-12 µl MAM-Ac or saline. Since MAM-Ac injections causes a reduction in food intake, a pair fed control group as well as a positive control was used.

The experimental procedure included post injection determinations of food intake, body and organ weights, body composition and reproductive performance. In addition, adrenals, pituitaries, ovaries and uteruses were histologically examined and the levels of LH and prolactin were determined in the blood and pituitary by RIA. Hypothalami were analyzed for PIF and LHRF activities.

Food intake dropped sharply following the injection of MAM-Ac. From a daily consumption of about 20 g, the intake dropped to 3 g one day post injection. Thereafter it increased until it was back to normal one week later. As a result of the reduced food consumption the rats lost about 12-18% of their initial body weight, which was restored only 10-12 days post injection. The same body weight loses and

body compositional changes were obtained in the MAM-Ac injected and pair fed rats.

As a response to MAM-Ac injections the adrenals became heavier and the thymuses lighter than in pair fed controls. Histological examination of the adrenals revealed the presence of hemorrhages and indistinct division of the different zones.

The estrous cycle was significantly prolonged ($p<0.001$) following the administration of MAM-Ac. Before injection all rats had cycles of 4-5 days. The first cycle after the injection was completed within 9.0-9.8 days by the MAM-Ac injected rats and within 5.2-5.4 days by the pair fed rats. The second and third cycles after injection were similar in length for all groups.

Reproductive performance as measured by the length of time from mating to pregnancy, % fertility and the number of born and weaned pups. All rats were pregnant within 9 days from mating and 10.8 pups per litter were born to MAM-Ac injected rats versus 10.5 to the pair fed controls.

Organ weights expressed per 100 g body weight were generally the same in all groups when analyzed according to the stages of the estrous cycle in which the rats were sacrificed. Since most of the rats treated with MAM-Ac were at diestrus 1 to 6 days post injection and since rats at diestrus have lighter uteruses, thus MAM-Ac injected rats had lighter uteruses during this time interval. The

ovaries and the pituitaries of the MAM-Ac injected rats began to decrease in weight from the 4th day on.

Histological examination of the ovaries revealed that for a period of approximately 8 days after the injection of MAM-Ac, the rats had ovaries filled primarily with well developed corpora lutea and unstimulated uteruses with dense endometrium, narrow lumen and few glands.

Gonadotropin metabolism as measured by RIA was impaired in rats injected with MAM-Ac. LHRF activity measured in the hypothalami of the MAM-Ac injected rats was lower when compared with the positive control. However there was no change in the PIF activity in the MAM-Ac injected rats when compared with both controls. The anterior pituitary of MAM-Ac injected rats sacrificed at diestrus was found to contain less prolactin and LH. In addition MAM-Ac injected rats sacrificed at proestrus and estrus contained less LH in their pituitaries. Since most of the MAM-Ac injected rats sacrificed 1 to 6 days post injection were at diestrus, pituitary LH was lower 1 to 3 days post injection and pituitary prolactin was lower 1 to 6 days post injection. Blood LH was lower in MAM-Ac injected rats sacrificed at metestrus and diestrus whereas blood prolactin levels were the same in MAM-Ac injected rats and in both controls sacrificed at the various stages of the cycle. As a result, 1 to 6 days after the injection blood LH was lower in MAM-Ac injected rats than in both controls whereas blood prolactin did not decrease.

The significance of the blood-brain barrier in preventing cycasin and MAM-Ac neurotoxicity was studied in rats and mice. The experimental procedure included the implantation of cycasin or MAM-Ac in the brain third ventricle of rats and mice, by stereotaxic instrument. Only 2 out of the 33 implanted mice and none of the 62 implanted rats showed neurological disorders. No gross lesions were observed in these animals.

The significance of the physiological stage of the developing brain in relation to MAM-Ac and cycasin toxicity was studied during the gestation in rats. Laparatomy was performed on pregnant rats during 13 to 20 days of gestation and cycasin or MAM-Ac was injected subcutaneously to each fetus. This procedure was found to be very stressful since only 30-41% of the rats undergoing laparatomy weaned pups and only 8-20% of the injected pups were weaned, regardless of the treatment given. At the age of 6 months the rats were examined for brain lesions and tumors. No gross lesions were observed in these rats and only 2.5% of the rats treated with MAM-Ac had liver or kidney tumors.

Conclusions

1. MAM-Ac injections caused disorders in the gonadotropin metabolism and sterility prevailed for approximately 10 days.

2. The sequence of events from injection time to the initiation of sterility and the primary organ affected are unknown.
3. The blood-brain barrier is not the mechanism by which neurological disorders are prevented in mature mice and rats.

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