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Development of Hepatic Lesions in Male Fischer-344 Rats Fed AIN-76A Purified Diet

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Development of Hepatic Lesions in Male Fischer-344 Rats Fed AIN-76A Purified Diet. MEDINSKY, M. A., POPP, J. A., HAMM, T. E., AND DENT, J. G. (1982). *Toxicol. Appl. Pharmacol.* **62,** 111–120. The suitability of the AIN-76A diet for Fischer-344 rats was investigated. This diet, proposed by the American Institute of Nutrition for use when a purified diet composed of refined ingredients and added vitamins and minerals is required, was tested in Sprague–Dawley rats. Male weanling Fischer-344 rats were fed three different lots of the AIN-76A diet from two suppliers. Increase in body weights and food consumption were compared to animals fed a cereal-based control diet. Animals were sacrificed at various intervals and tissues were taken for histopathological observation. By 8 weeks moderate to marked periportal lipidosis developed in livers of all rats fed the AIN-76A diet. Liver–body weight ratios over the 8-week period were significantly higher in rats fed AIN-76A diets compared to rats fed the control diet. However, growth rates of rats fed the AIN-76A diet were similar to growth rates of controls. Some rats fed the AIN-76A diet developed severe hemorrhagic lesions. The AIN-76A diet in its present form is not suitable for use with male Fischer-344 rats.

The need for a well-defined diet for toxicity or carcinogenicity studies in laboratory animals has been emphasized by the National Research Council Committee on Laboratory Animal Diet (Newberne *et al.*, 1978). Imbalance of ingredients, nutritional inadequacies, or contaminants can influence the response of animals to toxicants. Control of dietary variables leads to more reproducible results within a study and when comparing studies from different laboratories (Newberne *et al.*, 1978). The American Institute of Nutrition (AIN) Committee on Standards for Nutritional Studies formulated the AIN-76A Diet (Bieri *et al.*, 1977; Bieri, 1980) for this purpose and suggested that it be used when the experimental protocol requires a purified diet (e.g., for manipulation of trace nutrient levels). This diet is composed of commercially refined proteins, carbohydrates, and fat, with vitamin and mineral mixes added. Using growth rates, liver-to-body weight ratios, food efficiency, and reproduction as measures of nutritional adequacy, the AIN-76A diet was shown to be adequate for Sprague–Dawley rats when compared to results obtained with NIH-07, an open formula cereal-based diet (Bieri *et al.*, 1977).

In preliminary studies with male Fischer-344 rats fed the AIN-76A diet, we observed an increased mortality associated with hemorrhage in the nasal and thoracic cavities and in the epididymis. After 6 weeks on the diet, many rats had pale, friable livers and histopathologically significant hepatocellular lipidosis. The current study was designed to (1) determine if these observations were

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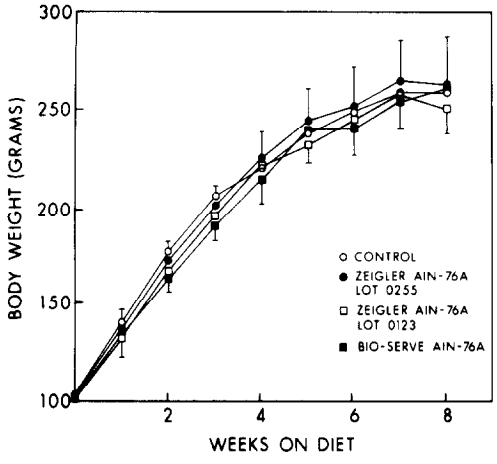


FIG. 1. Increase in body weight of male Fischer-344 rats fed either AIN-76A diet or Wayne Lab Blox (control) diet. Data points represent mean body weights. Error bars are standard deviations of the data.

reproducible with the AIN-76A diet obtained from different suppliers, (2) investigate the extent and morphology of the liver lesion, and (3) outline the time course for development of the lesion.

METHODS

Diets. Formulation of the AIN-76A[2] diet has been published (Bieri *et al.*, 1977; Bieri, 1980). Three pelleted lots of AIN-76A diet were obtained (Ziegler Bros., Inc., Gardners, Pa., Lots 0123 and 0255, and Bio-Serve, Inc., Frenchtown, N.J., Lot 1110) in 50-lb sealed boxes. The diet was stored frozen (ca. −4°C) in sealed plastic bags. Twenty-four hours before use, unopened plastic bags containing the diets were brought to room temperature (ca. 25°C).

Maintenance of animals. One hundred twenty male weanling (28-day-old) Fischer-344 rats (COBS CDF/CrlBR), obtained from Charles River Breeding Laboratories, Kingston, New York, were randomly placed three per cage in suspended wire-bottomed, stainless-steel cages and acclimated for 1 week prior to initiating the study. During this time rats were fed certified Wayne Lab-Blox (Allied Mills, Chicago, Ill.), a closed formula cereal-based diet, and tap water *ad libitum*. Ambient temperature of 21 ± 3°C, relative humidity of 50 ± 10%, and a 12-hr light:dark cycle were provided. Sera from sentinel rats from the same source, of the same age and strain, and maintained in the same animal rooms were tested (Microbiological Associates, Bethesda, Md.) for titers to Reovirus type 3, pneumonia virus of mice, Theiler's encephalomyelitis, Kilham rat virus, Toolan's H-1, Sendai, mouse adenovirus, lymphocytic choriomeningitis, and rat corona virus. Sera were tested from one rat per week for the duration of the study. These sera did not have titers to any of the above viruses.

At the end of the acclimation period rats were divided into four groups of 27 each, and one group of 12. One group of 27 continued receiving Wayne Lab Blox throughout the experiment (control). The other three groups of 27 rats were fed either Zeigler AIN-76A diet (Lot 0123), Zeigler AIN-76A diet (Lot 0255), or Bio-Serve AIN-76A diet. A group of 12 rats was fed a choline-devoid diet obtained from Bio-Serve (Biomix 1139; Lot 3129). The choline-devoid diet served as a positive control since rats fed this diet develop fatty centrilobular lipidosis (Harper, 1958). Rats from each group were housed 3 per cage and allowed free access to both respective diets and water.

Data collection. Animals were weighed at the beginning of the study and weekly thereafter. All three animals in each cage were weighed and the average body weight per rat was calculated. Food consumption was determined for each cage twice weekly. The rats were observed for general health, morbidity, and mortality three times weekly. Animals found in a moribund condition were euthanized and necropsied. Any animals found dead were stored at 4°C and necropsied within 24 hr.

At least three animals from each of the three groups fed the AIN-76A diet lots and from the Wayne Lab Blox (control) diet were necropsied 1, 2, 4, 6, or 8 weeks after initiation of the study. Three animals fed the choline devoid diet were necropsied after 1 or 2 weeks. At necropsy animals were weighed, anesthetized with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N.J.) and then killed by severing the abdominal aorta. Livers were excised, blotted, and weighed. Slices 3- to 5-mm-thick through the left lobe of the liver were frozen in liquid N_2. Histologic sections (4–5 μm) were cut from the block of frozen liver and stained with oil red O in propylene glycol (Luna, 1968). Sections were coded and examined independently and blindly by two

[2] Composition of the AIN-76A purified diet (for rats and mice) per kg diet: casein (85% protein), 200 g; DL-methionine, 3 g; cornstarch, 150 g; sucrose, 530 g; cellulose-type fiber, 50 g; corn oil, 50 g; choline, 0.57 g; thiamin, 4.7 mg; riboflavin, 6.0 mg; pyridoxine, 5.8 mg; nicotinic acid, 30 mg; pantothenic acid, 7.4 mg; folic acid, 2 mg; D-biotin, 200 μg; cyanocobalamin, 10 μg; vitamin A, 4000 IU; vitamin D, 1000 IU; vitamin E, 50 IU; vitamin K, 500 IU; calcium, 5.2 g; phosphorus, 4 g; sodium, 1.02 g; potassium, 3.6 g; magnesium, 0.5 g; manganese, 54 mg; iron, 35 mg; copper, 6 mg; zinc, 30 mg; iodine, 0.2 mg; selenium, 0.1 mg; chromium, 2.0 mg; chloride, 1.56 g; sulfate, 1.0 g.

TABLE 1

GROWTH AND FOOD EFFICIENCY OF RATS FED THE AIN-76A AND CLOSED FORMULA CEREAL-BASED DIETS[a]

Diet	No. rats	Initial weight[b] (g)	21-day weight[b] (g)	Avg. daily weight[b] (g)	g gain/g food[c]
Zeigler Lot 0123	27	103 ± 2.6	197 ± 6.4	4.5 ± 0.3	0.31 ± 0.01
Zeigler Lot 0255	27	103 ± 4.9	202 ± 6.4	4.7 ± 0.4	0.32 ± 0.01
Bio-Serve	27	101 ± 2.4	191 ± 7.2	4.3 ± 0.4	0.29 ± 0.02
Cereal based (control)	27	103 ± 3.4	206 ± 7.0	4.9 ± 0.4	0.28 ± 0.01

[a] Rats were housed three per cage; food consumption and body weight data were obtained for each cage.
[b] Data are mean ± SD.
[c] First 14 days.

of the authors (M.A.M., J.A.P.) for the degree of lipid accumulation, the sublobular distribution of the lipid accumulation, and the consistency of the lesion from one lobule to the next. Additional liver slices also were fixed in 10% neutral-buffered formalin, processed, and stained with hematoxylin and eosin. Other tissues collected for histopathology after 8 weeks on the diet were: brain, thoracic spinal cord, pituitary, thyroid and adrenal glands, heart, lung, spleen, right kidney, stomach, duodenum, pancreas, ileum, upper colon, prostate, thymus, esophagus, trachea, skull, cervical lymph nodes, submaxillary salivary glands, urinary bladder, and tests with epididymis. These tissues were fixed in 10% neutral-buffered formalin. Tissues from rats fed Zeigler diet Lot 0123 (8 weeks) were processed, stained with hematoxylin and eosin, and examined for histologic lesions.

After 8 weeks on the Bio-Serve AIN-76A diet, the 11 surviving rats were divided into two subgroups. The subgroup of 6 rats was continued on the AIN-76A diet; the other subgroup of 5 rats was switched to the Wayne Lab Blox (control) diet. These 11 rats, and 6 others that were fed the Wayne Lab Blox (control) diet during the entire study, were necropsied 16 weeks later. Liver tissue from these 17 rats was fixed in liquid nitrogen and processed for oil red O stain as described above and for hematoxylin and eosin stain of paraffin-embedded tissue.

All surviving rats fed AIN-76A diets obtained from Zeigler were sacrificed after 8 weeks on the diets. All surviving rats fed the choline-devoid diet were sacrificed after 2 weeks on the diet.

RESULTS

Growth of rats fed the AIN-76A diets over the 8-week period was the same as that of rats fed the control cereal-based diet (Fig. 1). Food efficiency and daily weight gain for animals in all four groups (Table 1) were similar.

Liver/body weight ratios of the animals fed the AIN-76A diet for 1 to 8 weeks were significantly higher ($p < 0.05$) than ratios for rats fed the control diet (Fig. 2). Percentage liver-body weight ratios (mean ± SEM) for rats fed the choline-devoid diet and sacrificed after 1 or 2 weeks were 6.4 ± 0.4 and 7.1 ± 0.3, respectively. These ratios were higher than those found for rats maintained on any of the AIN-76A diets or on the control diet for 8 weeks. The six rats

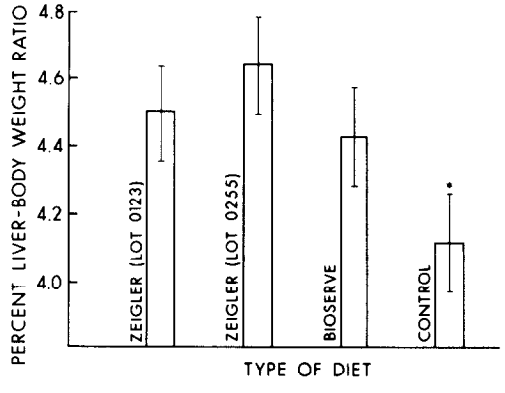
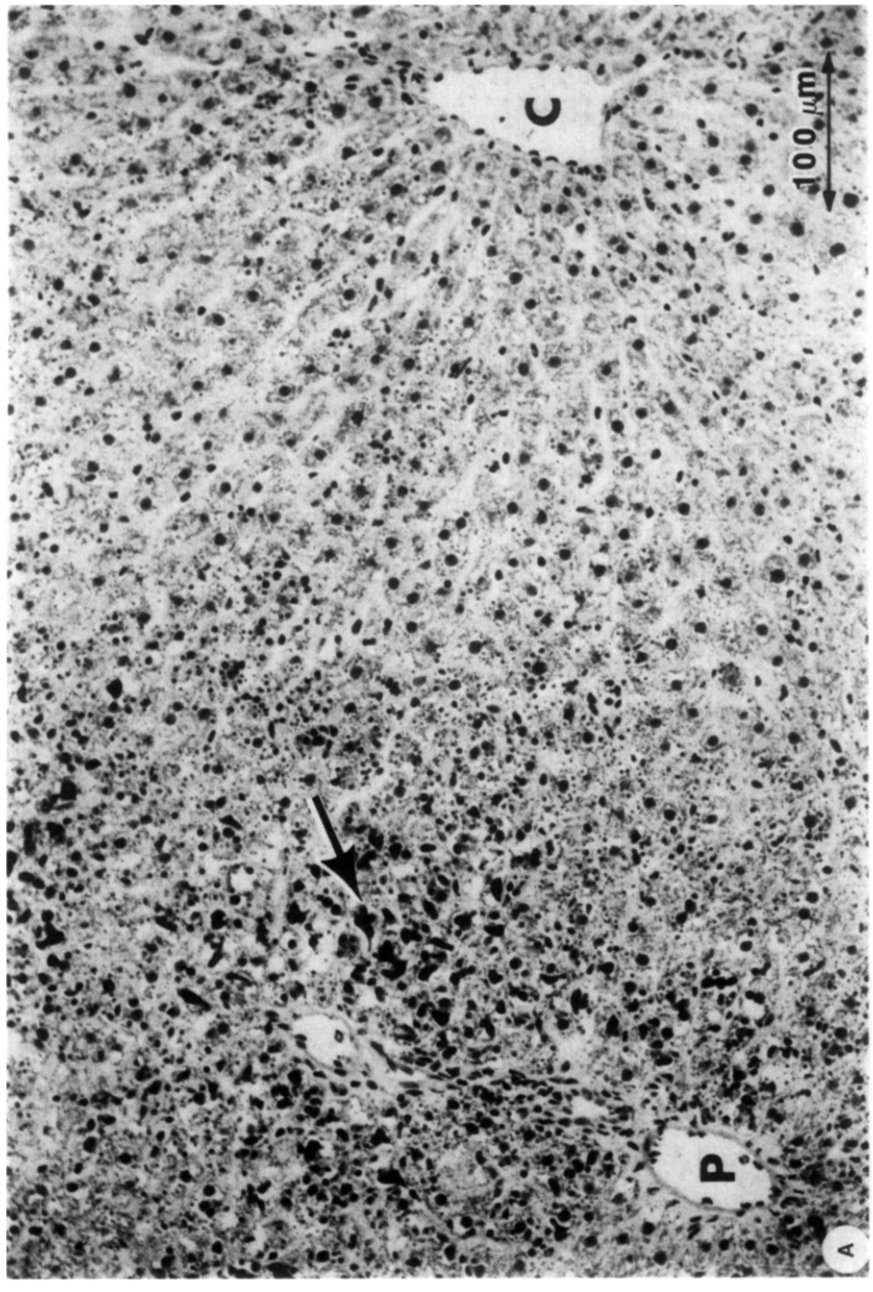


FIG. 2. Percentage liver-body weight ratios of rats fed either the AIN-76A diet or Wayne Lab Blox (control) diet. Bars represent the mean percentage liver-body weight ratios for rats sacrificed at various times during the 8-week study. Error bars indicate 95% confidence intervals on the means. Asterisk (*) indicates that the control diet produced a significantly lower liver-body weight ratio than did the three AIN-76A diets.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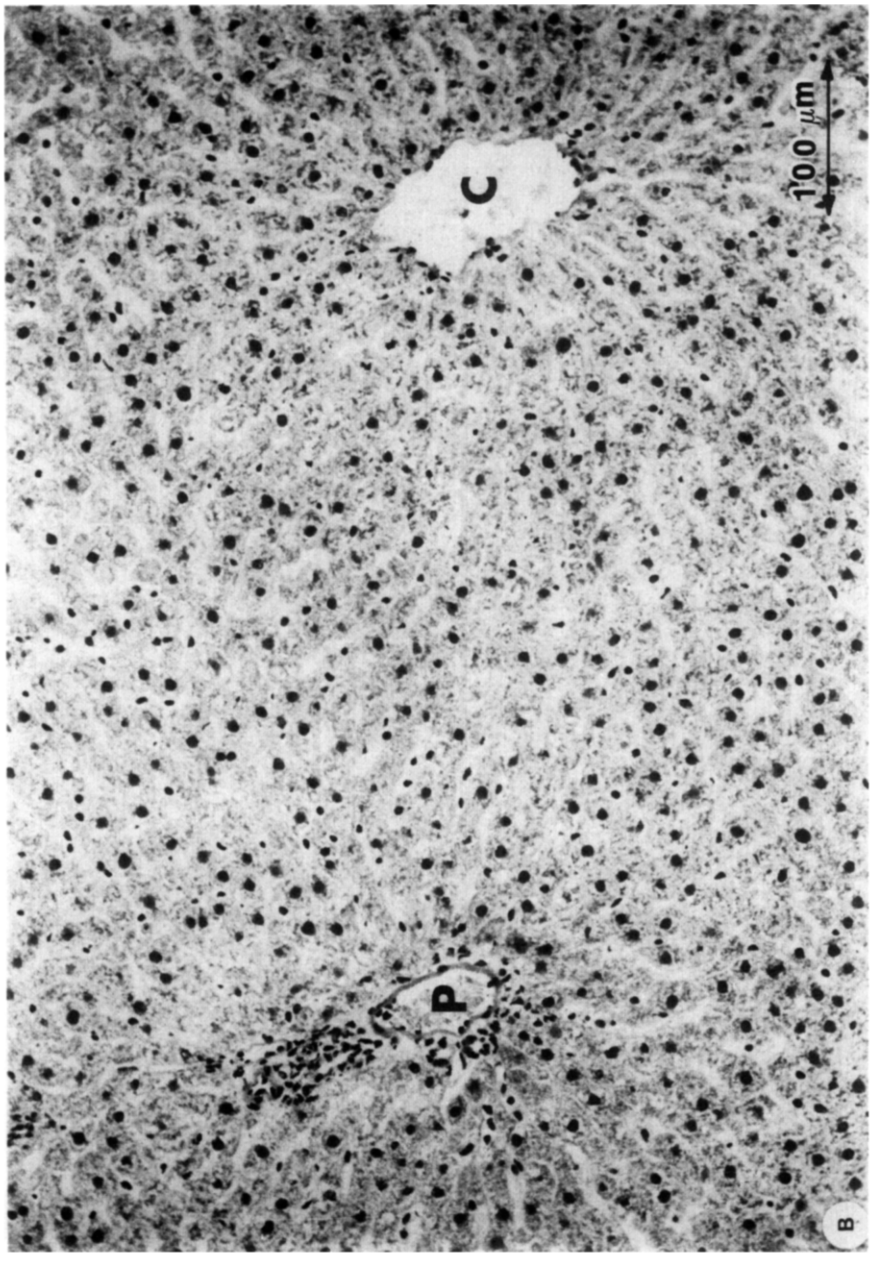

FIG. 3. Photographs of frozen fixed liver sections following stain with oil red O, ×200. (A) Stain (arrow) is found in periportal hepatocytes in liver of a rat maintained on Bio-Serve AIN-76A for 6 weeks; p, portal triad; c, central vein. (B) No stain was found in periportal hepatocytes in liver of a rat maintained on Wayne Lab Blox (control) diet for 6 weeks; p, portal triad; c, central vein.

fed the AIN-76A diet (Bio-Serve, Lot 3129) for 24 weeks had significantly higher ($p < 0.05$) percentage liver/body weight ratios (3.8 ± 0.2) than either the six rats fed the control diet for 24 weeks or the five rats fed AIN-76A diet for 8 weeks and then the control diet for 16 weeks (2.8 ± 0.1 for both groups).

Oil red O stain demonstrated hepatic lipidosis in rats fed any of the AIN-76A diets or the choline-devoid diet. In the AIN-76A diet groups, this lesion was most prominent in the hepatocytes surrounding the portal triads (Fig. 3A). Hepatic lipidosis was diffuse and affected all areas of the lobule in rats fed the choline-deficient diet for 1 or 2 weeks. Rats fed the cereal-based control diet had little or no accumulation of lipid (Fig. 3B).

Hepatic lipidosis became progressively more severe and more consistent from 1 to 8 weeks of feeding the AIN-76A diet. There were no differences in the degree, consistency, or lobular distribution of the lipid accumulation at any time among the three groups fed the AIN diets. After 1 or 2 weeks, very mild diffuse lipidosis was seen in some rats fed either AIN-76A diet or control diet. Livers from rats fed AIN-76A diet could not be distinguished from the livers of animals fed the control (Wayne Lab Blox) diet. Abnormal lipid accumulation was present in the periportal hepatocytes of 50% of the livers from rats fed the AIN-76A diets for 4 or 6 weeks. After 8 weeks of feeding AIN-76A diets, all of the livers had moderate to marked periportal lipidosis. The zone of lipidosis was relatively narrow and affected less than one-third of the lobule. Rats from the control group (Wayne Lab Blox) had a mild diffuse lipidosis that did not accumulate in the periportal hepatocytes.

Rats fed the Bio-Serve AIN-76A diet for 24 weeks also had a periportal distribution of hepatic lipidosis. Paraffin-embedded sections of liver tissue contained large cytoplasmic vacuoles in periportal hepatocytes. Oil red O stain of frozen liver sections indicated that these hepatocytes contained lipid. In addition the cytoplasm of the hepatocytes in the midzonal and centrilobular areas of the livers was foamy (Fig. 4). The cytoplasm of these cells also stained with oil red O.

Livers of rats fed control (Wayne Lab Blox) diet for 16 weeks, following 8 weeks of maintenance on AIN-76A (Bio-Serve), were indistinguishable from livers taken from the group fed control (Wayne Lab Blox) diet for 24 weeks. There was a diffuse distribution of very mild lipidosis with no evidence of periportal accumulation.

In animals fed AIN-76A diet (Zeigler Bros., Inc., Lot 0123), the only other microscopic lesions found were in the kidney and pancreas. In two of six animals necropsied at 8 weeks, the kidneys had several small foci of mineralization. One of the six rats had a mild interstitial nonsuppurative pancreatitis with extensive hemosiderin accumulation. One other rat had acute pancreatitis with a diffuse area of hemorrhage and hemosiderin deposition.

Gross hemorrhagic lesions were present in 2 of 27 rats fed the AIN-76A diet (Zeigler Bros., Inc. Lot 0123) for 5 weeks. One of these rats died and the other was moribund and killed. The hemorrhagic lesions were found in the intestines of the first rat and in the meninges of the second. Hemorrhagic petechiae were seen in 5 of 16 rats fed the Zeigler AIN-76A died (Lot 0123 or 0255) and examined at scheduled necropsies at 6 or 8 weeks. The petechiae occurred in the lymph nodes, epididymis, lungs, liver, pancreas, and intestines. No hemorrhagic lesions were seen in rats fed either AIN-76A from Bio-Serve or the control diet.

DISCUSSION

There are many metabolic alterations that may account for the pathogenesis of fatty liver. Lipid accumulation in hepatocytes can result from an increase in the synthesis of

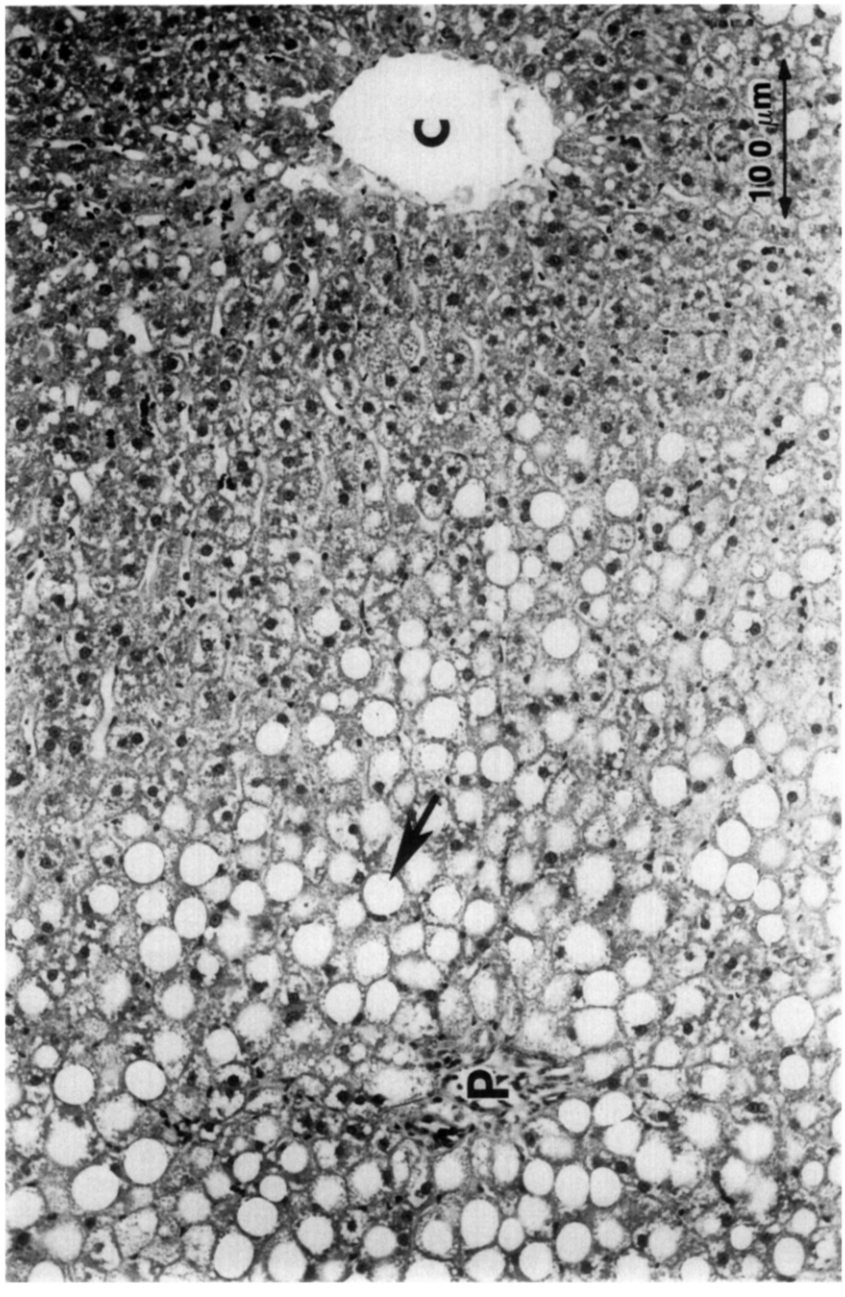

FIG. 4. Photograph of formalin-fixed, paraffin-embedded liver section stained with hematoxylin and eosin, ×200. Section taken from a rat fed Bio-Serve AIN-76A diet for 24 weeks. Comparable frozen sections stained with oil red O indicated that the unstained vacuoles in the periportal hepatocytes of the paraffin-embedded sections (arrows) had contained lipid. Lipid has also infiltrated the midzone and central areas as indicated by foamy cytoplasm which also stained with oil red O; p, portal triad; c, central vein.

triglycerides, with normal or decreased rate of secretion of lipoprotein into the blood. There may be one or more defects in the pathway leading to formation of secreted lipoproteins. Triglycerides may be synthesized in a part of the hepatocyte where they are unavailable for secretion (Plaa, 1980). Although the specific etiology of the lesion is still undetermined, the development of periportal lipidosis in this study demonstrated that the AIN-76A diet is not suitable for use with male Fischer-344 rats. Livers of rats fed this diet developed the lesions as early as 4 weeks and by 8 weeks all rats had moderate to marked lipid accumulation in hepatocytes surrounding the portal triads. The degree of lipidosis progressed for 6 months in rats continuously fed the AIN-76A diet. Periportal lipidosis is a characteristic lesion of the human nutritional disease, kwashiorkor (Davies, 1948; Trowell *et al.*, 1954). Lipid accumulation occurs during the first few months in the progression of this disease with hepatic fibrosis developing at later times. Presumably the major cause of kwashiorkor is lack of adequate protein of high quality, because feeding affected children a mixture of skim milk powder and sucrose is the usual therapy (Trowell *et al.*, 1954). In the present study we also saw regression of the lesions when rats fed Bio-Serve AIN-76A diet were given a control cereal-based diet.

Acute dietary deficiencies of certain essential amino acids such as valine, lysine, threonine, histidine, tryptophan, and methionine induce periportal lipidosis in laboratory animals (Sidransky and Baba, 1960; Sidransky and Farber, 1958; Adamstone and Spector, 1950). It appears that both the amount and quality of protein in the diet are factors in the development of lipidosis.

In the AIN-76A diet, the protein source, feed grade casein containing at least 85% protein, is low in sulfur amino acids. Consequently, the diet is supplemented with 0.3% methionine. Casein makes up 20% of the AIN-76 diet. Thus, neither the quality nor the amount of protein in the AIN-76A diet seems to be a factor in the development of periportal lipidosis reported in our study.

Administration of some halogenated hydrocarbons also causes an increase in liver lipids. Sublethal doses of the chlorinated insecticide mirex (200 mg/kg) produce periportal lipidosis within 6 days (Kendall, 1979). Chronic ingestion of low doses of mirex, photomirex, or Kepone (1 ppm) or Aroclor 1260 or Aroclor 1254 (20 ppm) over 28 days all caused perivenous and midzonal accumulation of lipid in the livers of rats (Chu *et al.*, 1980).

Casein, the most likely source for polychlorinated hydrocarbon contamination in the diets used in our study, was analyzed and nine chlorinated insecticides were detected at levels of 5 ppb or less.[3] Total polychlorinated biphenyl contamination was less than 10 ppb. These levels are well below those required to produce hepatic lipidosis. Thus, it is unlikely that halogenated hydrocarbons could be present in the AIN-76A diet in amounts sufficient to account for the lipidosis observed.

The AIN-76A diet is a defined purified formulation and some essential trace nutrients may not be added to the mineral mix. However, because of the presence of trace metals as contaminants in reagent grade chemicals, trace metal deficiencies are generally a problem only in studies carried out in ultraclean environments under carefully controlled conditions (Bieri *et al.*, 1977). Thus, it is unlikely that the periportal lipidosis in this study was the result of a trace element deficiency.

The similarities in food efficiency and growth rates seen for rats fed AIN-76A diet or the cereal-based control diet indicated that these criteria alone are not sufficient to determine the suitability of a diet. The significant increase in liver/body weight ratios

[3] Chlorinated insecticides found were DDE, DDD, DDT, Dieldrin, BHC, Lindane, HCB, Endrin, and Heptachlor epoxide. No other chlorinated insecticides were detected.

of rats fed AIN-76A diet for 24 weeks compared to controls suggested that the cereal-based (control) and purified (AIN-76A) diets were nutritionally different when used with male Fischer-344 rats. However, in other studies using female Sprague–Dawley rats fed AIN-76 diet or NIH-07 open formula cereal-based diet for 24 weeks, no differences in liver–body weight ratios were noted (Bieri *et al.*, 1977).

Hemorrhagic lesions found in 2 of 27 rats fed the AIN-76A manufactured by Zeigler Bros., Inc. may be because of the level of Vitamin K in the diet. This level, 500 μg/kg, as stated by the manufacturer, was recommended by the AIN (Bieri, 1980). The level of Vitamin K suggested in the original formulation was 50 μg/kg diet (Bieri *et al.*, 1977). After reports of hemorrhagic lesions (Roebuck *et al.*, 1979), this level was increased to 500 μg/kg (Bieri, 1980). The AIN also suggested that Vitamin K be added in the stabilized form, menadione sodium bisulfite. The levels of Vitamin K in the AIN-76A diet manufactured by Bio-Serve and the Wayne Lab Blox were 1500 and 2800 μg/kg, respectively, as stated by the manufacturers. No hemorrhagic lesions were found in animals maintained on either diet. Other investigators have found that male Fischer and Sprague–Dawley rats had a higher incidence of death because of butylated hydroxytoluene (BHT)-induced hemorrhages compared to Wistar or Donryu strains when 1.2% BHT was added to the diet for 3 weeks (Takahashi *et al.*, 1980). BHT, an antioxidant, was not present in the corn oil in the AIN-76A diets supplied by Zeigler or Bio-Serve. However, considering the increased susceptibility of Fischer rats to hemorrhagic lesions, it may be necessary to increase the Vitamin K levels in the present AIN-76A formulation for use with this strain.

Spontaneous intranephronic calculosis has been observed consistently in female Sprague–Dawley rats fed the AIN-76 purified diet for 18 days (Nguyen and Woodard, 1980). Rats fed a natural products diet for the same period of time did not develop nephrocalcinosis. We found small foci of mineralization in kidneys of two out of six male rats sacrificed after 8 weeks on the AIN-76 diet. This lower incidence seen in our study is not unexpected considering we used males only and females are more susceptible to nephrocalcinosis than males when fed purified diets (Cousins and Geary, 1966).

In summary, we report the consistent development of marked hepatic lipidosis in male Fischer-344 rats maintained for 24 weeks on the AIN-76A diet. We conclude that the present formulation of the AIN-76A diet is not appropriate for use with male Fischer-344 rats.

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