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Histochemical effects on liver from C57 BL/6 Jax mice fed selected Tweens

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HISTOCHEMICAL EFFECTS ON LIVER FROM C57 BL/6 JAX MICE FED SELECTED TWEENS

by

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INTRODUCTION

Various non-ionic surface active agents have been used in a wide variety of food products. Classified as food additives by the Food and Drug Administration because these agents are used in relatively small amounts whenever or wherever they develop technological advantage, they have proven to be particularly useful in food processing as emulsifiers in the following foods: (1) icings for baked sweet goods, (2) ice milk, ice cream, frozen custard and frozen fruit desserts, (3) cake mixes, (4) whipped vegetable oil toppings, (5) salad dressings, and (6) confectionery coatings.

The toxicology of some of these emulsifying agents has been studied extensively in experimental animals and human subjects. These investigations varied greatly in their objectives. Some were undertaken to determine the effects of these emulsifiers on growth, food utilization and metabolism, reproduction, physiological behavior, mortality, and histology of various organs. Time on diet among the experimental animals, type of experimental animal utilized, levels at which the emulsifiers were fed, and emulsifiers used varied greatly among the investigations.

Because of the widespread use of emulsifiers and the diversity in experimental conditions in previous investigations, the Department of Zoology and Entomology at Iowa State University has undertaken a series of investigations in which a specific strain of mice (Jax C57 BL/6) is fed diets containing specific concentrations of emulsifiers (Tween* 20

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or Tween 60) for varying periods of time. Studies in this over-all investigation include the following: (1) changes with age in normal histology and glycogen content of liver tissue from the C57 BL/6 Jax mouse, (2) reproductive ability of mice on long term feedings of emulsifiers, (3) relationship between anesthesia time and chronic feeding of emulsifiers to C57 BL/6 Jax mice, (4) effect on certain hematological values of C57 BL/6 Jax mice fed emulsifiers for varying periods of time, and (5) chronic effects upon the histology and histochemistry of the liver of Tween-fed C57 BL/6 Jax mice.

The present study was concerned with the last of the above enumerated phases, namely, liver histological and histochemical manifestations in C57 BL/6 Jax mice fed Tweens for varying periods of time. Selection of this aspect of the over-all investigation of Tween-fed mice was based upon the following reasons: (1) deficiency of publications concerning the microscopic findings of effects of these surface active agents upon the liver, (2) histological and histochemical changes in the liver often reflect alterations in the metabolism of the organism, and (3) previous histological investigation of the livers of normal, older C57 BL/6 Jax mice indicated certain ageing effects which might be initiated earlier in animals fed diets containing Tweens.

The objectives of this study were to determine in Tween-fed mice the following: (1) in vitro liver glycogen content and (2) changes in liver histology.

REVIEW OF LITERATURE

Liver Histology

The mammalian liver is a compound tubular gland incompletely invested by an outer tunica serosa within which a fibrous connective tissue forms a thin capsule, the capsule of Glisson. At the hilus the capsule is thickened and extends into the liver to form an internal fibrous connective tissue framework. The internal stroma delimits small subdivisions of liver parenchyma known as hepatic lobules.

The original concept of the structure of the liver was based upon the anatomical unit of the lobule which was described as a cylindrical or polyhedral structure about one millimeter in diameter and two millimeters in length. The lobule itself was made up of a series of hepatic cords radially arranged about a central vein. Although the lobule of the liver was first recognized grossly very early in the history of microscopic anatomy, its true histological architecture is still questioned by some investigators. Much was written in the early part of the twentieth century regarding the liver's morphological organization: Mall (1906), Mann and Magath (1922a), Opie (1925), Higgins and Murphy (1930). In 1932 Cowdry prepared a review of the literature.

Elias (1949) described a modified concept of the structure of the liver. He claimed that the liver is a continuous mass of hepatic cells which are arranged in plates penetrated by the sinusoids of the liver. Between the cells run the intra-lobular bile canaliculi.

Since this modification by Elias of liver organization, many new

theories of liver structure have been advanced. Riddell (1961) presented an excellent summary of these newer concepts of liver histology and organization.

Changes in Mouse Liver Histology with Age

Many investigations of age changes in tissues have concentrated on the regulatory mechanisms, such as the nervous system and the endocrine organs. Very little has been done to ascertain what age changes occur in mouse liver histology.

Andrew (1941) noted a definite and consistent difference in the histological picture of the liver in young, middle age, and senile C57 Jax mice. The nuclei of the liver cells of young animals were very uniform in size and appearance, and all were spheroid. Among the middle age animals there were more variation in nuclear size and form, and a tendency toward a clear zone between nucleus and cytoplasm. There was a greater degree of variability in size and form of the nuclei in liver cells of senile animals. Giant nuclei (20 microns or more in diameter) which often contained vacuoles were noted in hepatic cells from the senile animals. The perinuclear clear space was a conspicuous feature of the cell of the senile liver.

Andrew *et al.* (1943) reported further that the most constant and conspicuous age changes in liver histology of C57 Jax mice were in the nuclei of the hepatic cells. There were numerous giant nuclei which contained multiple nucleoli in the senile mouse. Collections of connective tissue cells and lymphocytes were found as periportal infiltrations in

the livers of senile mice. No change in amount or character of the connective tissue of the liver in old age was noted.

Liver Glycogen

Much has been published regarding the distribution of glycogen in the hepatic cell. Ehrlich (1883) established the diffuse distribution of glycogen in the liver cell, and Lewis (1921), by means of tissue culture cells, confirmed this work.

Evidence regarding glycogen distribution in the lobes of the liver is conflicting. Bartlett et al. (1914) believed the relative amounts of glycogen in the different lobes of the liver must vary from time to time according to the stage of digestion. However, Dowler and Mottram (1918) stated that the lobes of the liver of several different experimental animals have a different and unpredictable glycogen content. They felt that the distribution of glycogen was irregular and followed no fixed plan. According to these investigators the probable factors that influenced the partition of metabolic products among the different lobes were localized constriction, incomplete mixing of the blood in the portal vein, and variance among the cells for a certain type of work at a given time.

Contrary results have also been reported regarding the glycogen deposition within the hepatic lobule. Noel (1923) found that glycogen appeared first in the cells adjacent to the central vein. Cells about the peripheral portion of the lobule were the last ones to accumulate glycogen. Following ingestion of sufficient carbohydrates, glycogen became rather evenly distributed throughout the cells of the hepatic lobule.

According to this investigator the withdrawal of glycogen took place in a reverse order.

Forsgren (1935) reported that the deposition of glycogen began in the interior of the lobule and advanced toward the periphery. According to this study glycogen was present in large amounts during the night, whereas the largest amount of bile was secreted during the afternoon. This cycle was characteristic for rabbits, rats, and mice.

Higgins and Murphy (1930) and Deuel et al. (1938) found that glycogen deposited in the liver in the diurnal cycle was largely alimentary in origin.

Deane (1944) found that with both controlled and uncontrolled feeding, glycogen appeared to be deposited shortly after the time of eating. In disagreement with the descriptions of previous workers, Deane found that glycogen was deposited initially in the peripheral zone of the hepatic lobules, then more centrally.

The studies of Mann and Magath (1922a, 1922b, 1922c) clarified the vital function of the liver in relation to carbohydrate metabolism. In a series of studies the effects of the total removal of the liver were observed. Best (1934) also reviewed the role of the liver in the metabolism of carbohydrate and fat.

The formation of glycogen from glucose in fed and fasted rats was studied by Stetten and Boxer (1944). A constant concentration of deuterium oxide was maintained in the body fluids and the extent to which heavy hydrogen was incorporated into liver and carcass glycogen was determined.

Soskin (1944) reviewed the general role of the liver in regulating the blood sugar level.

Kleinfeld and Koulish (1957) reported the appearance of structures in the nuclei of abnormal mouse liver which were limited by a basophilic membrane-like boundary and contained glycogen. These structures were referred to as nuclear inclusions. Chips and Duff (1942) reported that these nuclear inclusions were fairly common in human liver. Wilson (1954) found similar nuclear inclusions in normal mouse liver and in the liver of mice subjected to various experimental treatments which produced liver injury. In a study dealing with senile changes in the liver of the mouse, Andrew et al. (1943) found these nuclear inclusions and explained their appearance as the result of some disturbance of the cell metabolism.

Effects of Surface Active Agents on the Liver

Investigations dealing with the effects of feeding non-ionic, surface active agents on the liver of an experimental animal vary in their results. Brush et al. (1957) fed Tween 60 at two, five, and 10 percent levels to mice for four months and found no pathological conditions in the liver.

Fitzhugh et al. (1959) reported questionable fatty changes of a very slight degree in the livers of rats fed Tween 60 at 25 percent levels for 12 weeks.

In an investigation dealing with experimental development of liver necrosis by feeding a necrogenic diet to rats, Gyorgy et al. (1958) significantly delayed the development of liver necrosis by mixing Tween 60

at five or 10 percent levels to the diet. The reason Tween 60 may not enhance development of hepatic necrosis was not offered, however, it was suggested that stearic acid may not enhance the development of hepatic necrosis as other dietary fats are known to do.

Harris et al. (1951) found no significant hepatic change after feeding weanling rats Tween 20, polyoxyethylene monolaurate (G-2129), and polyoxyethylene monostearate (Myrj 45) at a level of 25 percent of a synthetic diet for 70 days. However, the livers of rats given sorbitan monolaurate (Span 20) at the same concentration and period of time as above showed necrosis of the central parts of the lobule with or without fibroblastic proliferation. Fat globules were observed in many liver cells.

Kruesi and Van Itallie (1956) conducted a study dealing with the injurious effect on the livers of patients with pre-existing liver disease when three to six grams of Myrj 45 (polyoxyethylene-8-monostearate) were taken by mouth for 66 days. The patients continued to convalesce normally, both clinically and in terms of liver function.

No consistent pathologic changes were observed by Orten and Dajani (1957) in the livers of hamsters fed Myrj 45 for 28 weeks.

A similar conclusion was reported by Oser and Oser (1957) when hamsters were fed Myrj 45 at various levels for four weeks. However, in a study reported by Hsi et al. (1950), weanling hamsters fed Myrj 45 at 15 percent level from two to 10 weeks exhibited damage to their livers. The height of the damage was reached at four weeks and remained at this level without noticeable acclimation. The injuries to the liver consisted

of necrotic lesions of relatively small areas which occurred peripherally.

Krehl et al. (1955) reported no significant changes in liver histology among rats fed Myrj 45 for one year.

PAS Reaction for Histochemical Demonstration of Glycogen

The PAS (periodic acid-Schiff) reaction is based upon the use of periodic acid as an oxidant which breaks C-C bonds in various structures where they are present as 1:2 - glycol groups and converts them into dialdehydes. Further, these aldehydes are localized by combination with Schiff's reagent to give a substituted dye which is red in color.

According to Hotchkiss (1948) after the use of aqueous fixatives only substances of high molecular weight remained in the tissues in sufficient quantity to give a positive result. Such substances were polysaccharides, hyaluronic acid, mucoproteins, and mucins.

Since this reaction is not specific for glycogen, it is necessary to remove the glycogen in control sections and take them through the same sequence of treatment. Differences in staining reaction between control and untreated sections are presumed to be due to glycogen.

Prompt fixation is required for histochemical demonstration of glycogen (Kugler and Wilkinson, 1959). Investigations dealing with histochemical demonstration of glycogen vary in the type of fixative utilized. Hale (1957) recommended freeze-drying preservation followed by freeze-substitution or acetic-formalin-alcohol.

Since glycogen tends to migrate to one side of the cell when aqueous fixatives are utilized, Mancini (1948) recommended freeze-drying.

Lillie (1947) pointed out that since freezing techniques may be impractical in some laboratories, prompt fixation with Carnoy's fluid, alcoholic formalin or acetic-alcohol-formalin, gives good preservation of glycogen.

In addition to periodic acid, chromic acid and permanganate are two other reagents that have been utilized for oxidation of 1:2 - glycol groupings to aldehyde. Lillie (1951) reported that these two oxidants oxidize aldehydes to carboxylic acid if exposure is prolonged to several hours.

There must be present in tissue a minimal quantity of glycogen before it can be demonstrated histochemically (Kugler and Wilkinson, 1959). Good correlation between chemically and histologically determined glycogen concentrations of liver was obtained by Deane et al. (1946). The histological and biochemical results were linearly related up to a value of five percent of glycogen content.

EXPERIMENTAL PROCEDURE

Experimental Animals

Mice of the C57 BL/6 Jax strain were used throughout the study. A colony of mice of this strain was maintained by brother-sister matings of animals originally obtained from Jackson Memorial Laboratory at Bar Harbor, Maine. Selection of this strain of mice was based upon their low incidence of tumor development and long life span accompanied by few degenerative changes with age. In all cases, the mice used in this study were weaned at 21 days and fed Purina Mouse Breeder Chow* in pellet form until eight weeks old. An "equilibration" period of five weeks was allowed after weaning.

Mice utilized in each experiment, randomly grouped into fives according to sex, were placed in plastic cages provided with a perforated steel lid, and wood shavings for bedding. Animal room temperature was maintained at 74-76° F. The animal room was darkened by use of black shades, and artificial light was utilized only during the daily period of animal care.

All possible cleanliness was maintained in the cages, food

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*Contents as stated on container:

Guaranteed analysis:	Crude protein not less than	17.0%
	Crude fat not less than	11.0%
	Crude fiber not more than	2.0%
	Nitrogen Free Extract not less than	52.0%
	Ash not more than	5.5%

Ingredients: dried skimmed milk, ground wheat, brewers' dried yeast, corn oil, animal fat (preserved with butylated hydroxyanisole), vitamin A feeding oil, D activated plant sterol, 1.4% salt, 0.13% iron citrate.

receptacles and watering apparatus. Weekly, each group of five mice was weighed, and placed in a clean cage provided with fresh bedding, clean food receptacle and watering apparatus. Food and water were supplied ad libitum. Throughout the week food and water were replenished as needed. Soiled cages, food receptacles and watering apparatus were washed with hot water and detergent, rinsed in distilled water, immersed in a disinfecting solution (Hytron) and finally rinsed in distilled water. The clean items were allowed to air dry.

Experimental Diets

The non-ionic, water-soluble, surface active agents used in this study were polyoxyethylene sorbitan monolaurate (Tween 20) and polyoxyethylene sorbitan monostearate (Tween 60).

These Tweens are fatty acid esters of anhydrosorbitols which have been solubilized by etherifying the free hydroxyl groups with ethylene oxide. One fatty acyl residue is present per mole of each of the possible anhydrosorbitols produced. Physically Tween 20 and Tween 60 are characterized as yellow, oily liquids at room temperature. This physical characteristic was the basic reason for selection of these surface active agents. Tween 20 and Tween 60 in liquid form were readily blended with the mouse food.

The following five diets were used: a control of Purina Mouse Breeder Chow in meal form (same ingredients and analysis as pellet form), and experimental diets of Purina Mouse Breeder Chow in meal form containing, in terms of percentage weight, five percent Tween 20 or Tween 60,

or 10 percent Tween 20 or Tween 60.

The diets were mixed in 1000-gram portions. Tweens were thoroughly blended by hand into the meal form of mouse food, and, finally, in order to insure uniformity of the Tween within the diet, each portion was mixed at least 10 minutes in a feed mixer. The diets were kept in large covered crocks at the animal room temperature.

Experimental Plan

Nine experiments based upon period of time upon the diets were undertaken in this study. The periods of time were the following weeks: 10, 18, 29, 34, 52, 54, 79, 93, and 104. In most of these experiments mice were divided into five groups, 10 animals each (five males and five females), and were fed a control diet or diets containing five percent levels of Tween 20 or Tween 60, or 10 percent levels of Tween 20 or Tween 60. In the experiment in which the animals were fed 79 weeks, only male animals were used, and only female animals were utilized in the experiment based upon 104 weeks of feeding time. Mice of the same age and sex were not available at the same time when these two experiments were initiated.

In an additional investigation 10 control animals (five males and five females) were killed at eight weeks of age. This experiment was conducted to study the liver histology and determine the relative liver glycogen content of animals which were the initial age of all animals in each experiment.

Preparation of Tissue

Throughout the study, food was removed from the animals 10 hours before killing, and each animal was weighed immediately before death. Mice were killed between 8:00 A.M. and 9:00 A.M. in order to minimize any diurnal variation effect. Mice were killed by striking the back of the head upon a laboratory table. A sample of the anterior region of the median liver lobe was removed immediately and placed in fresh Carnoy's fixative (six parts absolute ethanol, three parts chloroform, one part glacial acetic acid) for three hours. While no attempt was made to measure the exact location of the extirpation, generally a sample of liver was removed from the same location on each mouse liver. Less than one minute of time was involved from killing to placing of tissue into fixative.

After fixation the tissue was dehydrated in two changes of absolute ethanol, cleared in xylene, and embedded in a mixture of 90 percent paraffin, 10 percent bayberry wax at 58° C.

Sections of each liver cut at four and seven microns were stained by the periodic acid-Schiff (PAS) technique and counterstained with Mayer's hemalum (Lillie, pp. 123-124, 1954). Similar sections of each liver were treated with malt diastase for 45 minutes at 35-45° C. and then stained in the same manner. These enzyme-treated tissues served as histochemical checks and were also used for histological observations.

Preparation of the Schiff reagent was according to the "cold Schiff" procedure (Lillie, p. 156, 1954). One gram of basic fuchsin and

1.9 g. $Na_2S_2O_5$ were dissolved in 100 cc. 0.15 N HCl. The solution was shaken well and allowed to stand overnight at room temperature. The solution was then shaken with 500 mg. of fresh activated charcoal and filtered. Distilled water was added to the colorless solution to restore the original 100 cc. volume. If the solution possessed a yellow color after the first filtration, a fresh lot of activated charcoal was obtained and the decolorization by charcoal was repeated. When not in use the solution was refrigerated. Whenever a white precipitate or pink color appeared in the solution, it was discarded.

A 0.1 percent aqueous solution of malt diastase held at pH 6.0 with a phosphate buffer was prepared for digestion of the control slides.

Determination of Relative Liver Glycogen

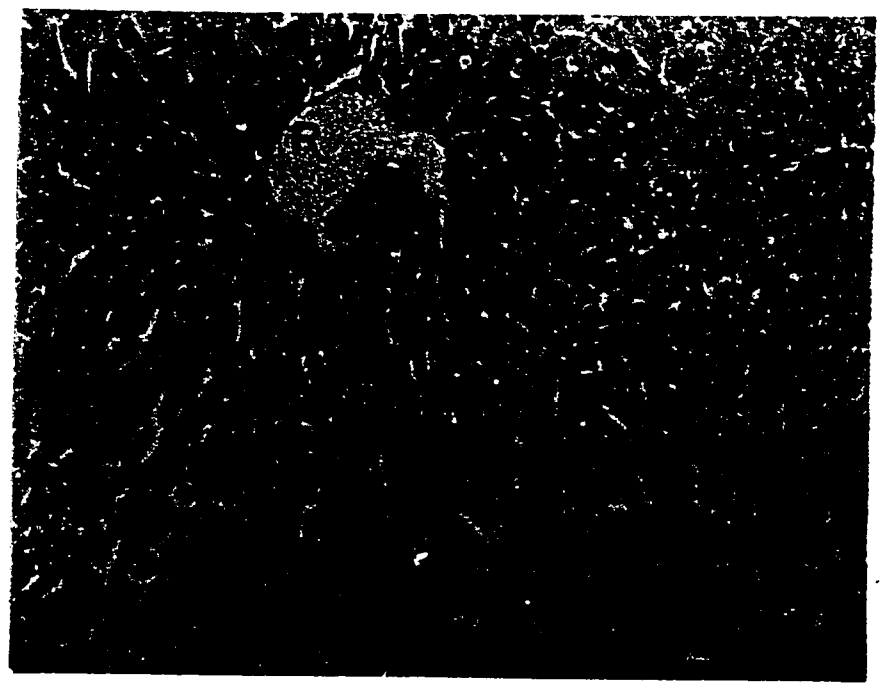
Liver tissue sectioned at seven microns, stained by the periodic acid-Schiff technique and counterstained with Mayer's hemalum along with control slides (diastase-treated) were utilized in determination of the relative liver glycogen. Each liver was graded numerically from one to five according to the total amount of glycogen present in the lobules. Only livers containing glycogen in hepatic cells immediately adjacent to periportal regions and in a few hepatic cells scattered throughout the lobules were assigned a value of one. A relative value of two was given to livers which possessed glycogen in hepatic cells adjacent to the periportal regions and approximately midway in the intermediate zones of the lobules. Whenever livers exhibited hepatic cells with uniform moderate concentration of glycogen in all regions of the lobules, a value

of three was assigned. Extensive concentration of glycogen in hepatic cells adjacent to periportal regions and moderate concentration in all other regions of the lobules was the concentration of livers which possessed a relative value of four. A relative value of five was assigned to those livers which possessed extensive concentration of glycogen in hepatic cells in all regions of the lobules. (See Figures 1 through 6.)

Plate I

Figure 1. Cross section of liver of male C57 BL/6 Jax mouse fed Tween 60 diet at 10 percent level for 54 weeks. Liver assigned value of one for relative glycogen concentration. CV, central vein; PR, portal region. Seven microns, PAS and hemalum, X 160

Figure 2. Cross section of liver of male C57 BL/6 Jax mouse fed Tween 60 diet at 10 percent level for 54 weeks. Liver assigned value of two for relative glycogen concentration. CV, central vein; PR, portal region. Seven microns, PAS and hemalum, X 160




CV

PLATE II

Figure 3. Cross section of liver of male C57 BL/6 Jax mouse fed control diet for 52 weeks. Liver assigned value of three for relative glycogen concentration. CV, central vein; PR, portal region. Seven microns, PAS and hemalum, X 160

Figure 4. Cross section of liver of female C57 BL/6 Jax mouse fed control diet for 18 weeks. Liver assigned value of four for relative glycogen concentration. Note granulation of glycogen in portal region. CV, central vein; PR, portal region. Seven microns, PAS and hemalum, X 160

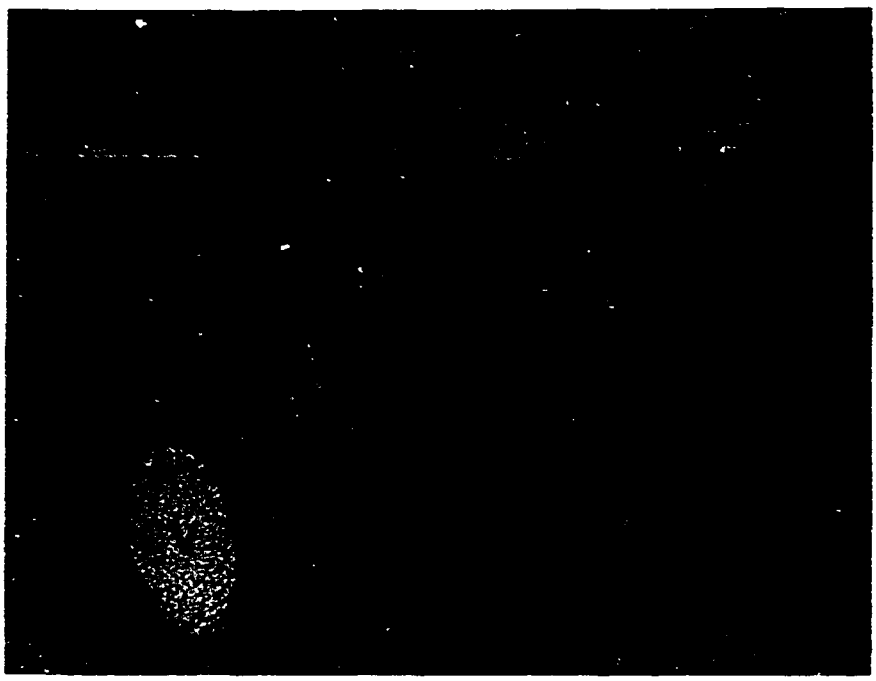
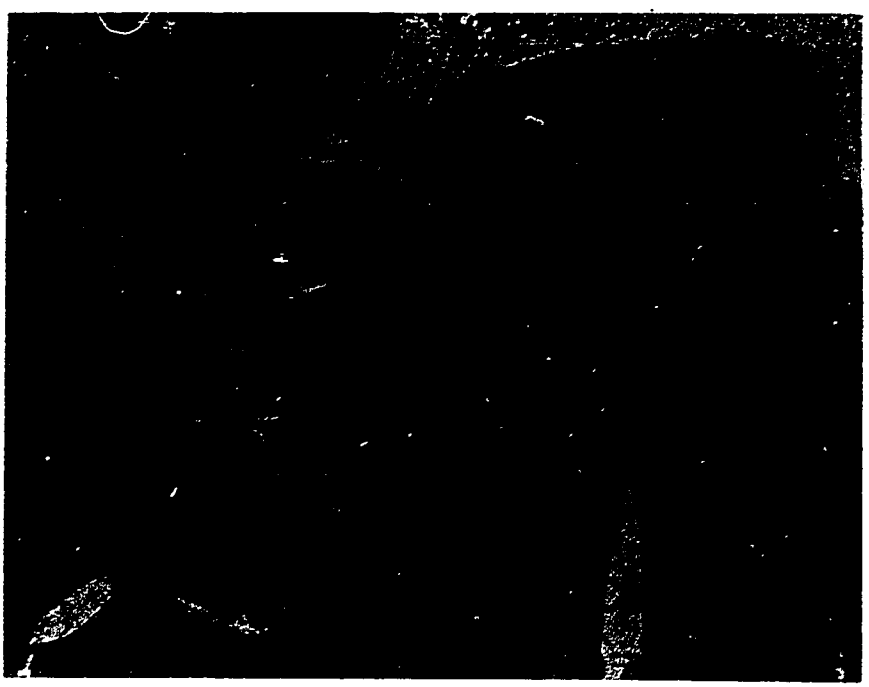


Plate III

Figure 5. Cross section of liver of male C57 BL/6 Jax mouse fed control diet for 10 weeks. Liver assigned value of five for relative glycogen concentration. Note homogeneous coloration. CV, central vein; PR, portal region. Seven microns, PAS and hemalum, X 160

Figure 6. Cross section of liver of female C57 BL/6 Jax mouse fed Tween 20 diet at five percent level for 18 weeks. Liver assigned value of five for relative glycogen concentration. Note granulation of glycogen in cytoplasm of hepatic cells. CV, central vein; PR, portal region. Seven microns, PAS and hemalum, X 160


P
CV



RESULTS

Macroscopic Observations

Macroscopic examination of livers at necropsy revealed no evidence of pathology among animals in all experiments.

The individual body weights of the animals at the time of killing along with the mean body weights are summarized in Table 1. Generally among all animals there was an increase in mean body weight through 54 weeks on the diet.

Microscopic Observations

In the following descriptions the branches of the hepatic vein which are located in the center of lobules are referred to as central veins and branches of the portal vein located at the periphery of the lobules as interlobular veins, regardless of size. Branches of the hepatic artery will be referred to as hepatic arteries. Sites seen in cross section at the periphery of the lobules which reveal a hepatic artery, a branch of the bile duct and an interlobular vein together with connective tissue that immediately surrounds them constitute a portal region. While the parenchyma of the liver consists of the bile duct cells and the epithelial cells which make up the so-called liver cords, the term, hepatic cells, will be used to refer to the epithelial cells of the liver cords. The connective tissue cells will be regarded as the connective tissue stroma of the liver.

One of the most conspicuous qualitative features among the liver

Table 1. Body weights of C57 BL/6 Jax mice at time of killing

Diet	Time on diet (weeks)	Initial number of animals and sex	Body weights of surviving animals (gm.) 1	2	3	4	5	Mean body weight (gm.)	Standard deviation
Control	0	5M	25	21	22	20	23	22.0	1.9
		5F	18	17	15	17	19	17.2	1.5
	10	5M	25	30	29	25	30	27.8	2.6
		5F	24	25	24	24	24	24.2	0.1
	18	5M	25	26	31	28	27	27.4	2.3
		5F	25	24	25	22	24	24.0	1.3
	29	6M	32	30	35	--	--	32.3	2.5
		6F	22	22	23	25	24	23.2	1.3
	34	5M	30	32	30	29	30	30.2	1.1
		5F	25	25	24	24	23	24.2	0.3
	52	5M	32	34	35	41	36	35.6	3.4
		5F	34	30	30	28	29	30.2	2.3
	54	5M	36	47	39	30	45	39.4	7.1
		5F	35	30	25	--	---	30.0	5.0
	79	5M	34	45	38	30	--	36.8	6.4
	93	5M	25	--	--	--	--	25.0	0.0
		5F	17	--	--	--	--	17.0	0.0
	104	5F	30	26	--	--	--	28.0	4.0
Tween 20 5%	10	5M	27	27	25	25	26	26.0	1.0
		5F	16	16	21	22	21	19.2	3.0
	18	5M	29	29	29	31	--	29.5	1.0
		5F	25	25	24	25	24	24.6	0.6
	29	6M	30	29	30	29	30	29.6	0.6
		5F	25	24	25	25	23	24.4	0.9
	34	5M	30	30	29	30	30	29.8	0.5
		5F	28	27	28	25	--	27.0	1.4

Table 1. Continued

Diet	Time on diet (weeks)	Initial number of animals and sex	Body weights of surviving animals (gm.) 1	2	3	4	5	Mean body weight (gm.)	Standard deviation
Tween 20 5%	52	5M	30	35	37	35	35	34.4	2.6
		5F	30	32	25	30	--	29.3	3.0
	54	5M	32	28	36	--	--	32.0	4.0
		5F	30	25	28	28	29	28.0	1.9
	79	6M	30	30	35	31	--	31.5	2.4
	93	5M	32	30	--	--	--	31.0	1.4
		5F	45	40	--	--	--	32.5	10.6
	104	5F	35	33	30	29	34	32.2	2.6
Tween 20 10%	10	5M	25	25	25	24	25	24.8	0.5
		5F	19	22	22	20	23	21.2	1.6
	18	4M	25	24	26	--	--	25.0	1.0
		5F	22	24	21	21	20	21.6	1.5
	29	6M	30	30	29	30	30	29.8	0.5
		5F	24	25	24	25	26	24.8	0.8
	34	5M	32	30	32	30	22	29.2	4.2
		5F	22	25	25	26	24	24.4	1.5
	52	5M	34	31	34	32	32	32.6	1.3
		5F	25	30	32	29	25	28.2	3.1
	54	5M	29	32	26	35	--	30.5	3.4
		5F	35	35	37	38	--	36.2	1.5
	79	6M	32	35	--	--	--	33.2	2.1
	93	5M	--	--	--	--	--	--	--
		5F	22	25	--	--	--	23.5	2.1
	104	5F	34	30	31	33	--	32.0	1.8

Table 1. Continued

Diet	Time on diet (weeks)	Initial number of animals and sex	Body weights of surviving animals (gm.) 1	2	3	4	5	Mean body weight (gm.)	Standard deviation
Tween 60 5%	10	5M	26	30	29	26	30	28.2	2.1
		5F	20	24	22	23	24	22.6	1.7
	18	4M	25	28	29	28	--	27.5	1.7
		5F	22	22	22	24	25	23.0	1.4
	29	6M	31	28	30	30	31	30.0	1.2
		5F	25	25	24	25	25	24.8	0.5
	34	5M	32	34	34	29	32	32.2	2.1
		5F	31	34	36	32	34	33.4	2.0
	52	5M	27	35	37	31	30	32.0	4.0
		5F	28	29	29	26	27	27.8	1.3
	54	5M	45	35	34	31	25	34.0	7.3
		5F	35	45	35	32	--	36.8	5.7
	79	5M	30	25	28	28	25	27.2	2.2
	93	5M	--	--	--	--	--	---	--
		5F	--	--	--	--	--	---	--
	104	5F	25	--	--	--	--	25.0	0.0
Tween 60 10%	10	5M	27	25	27	27	--	26.5	1.0
		4F	21	24	24	20	--	22.2	2.1
	18	5M	25	25	25	25	27	25.4	0.9
		5F	22	21	19	20	22	20.6	1.3
	29	6M	30	30	31	30	30	30.2	0.5
		6F	29	34	29	28	--	30.0	2.7
	34	5M	29	29	30	30	32	30.0	1.1
		4F	25	25	28	29	--	26.8	2.1
	52	5M	32	32	34	34	33	33.0	1.0
		5F	29	25	22	25	25	25.2	2.5

Table 1. Continued

Diet	Time on diet (weeks)	Initial number of animals and sex	Body weights of surviving animals (gm.) 1	2	3	4	5	Mean body weight (gm.)	Standard deviation
Tween 60 10%	54	5M	32	40	38	43	35	37.6	4.3
		5F	40	29	30	40	--	34.6	1.9
	79	5M	32	32	33	--	--	32.3	0.6
	93	5M	29	--	--	--	--	29.0	0.0
		5F	30	--	--	--	--	30.0	0.0
	104	5F	24	25	25	24	--	24.5	0.6

sections of control and Tween-fed animals was the change in regularity in the shape, size and general appearance of the nuclei of the hepatic cells with increasing age. Among all of the senile animals (animals on diets from 79 to 104 weeks) many of the hepatic nuclei were much elongated in outline, some had angular shapes, and some appeared to have wrinkled, distorted nuclear membranes. (See Figure 7.)

Among the young and middle aged animals the nuclei were, in general, uniformly spherical in form and varied little in size within each animal or from animal to animal. (See Figure 8.)

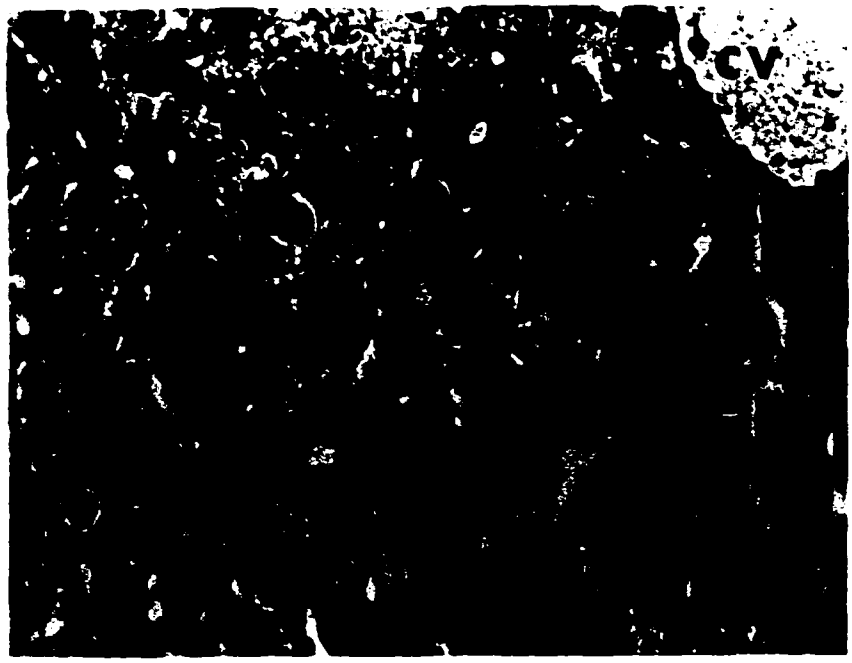
In the senile animals, regardless of diet, many of the nuclei of the hepatic cells may well be called "giant nuclei", because they generally measured twice or more (20-30 microns) the diameter of the other hepatic cell nuclei in the same section. (See Figures 9 and 10.)

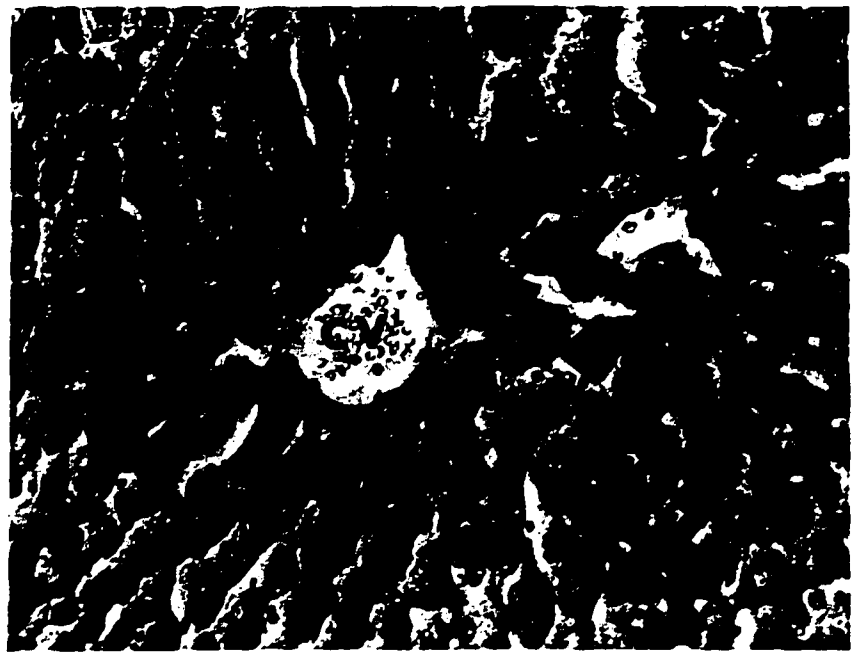
At least one or two hepatic cells were observed in mitosis in each

Plate IV

Figure 7. Cross section of central vein region in liver of female C57 BL/6 Jax mouse fed Tween 60 diet at 10 percent level for 104 weeks. Note irregularity in size and distorted shape of hepatic cell nuclei. CV, central vein. Seven microns, PAS and hemalum, X 400

Figure 8. Cross section of central vein region in liver of male C57 BL/6 Jax mouse fed control diet for 10 weeks. Note regularity in size and round form of hepatic cell nuclei. CV, central vein. Seven microns, PAS and hemalum, X 400


CV


CV

mouse among the animals fed 10 and 18 weeks on control or Tween diets.

Sporadic (less than 0.1 percent of nuclei in all cases) intranuclear bodies were observed. In liver sections prepared by the PAS procedure with prior enzymatic treatment, these intranuclear bodies appeared as clear regions, round or oval in shape, and bound by a limiting membrane. Often they were relatively large, solitary bodies occupying a considerable part of the volume of the nucleus. Among liver sections prepared by the PAS technique without enzymatic treatment, these intranuclear bodies presented PAS positive material. (See Figure 11.)

The location of hepatic cells with intranuclear glycogen bodies varied within the lobules.

Among the liver tissue of 74 control animals (38 males and 36 females) examined in all experiments, intranuclear bodies were found in liver from 16 animals (12 males and four females). Within the 68 animals (33 males and 35 females) fed Tween 20 at the five percent level which were examined in all experiments, liver from 37 animals (21 males and 16 females) possessed hepatic cells with intranuclear bodies, while 18 animals (12 males and six females) in all experiments in which Tween 20 at the 10 percent level was fed possessed livers with intranuclear bodies in hepatic cells. Liver from 64 animals (29 males and 35 females) fed Tween 20 at the 10 percent level were examined. Twenty six (21 males and five females) among the 64 animals (34 males and 30 females) examined which were fed Tween 60 at the five percent level in all experiments and 26 animals (17 males and nine females) among the 65 animals (33 males and 32 females)examined which were fed Tween 60 at the 10 percent level in

Plate V

Figure 9. (Upper left) Hepatic cell possessing large nucleus in liver of male C57 BL/6 Jax mouse fed Tween 20 diet at 10 percent level for 79 weeks. Note intranuclear body with small amount of glycogen within and limiting membrane surrounding this structure. BN, binucleate cell; IN, intranuclear glycogen body. Seven microns, PAS and hemalum, X 1276

Figure 10. (Upper right) Hepatic cell with "giant" nucleus in liver of male C57 BL/6 Jax mouse fed Tween 20 diet at 10 percent level for 79 weeks. Note cross section of sinusoid, appearing as a small triangular white area, and smaller amount of cytoplasmic glycogen (lighter appearing than cytoplasm of other hepatic cells) in hepatic cell with large nucleus. GN, "giant" nucleus; S, sinusoid. Seven microns, PAS and hemalum, X 339

Figure 11. Cross section of liver from male C57 BL/6 Jax mouse fed Tween 20 diet at 10 percent level for 52 weeks showing large intranuclear glycogen body in hepatic cell. Crimson-stained glycogen is represented by black intranuclear mass. IN, intranuclear glycogen body. Seven microns, PAS and hemalum, X 1276


IN


S

all experiments possessed livers with intranuclear bodies within hepatic cells. In all of the experiments the incidence of animals in terms of percentage with intranuclear bodies within hepatic cells was the following for each diet: (1) control, 22 percent, (2) Tween 20 - five percent, 54 percent, (3) Tween 20 - 10 percent, 28 percent, (4) Tween 60 - five percent, 41 percent, and (5) Tween 60 - 10 percent, 40 percent.

Intranuclear bodies in hepatic cells were first observed among the control animals at 18 weeks of feeding time. These intranuclear bodies were first observed at 10 weeks feeding time among animals fed the following diets: five percent Tween 20, five percent Tween 60 and 10 percent Tween 60. Animals on the Tween 20 diet at the 10 percent level were first observed to possess these intranuclear bodies at 18 weeks of feeding time. (See Table 2.)

The presence of wide-spread pericentral infiltration of the hepatic cells by masses of lymphocytes and connective tissue elements was observed. In the regions where such infiltration occurred, there appeared to be an actual destruction of the hepatic cells and their replacement by masses of connective tissue cells on a framework of delicate fibers. (See Figure 12.)

In those liver sections in which pericentral infiltration was observed more frequently and the masses of connective cells occupied a considerable area around the central veins, this condition was classed as "marked". This condition can be classed as "marked" among animals fed each diet for the following periods of feeding time: 52, 54, 79, 93 and 104 weeks. (See Figure 13.)

Table 2. Summary of C57 BL/6 Jax mice with intranuclear glycogen bodies in hepatic cells

Age (weeks)	Time on diet (weeks)	Sex of animals examined	Diet				
			Control	Tween 20 5%	Tween 20 10%	Tween 60 5%	Tween 60 10%
			n[a]	n	n	n	n
8	0	Male	0 (5)[b]	- -	- -	- -	- -
8	0	Female	0 (5)	- -	- -	- -	- -
18	10	Male	0 (5)	0 (5)	0 (5)	1 (5)	0 (4)
18	10	Female	0 (5)	1 (5)	0 (5)	0 (5)	1 (4)
26	18	Male	1 (5)	1 (4)	0 (3)	1 (4)	1 (5)
26	18	Female	0 (5)	0 (5)	1 (5)	0 (5)	0 (5)
37	29	Male	0 (3)	2 (5)	5 (5)	5 (5)	2 (5)
37	29	Female	0 (5)	2 (5)	0 (5)	2 (5)	0 (5)
42	34	Male	4 (5)	5 (5)	3 (5)	5 (5)	3 (5)
42	34	Female	0 (5)	2 (4)	1 (5)	0 (5)	1 (4)
60	52	Male	1 (5)	5 (5)	1 (5)	4 (5)	4 (5)
60	52	Female	0 (5)	3 (4)	0 (5)	0 (5)	1 (5)
62	54	Male	3 (5)	2 (3)	1 (4)	2 (5)	3 (5)
62	54	Female	1 (3)	2 (5)	1 (4)	2 (4)	3 (4)
87	79	Male	2 (4)	4 (4)	2 (2)	3 (5)	3 (3)
101	93	Male	1 (1)	2 (2)	(0)	(0)	1 (1)
101	93	Female	1 (1)	2 (2)	1 (2)	(0)	1 (1)
112	104	Female	2 (2)	4 (5)	2 (4)	1 (1)	2 (4)

[a]The number of animals with intranuclear glycogen bodies in hepatic cells.

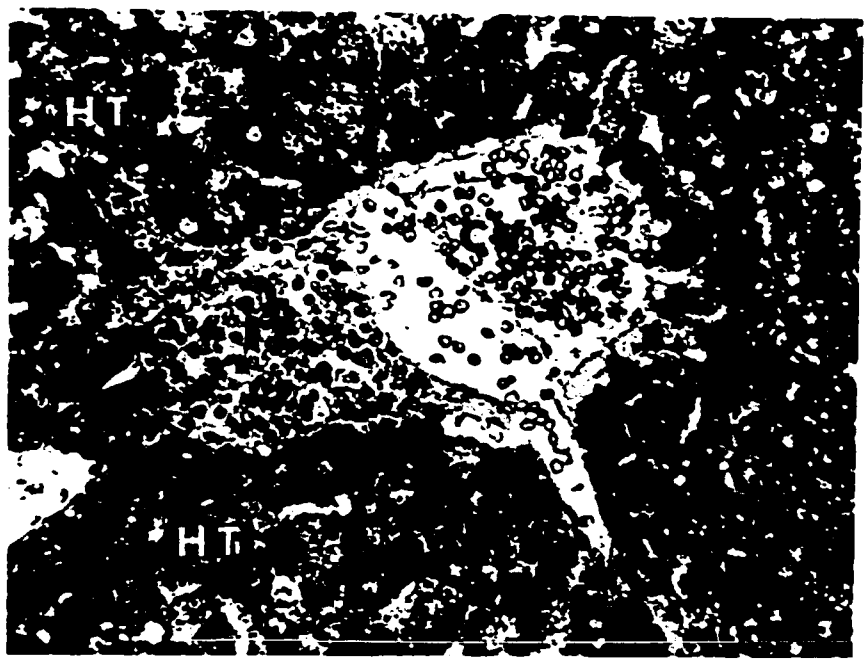
[b]The number in parentheses represents the number of animals examined.

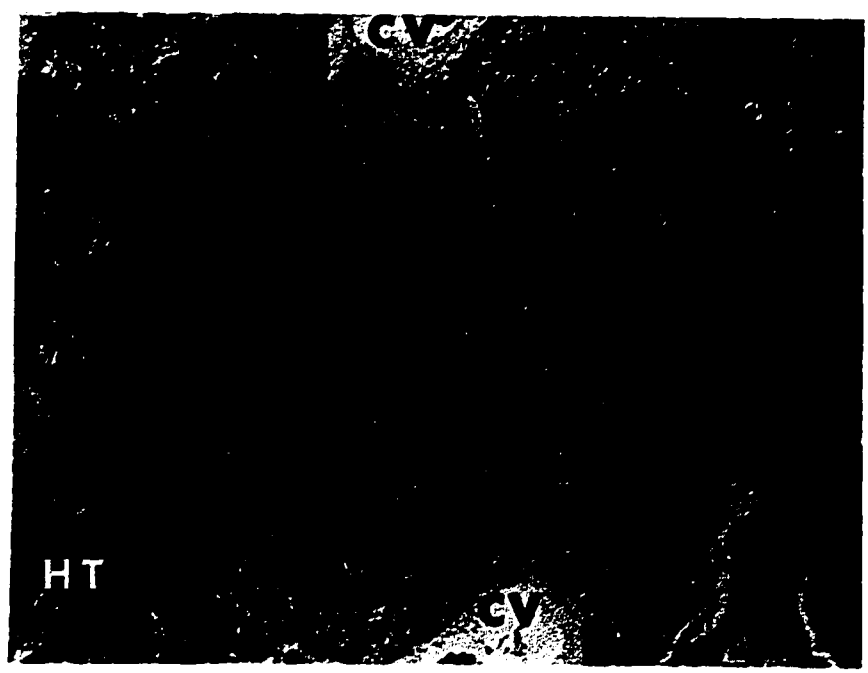
Among all the animals (65) examined which were fed Tween 60 at the 10 percent level, 28 animals, or 43 percent, showed the presence of pericentral infiltration of the hepatic cells by nonhepatic tissue. Animals fed this diet exhibited the highest incidence of pericentral infiltration.

Plate VI

Figure 12. Pericentral infiltration in liver of female C57 BL/6 Jax mouse fed Tween 20 diet at five percent level for 34 weeks. CV, central veins; IT, infiltrating tissue; HT, hepatic tissue. Seven microns, PAS and hemalum, X 400

Figure 13. "Marked" pericentral infiltration in liver of female C57 BL/6 Jax mouse fed Tween 60 diet at 10 percent level for 52 weeks. Note region between two central veins completely infiltrated with nonhepatic tissue. CV, central veins; IT, infiltrating tissue; HT, hepatic tissue. Seven microns, PAS and hemalum, X 160


HT
HT


CV
HT
CV

The number of animals in all other experiments on other diets that possessed pericentral infiltration were the following: (1) control, 17 of 74 animals (23 percent), (2) Tween 20 - five percent, 13 of 68 animals (19 percent), (3) Tween 20 - 10 percent, 10 of 64 animals (16 percent), and (4) Tween 60 - five percent, 14 of 68 animals (21 percent). (See Table 3.)

Among the animals summarized in Table 4 was a condition observed in the hepatic cells which will be referred to as cytoplasmic vacuolation. In liver sections prepared by the PAS technique without prior enzymatic treatment this cytoplasmic vacuolation was characterized by the following appearance. The cytoplasm of hepatic cells contained an empty, open, round space, or spaces among a few cells. In cells with a large vacuole the nucleus and cytoplasm were displaced laterally and appeared like a crescent-shaped figure at the periphery of the cell. If the nucleus was only slightly compressed, it had almost its normal appearance but if squeezed excessively, the nucleus was flattened and appeared to stain more deeply. The cytoplasm in hepatic cells with vacuolation presented PAS positive material in all cases. (See Figure 14.)

The composition of the contents of the vacuoles was not determined in the present study.

In liver sections prepared for another study in which mitochondria in the hepatic cells were stained, there appeared to be a decrease in the number and staining intensity of mitochondria within hepatic cells which possessed cytoplasmic vacuolation. There was a very close topographical relation of the mitochondria to the vacuoles present in the cells.

Table 3. Summary of C57 BL/6 Jax mice with pericentral infiltration of hepatic cells with connective tissue cells

Age (weeks)	Time on diet (weeks)	Sex of animals examined	Diet				
			Control	Tween 20 5%	Tween 20 10%	Tween 60 5%	Tween 60 10%
			n[a]	n	n	n	n
8	0	Male	0 (5)[b]	- -	- -	- -	- -
8	0	Female	0 (5)	- -	- -	- -	- -
18	10	Male	0 (5)	0 (5)	0 (5)	0 (5)	0 (4)
18	10	Female	0 (5)	0 (5)	0 (5)	0 (5)	0 (4)
26	18	Male	2 (5)	0 (4)	0 (3)	0 (4)	2 (5)
26	18	Female	2 (5)	1 (5)	0 (5)	0 (5)	0 (5)
37	29	Male	0 (3)	0 (5)	0 (5)	1 (5)	2 (5)
37	29	Female	1 (5)	0 (5)	0 (5)	2 (5)	3 (5)
42	34	Male	0 (5)	1 (5)	0 (5)	1 (5)	3 (5)
42	34	Female	3 (5)	1 (4)	1 (5)	1 (5)	2 (4)
60	52	Male	1 (5)	1 (5)	3 (5)	1 (5)	3 (5)
60	52	Female	1 (5)	0 (4)	1 (5)	1 (5)	3 (5)
62	54	Male	3 (5)	0 (3)	1 (4)	0 (5)	3 (5)
62	54	Female	1 (3)	2 (5)	2 (4)	3 (4)	3 (4)
87	79	Male	3 (4)	2 (4)	1 (2)	3 (5)	1 (3)
101	93	Male	0 (1)	1 (2)	(0)	(0)	0 (1)
101	93	Female	0 (1)	2 (2)	0 (2)	(0)	0 (1)
112	104	Female	0 (2)	2 (5)	1 (4)	1 (1)	3 (4)

[a]The number of animals with pericentral infiltration.

[b]The number in parentheses represents the number of animals examined.

Table 4. Summary of C57 BL/6 Jax mice with cytoplasmic vacuolation of hepatic cells

Age (weeks)	Time on diet (weeks)	Sex of animals examined	Diet									
			Control		Tween 20 5%		Tween 20 10%		Tween 60 5%		Tween 60 10%	
			n[a]		n		n		n		n	
8	0	Male	0	(5)[b]	-	-	-	-	-	-	-	-
8	0	Female	0	(5)	-	-	-	-	-	-	-	-
18	10	Male	0	(5)	0	(5)	0	(5)	0	(5)	0	(4)
18	10	Female	0	(5)	3	(5)	0	(5)	0	(5)	0	(4)
26	18	Male	0	(5)	1	(4)	3	(3)	4	(4)	0	(5)
26	18	Female	0	(5)	2	(5)	2	(5)	1	(5)	0	(5)
37	29	Male	1	(3)	3	(5)	1	(5)	1	(5)	2	(5)
37	29	Female	4	(5)	4	(5)	3	(5)	1	(5)	3	(5)
42	34	Male	4	(5)	4	(5)	2	(5)	0	(5)	0	(5)
42	34	Female	4	(5)	4	(4)	4	(5)	4	(5)	3	(4)
60	52	Male	2	(5)	4	(5)	4	(5)	4	(5)	3	(5)
60	52	Female	3	(5)	4	(4)	3	(5)	3	(5)	4	(5)
62	54	Male	5*[c]	(5)	3*	(3)	1	(4)	3	(5)	5*	(5)
62	54	Female	3*	(3)	3*	(5)	4*	(4)	4*	(4)	4*	(4)
87	79	Male	0	(4)	3	(4)	0	(2)	4	(5)	0	(3)
101	93	Male	0	(1)	1	(2)		(0)		(0)	0	(1)
101	93	Female	1	(1)	1	(2)	0	(2)		(0)	1*	(1)
112	104	Female	1	(2)	4*	(5)	4*	(4)	0	(1)	1	(4)

[a]The number of animals with cytoplasmic vacuolation in hepatic cells.

[b]The number in parentheses represents the number of animals examined.

[c]The asterisk represents "marked" vacuolation of hepatic cells in liver sections examined.

If hepatic cells in a liver section possessed cytoplasmic vacuolation at all, they were located centrally in the lobules. In liver sections in which many hepatic cells were characterized by very pronounced cytoplasmic vacuolation and were located in all regions of the lobules, the condition was classed as "marked". At 54 weeks on the diets, most of the animals in all diet groups possessed "marked" cytoplasmic vacuolation of the hepatic cells in the liver sections examined. (See Figure 15.)

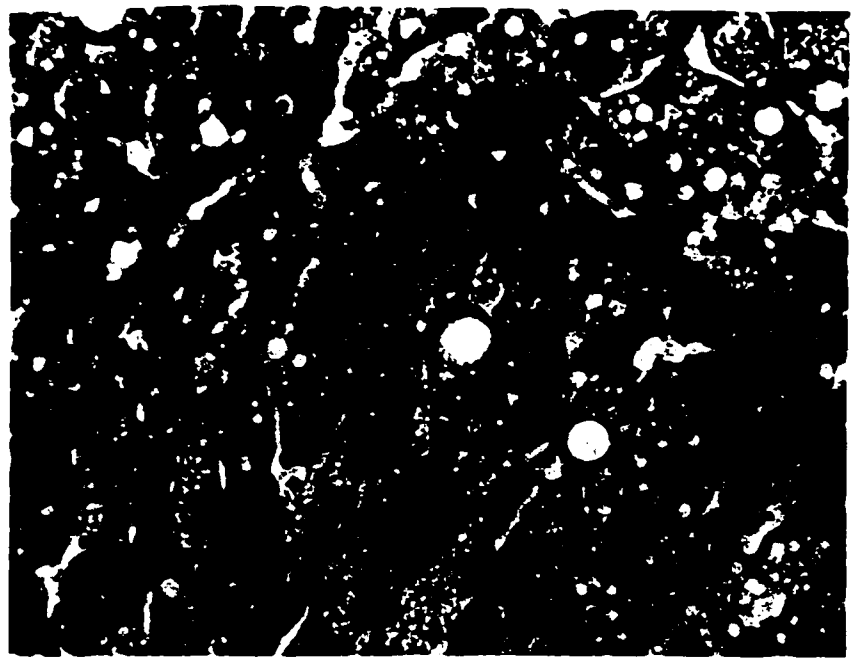
In all experiments the incidence of animals with cytoplasmic vacuolation of hepatic cells was the following for each diet: (1) control, 28 of 74 animals (38 percent), (2) Tween 20 - five percent, 44 of 68 animals (65 percent), (3) Tween 20 - 10 percent, 31 of 64 animals (48 percent), (4) Tween 60 - five percent, 29 of 64 animals (45 percent), and (5) Tween 60 - 10 percent, 26 of 65 animals (40 percent). (See Table 4.)

Necrotic lesions of relatively small areas within the hepatic tissues were observed with varying frequency among the animals. (See Table 5.) In all experiments the incidence of animals with focal necrosis of hepatic cells was the following for each diet: (1) control, 12 of 74 animals (16 percent), (2) Tween 20 - five percent, 24 of 68 animals (35 percent), (3) Tween 20 - 10 percent, 36 of 64 animals (57 percent), (4) Tween 60 - five percent, 46 of 64 animals (72 percent), and (5) Tween 60 - 10 percent, 52 of 65 animals (80 percent). Among the animals fed Tween 60 at five and 10 percent levels, necrosis of hepatic cells was observed first at 10 weeks on the diet. Animals fed Tween 20 at the 10 percent level exhibited mild necrosis of hepatic cells first at 29 weeks on the diet and among animals fed this same Tween at the five percent level,

Plate VII

Figure 14. Hepatic cells with cytoplasmic vacuolation in liver of female C57 BL/6 Jax mouse fed Tween 60 diet at 10 percent level for 93 weeks. Note cytoplasmic vacuoles, appearing as small round, white areas within hepatic cells. Seven microns, PAS and hemalum, X 400

Figure 15. Central vein region in liver of male C57 BL/6 Jax mouse fed Tween 60 diet at five percent level for 54 weeks showing region of excessive cytoplasmic vacuolation of hepatic cells. CV, central vein; RV, region of cytoplasmic vacuolation of hepatic cells. Seven microns, PAS and hemalum, X 160



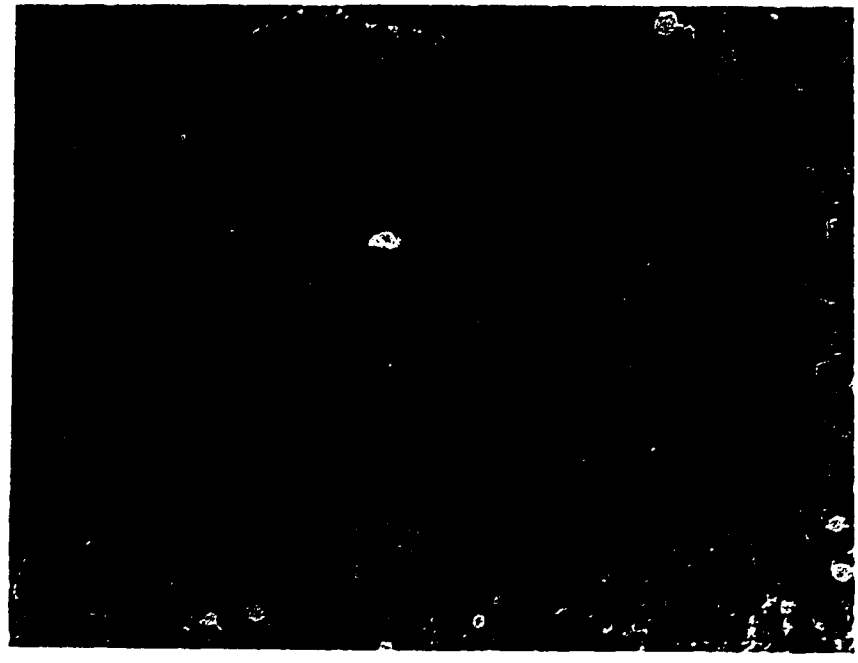


Table 5. Summary of C57 BL/6 Jax mice with necrosis of hepatic cells

Age (weeks)	Time on diet (weeks)	Sex of animals examined	Diet				
			Control	Tween 20 5%	Tween 20 10%	Tween 60 5%	Tween 60 10%
			n[a]	n	n	n	n
8	0	Male	0 (5)[b]	- -	- -	- -	- -
8	0	Female	0 (5)	- -	- -	- -	- -
18	10	Male	0 (5)	0 (5)	0 (5)	3 (5)	2 (4)
18	10	Female	0 (5)	0 (5)	0 (5)	4 (5)	2 (4)
26	18	Male	0 (5)	0 (4)	0 (3)	2 (4)	3 (5)
26	18	Female	0 (5)	0 (5)	0 (5)	3 (5)	4*[c] (5)
37	29	Male	0 (3)	0 (5)	2 (5)	4 (5)	2 (5)
37	29	Female	0 (5)	0 (5)	3*(5)	3 (5)	5 (5)
42	34	Male	0 (5)	3 (5)	4 (5)	1 (5)	4 (5)
42	34	Female	0 (5)	3 (4)	5 (5)	4 (5)	4 (4)
60	52	Male	1 (5)	0 (5)	4 (5)	5 (5)	4 (5)
60	52	Female	3 (5)	1 (4)	3 (5)	3 (5)	4* (5)
62	54	Male	1 (5)	1 (3)	3 (4)	4*(5)	5 (5)
62	54	Female	2 (3)	5 (5)	4*(4)	4*(4)	4 (4)
87	79	Male	1 (4)	3*(4)	2 (2)	5 (5)	3* (3)
101	93	Male	1 (1)	2 (2)	(0)	(0)	1 (1)
101	93	Female	1 (1)	1 (2)	2 (2)	(0)	1 (1)
112	104	Female	2 (2)	5*(5)	4 (4)	1 (1)	4 (4)

[a]The number of animals with necrosis of hepatic cells.

[b]The number in parentheses represents the number of animals examined.

[c]The asterisk represents "marked" necrosis of hepatic cells.

mild necrosis was first observed at 34 weeks on the diet. Mild necrosis of hepatic cells was first observed among control animals at 52 weeks on the diet, and generally the degree of necrosis was slight in all other control animals fed for longer periods of time.

These necrotic regions generally occurred peripherally in the liver sections, but in those animals in which the frequency of these lesions was greater (areas in Table 5 marked with an asterisk), they were also more deeply located.

The necrotic cells were pyknotic and had no detectable glycogen in the cytoplasm. Many small areas of necrosis were infiltrated with leucocytes. (See Figure 16.)

In all cases the bile ducts, hepatic arteries, and interlobular veins appeared normal histologically. No changes in the amount of connective tissue in the liver sections were observed in all of the experiments.

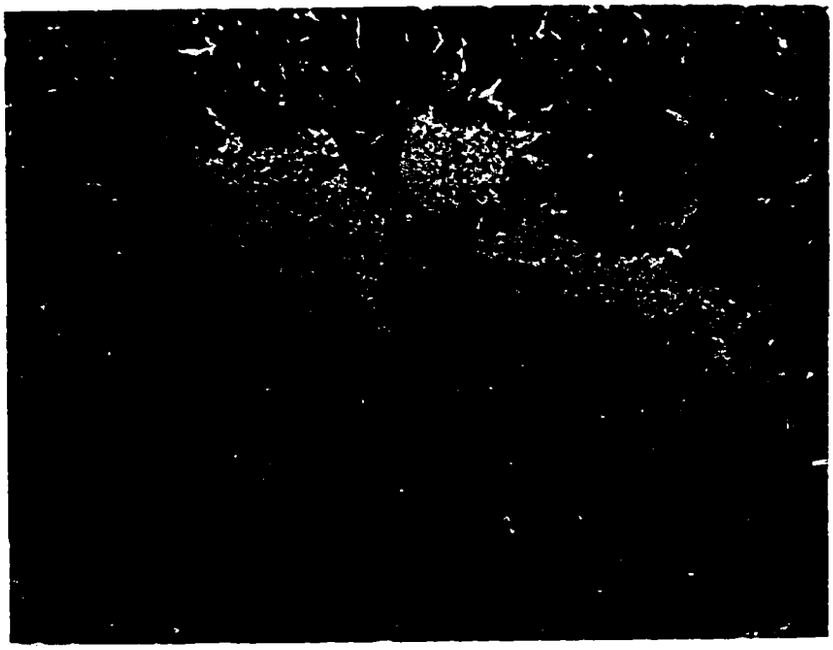
Comparison between liver sections prepared by the PAS technique with or without prior enzymatic treatment was undertaken to determine the relative concentrations of glycogen. Collagen in connective tissue and the elastic membrane of arteries were PAS positive, even after enzymatic treatment.

The PAS technique revealed the glycogen, if present, in hepatic cells as crimson granules of varying sizes and as a crimson homogeneous coloring. (See Figure 17.) Tissue culture studies have shown the glycogen to be actually in solution in the cytoplasm of the living cells and, therefore, its granular appearance in stained fixed tissue should be regarded as a constant fixation artifact (Maximow and Bloom, p. 422,

Plate VIII

Figure 16. Intralobular necrotic lesion in liver of male C57 BL/6 Jax mouse fed Tween 60 diet at five percent level for 54 weeks. Note lack of detectable glycogen within hepatic cells in necrotic region as contrasted to adjacent hepatic cells with cytoplasmic glycogen. CV, central veins. Seven microns, PAS and hemalum, X 160

Figure 17. Glycogen granules in hepatic cells of female C57 BL/6 Jax mouse fed Tween 20 diet at five percent level for 54 weeks. Note cross section of portal region in which several hepatic cells possess cytoplasmic vacuolation, appearing as white, round areas in hepatic cells. IV, interlobular vein; B, bile duct; H, hepatic artery. Seven microns, PAS and hemalum, X 400



1938).

After enzymatic treatment the crimson granules and homogeneous coloring were removed.

Variation in the types of coloration existed among the mice; some showed both types, some showed only one or the other. If granulation was present, it was generally periportal in location.

Since this variation in type of coloration existed among the animals, estimation of the relative glycogen concentration was only approximate.

In livers containing large amounts of glycogen, it was found evenly distributed throughout the liver lobule. When glycogen was present at all it was generally found to be most abundant in the periportal portions of the lobule. There was a tendency for glycogen to be concentrated in the same side of each cell at the edges of tissue. (See Figure 18.) This was probably due to the fixative having driven the glycogen ahead of it as it penetrated the tissue (Mallory, p. 126, 1938).

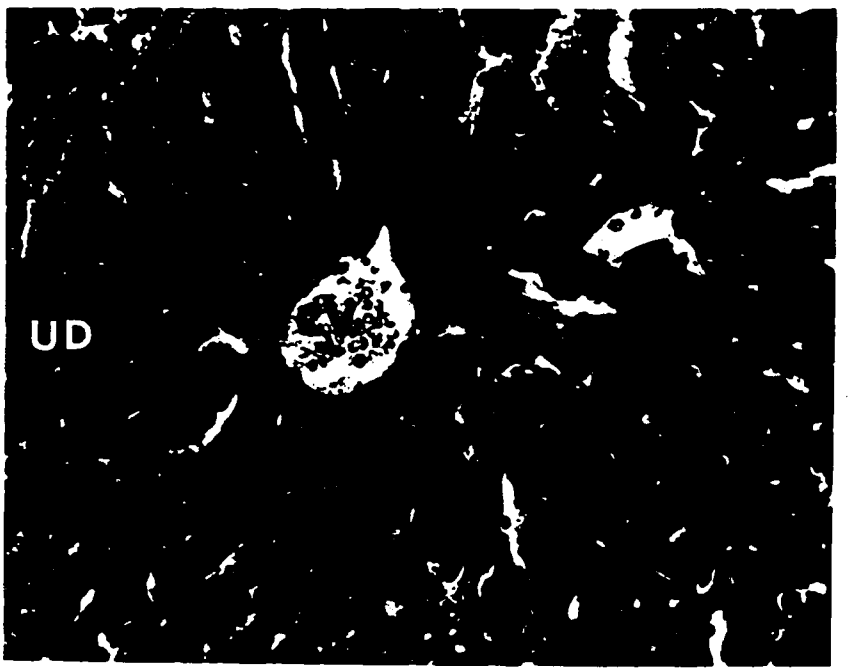
In liver sections with large amounts of glycogen, prepared by the PAS technique with prior enzymatic treatment, the hepatic cells possessed a characteristic appearance. The cytoplasm was pale and fluffy in appearance instead of its more usual homogeneous character. No discernible change in the appearance of the cytoplasm was observed in liver sections with lesser amounts of glycogen. (See Figure 19.)

Estimation of the relative concentration of glycogen for each animal and determination of the mean relative concentration for each cage of surviving animals in each feeding period for each diet revealed the following results. The highest mean relative concentration of glycogen

Plate IX

Figure 18. Cross section of central vein region in liver of male C57 BL/6 Jax mouse fed control diet for 10 weeks. Note diffusion artifact in area to one side of vessel in which uneven glycogen distribution across each cell is shown. CV, central vein; UD, uneven distribution of glycogen. Seven microns, PAS and hemalum, X 400

Figure 19. Diastase-treated, cross section of portal region in liver of female C57 BL/6 Jax mouse fed Tween 20 diet at 10 percent level for 10 weeks showing pale, fluffy cytoplasm of hepatic cells indicative of rich glycogen content. IV, interlobular vein; B, bile duct. Seven microns, diastase-PAS, and hemalum, X 400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among the male control animals was at 10 weeks feeding time, the lowest at 34 weeks. Among the male control animals in all experiments, the greatest variation in mean relative glycogen concentration within a specific group was in the following feeding periods: 0, 18 and 34 weeks. Female control animals had the highest mean relative concentration at 10 weeks feeding time, the lowest at 93 weeks. Variation among female control animals was the greatest at 54 weeks on the diet. (See Figures 20 and 21.)

Male animals fed Tween 20 at the five percent level presented the highest mean relative concentration of glycogen at 18 weeks on the diet; females on the same diet had the highest mean concentration at 10 weeks. At 79 weeks of feeding time the male animals fed Tween 20 at the five percent level showed the greatest variation while among the females it was at 54 weeks on the diet. (See Figures 20 and 21.)

The highest mean relative concentration of glycogen for the male and female animals fed Tween 20 at the 10 percent level was at 10 weeks on the diet. The lowest mean relative concentration for males on this diet was at 54 weeks feeding time; 93 weeks for the females. The greatest variation among the males occurred at the following periods of time on the diet: 29 and 34 weeks. The females showed the greatest variation at 18 weeks on the diet. (See Figures 20 and 21.)

Among the animals fed Tween 60 at the five percent level the highest mean relative glycogen concentration for both the males and females was at 10 weeks on the diet, and the lowest mean relative concentration was at 34 weeks on the diet for the males and 104 weeks for the females. The

Figure 20. Comparison of mean relative glycogen content in liver among male C57 BL/6 Jax mice fed control, Tween 20 - five percent, and Tween 20 - 10 percent diets for varying periods of time

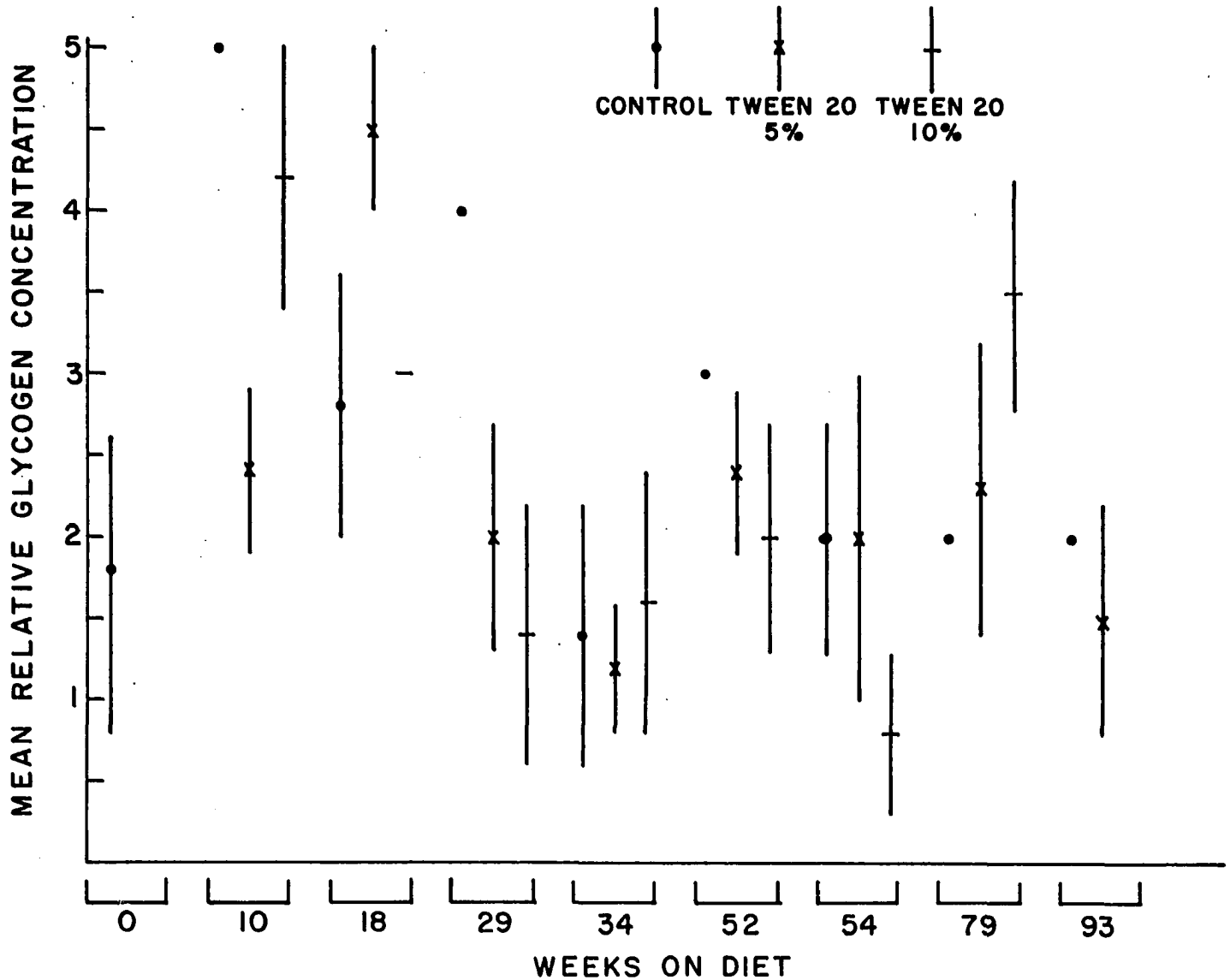
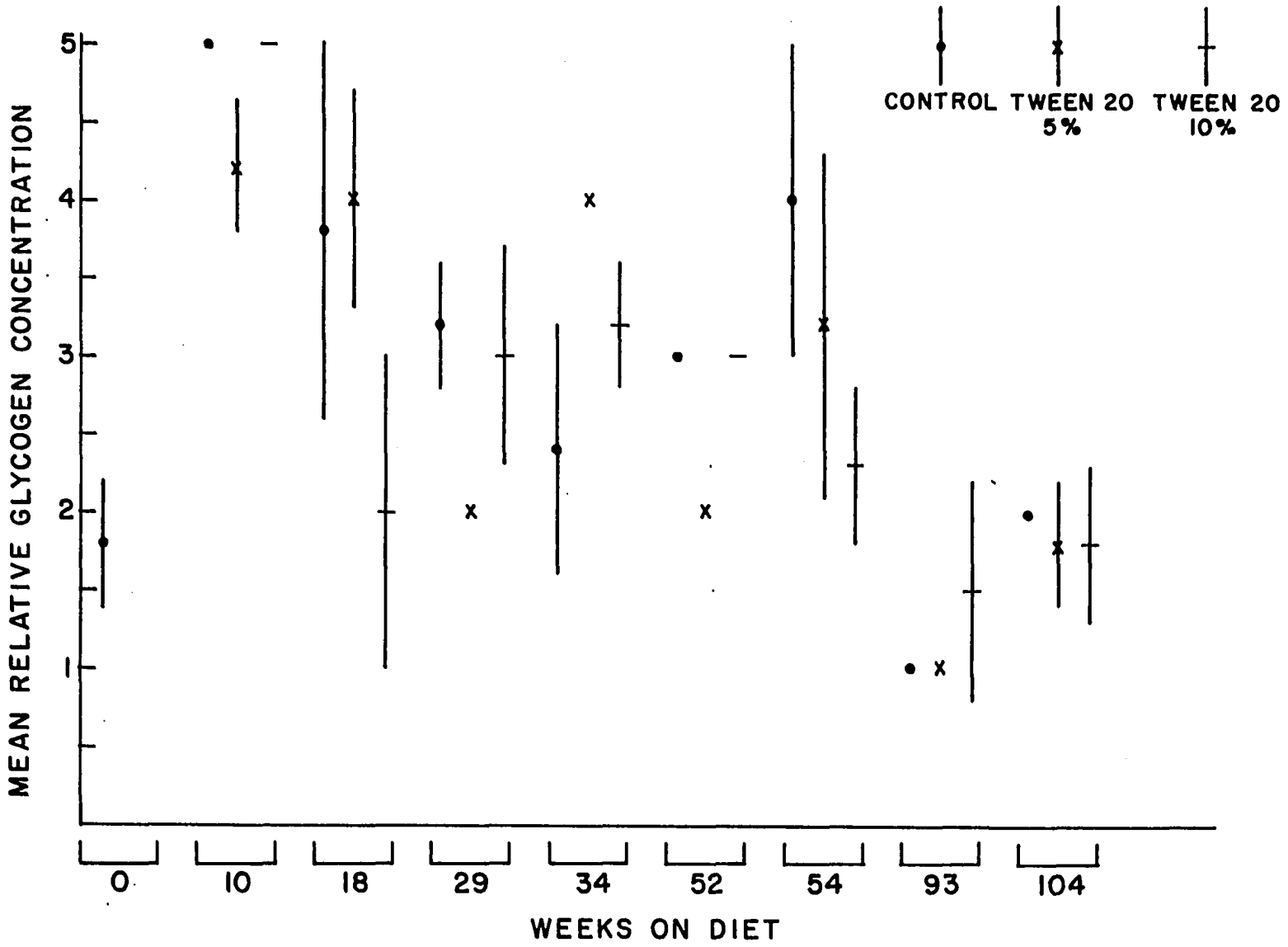

CONTROL
TWEEN 20 5%
TWEEN 20 10%
MEAN RELATIVE GLYCOGEN CONCENTRATION
5
4
3
2
1
0
10
18
29
34
52
54
79
93
WEEKS ON DIET

Figure 21. Comparison of mean relative glycogen content in liver among female C57 BL/6 Jax mice fed control, Tween 20 - five percent, and Tween 20 - 10 percent diets for varying periods of time


MEAN RELATIVE GLYCOGEN CONCENTRATION
5
4
3
2
1
CONTROL
TWEEN 20 5%
TWEEN 20 10%
0
10
18
29
34
52
54
93
104
WEEKS ON DIET

males showed the greatest variation at 79 weeks while it was 54 weeks for the females. (See Figures 22 and 23.)

The male animals fed Tween 60 at the 10 percent level showed the highest mean relative concentration of glycogen at 79 weeks on the diet, the females at 18 weeks. The lowest mean relative concentration for males on this diet was at 34 weeks feeding time and 54 weeks for the females. The males and females both showed the greatest variation at 10 weeks on the diet. (See Figures 22 and 23.)

Figure 22. Comparison of mean relative glycogen content in liver among male C57 BL/6 Jax mice fed control, Tween 60 - five percent, and Tween 60 - 10 percent diets for varying periods of time

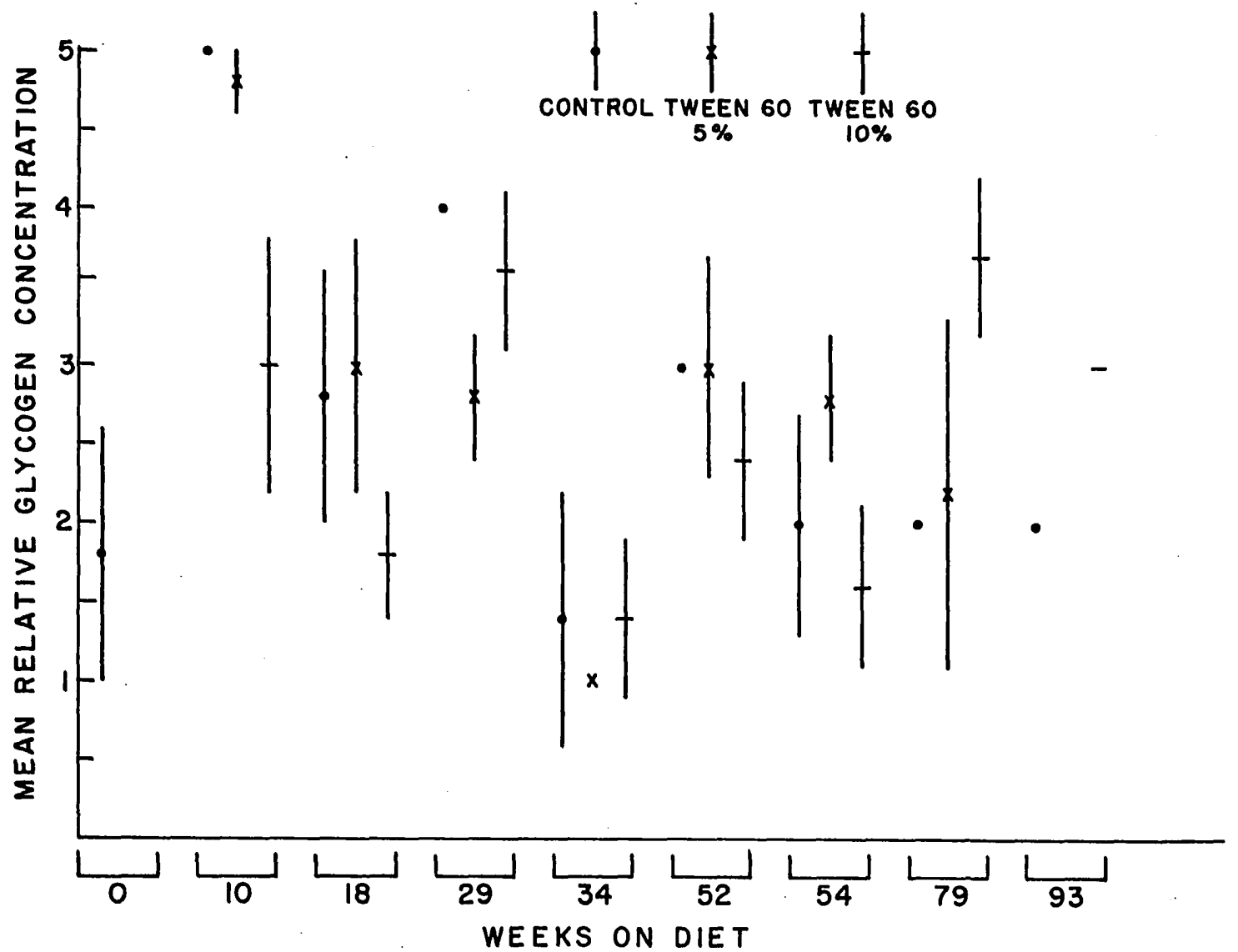
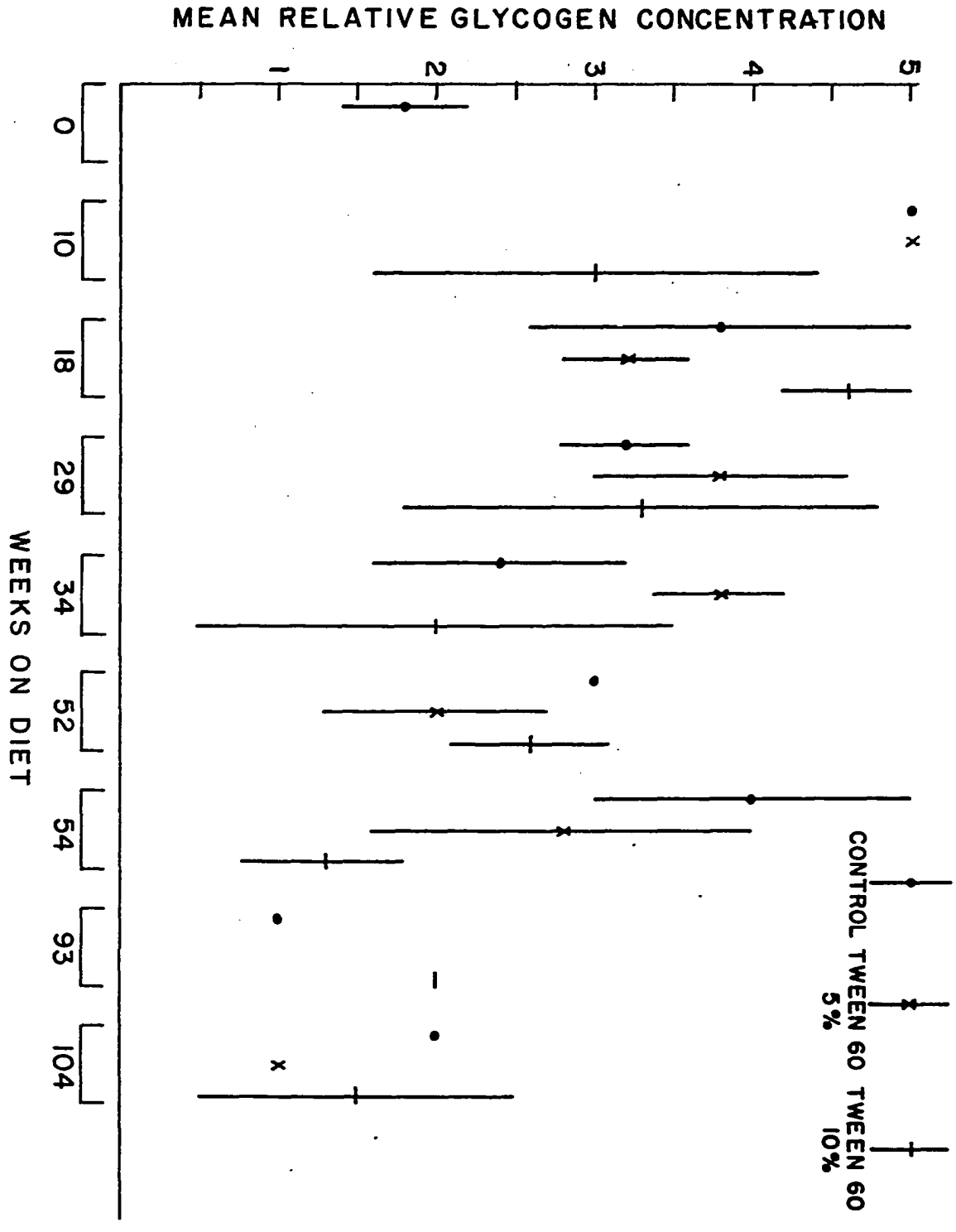

CONTROL
TWEEN 60 5%
TWEEN 60 10%
MEAN RELATIVE GLYCOGEN CONCENTRATION
5
4
3
2
1
0
10
18
29
34
52
54
79
93
WEEKS ON DIET

Figure 23. Comparison of mean relative glycogen content in liver among female C57 BL/6 Jax mice fed control, Tween 60 - five percent, and Tween 60 - 10 percent diets for varying periods of time


MEAN RELATIVE GLYCOGEN CONCENTRATION
1
2
3
4
5
WEEKS ON DIET
0
10
18
29
34
52
54
93
104
CONTROL
TWEEN 60 5%
TWEEN 60 10%

DISCUSSION

It should be emphasized that microscopic findings in hepatic tissue are significant only for a given set of conditions such as sex of the animal, diet, age, time of killing, and method used in killing. Previous investigators as well as the present author have been cognizant of the normal cyclic cytological and histochemical changes correlated with the processes of storage and secretion within the hepatic cell and lobule. Thus a variable microscopic picture is frequently within the realm of normal physiological variation in hepatic tissue. In light of the variation that may occur in normal hepatic tissue, the microscopic findings observed in the present study should be evaluated accordingly.

Some of the microscopic findings described in "results" may be due to dietary imbalance when emulsifiers with non-utilizable polyol portions are fed in the diet, rather than to the toxicity inherent in the compounds tested. Other changes may be due to a direct toxic effect of some portion of the compounds used. Also, many of the microscopic findings may be ageing effects which might be initiated earlier due to the toxicity of the Tweens acting directly or indirectly upon the hepatic tissue.

It may be assumed that Tween 60 is hydrolyzed by intestinal lipase to its component fatty acid (stearic acid) and corresponding polyhydric alcohol moiety (Wick and Joseph, 1956). After oral feeding to rats of polyoxyethylene sorbitan monostearate (Tween 60) labeled with C^{14} in the polyol portion, Wick and Joseph (1956) reported practically complete elimination of the polyol moiety from the animals. Six to 10 percent of the polyol moiety was excreted in the urine, two to seven percent

recovered in the expired carbon dioxide and the remainder was recovered in the feces.

The fate of ingested polyoxyethylene sorbitan monolaurate (Tween 20) has not been determined.

Focal necrosis of hepatic cells appeared to be one of the more apparent changes associated with feeding Tweens. This was especially true for the animals fed the Tween 60 diets. Not only was the frequency of animals with hepatic necrosis greater among the animals fed Tween 60, but the occurrence of necrotic lesions was generally more frequent at the shorter feeding periods. Necrosis was first observed in animals fed the Tween 60 diets for 10 weeks (18 weeks of age).

The high frequency of Tween 20 diet animals exhibiting necrosis of hepatic cells appears to be indicative of a Tween effect. The first appearance of these necrotic regions among animals which were 37 weeks of age indicates that the effect of Tween 20 in inducing necrosis of hepatic tissue was less effective upon younger tissue than Tween 60.

There are many causes for hepatic necrosis of focal distribution. In general, anoxia, toxic substances, and deficiency of foodstuffs are the main factors for inducing this type of necrosis in which the necrotic regions are distributed throughout the organ without regard for lobular architecture. Information from the present study does not indicate which of the above factors may be responsible for this type of necrosis.

While the frequency among the Tween-fed animals with intranuclear glycogen bodies in hepatic cells was greater than in the control animals, the low frequency (less than 0.1 percent) of these bodies in the hepatic

cells in the liver tissue examined probably does not represent a significant effect upon the over-all functioning of the liver.

However, the occurrence of these intranuclear glycogen bodies is noteworthy. These bodies may be an indication of disturbance of the nuclear membrane. The nuclear membrane may develop an inpocketing in which cytoplasm with glycogen is enclosed in the nucleus. An investigation utilizing an electron microscope would be necessary to determine if the limiting membranes surrounding these intranuclear bodies were actually part of the nuclear membrane, and if other cytoplasmic elements other than glycogen were present within the intranuclear bodies. It may be that these bodies are not areas of enclosed cytoplasm but the glycogen may be synthesized *in situ* and not transported from a glycogenetic region of the cytoplasm.

While Andrew *et al.* (1943) did not utilize the same histological techniques, the intranuclear inclusion bodies found in senile liver tissue from C57 mice were apparently of the same nature as the intranuclear bodies described in the present study. They found these intranuclear inclusion bodies only in liver cells of senile animals (586 days through 719 days of age), and described them as a senile change in the liver parenchyma.

In the present study this phenomenon was observed frequently among the older control animals, but it was also observed in a male control animal that was 182 days of age. Further, the data indicate that this senile change is initiated earlier in the *Tween*-fed animals. There was a high frequency of middle age, *Tween*-fed animals that possessed these

intranuclear bodies.

These intranuclear bodies are also probably of the same nature as the bodies discovered by Findlay (1932) in the livers of adult mice of the Claxton strain. The degree of incidence of the inclusions varied in different animals from 0.2 to 4.16 percent of the total number of cells. The ages of the mice were not given.

Findlay (1932) described the bodies as varying in size from about eight to 80 or 90 microns in diameter. This enormous body as indicated by the latter figure is difficult to conceive to be contained within a mouse liver nucleus.

Andrew (1941) observed the condition of infiltration of lymphocytes and connective tissue cells around the portal regions in the liver of senile C57 mice (586 days through 719 days of age). In those areas of infiltration the hepatic cells were missing. In the present study this same condition was observed, but in a different location and at a younger age. The infiltrating masses of cells were pericentral in location and were found in control animals that were 182 days of age. However, among the control animals (420 through 609 days of age) in the present study this phenomenon was observed more frequently and the areas of pericentral infiltration were larger in size. This appears to be a condition that becomes more pronounced among older animals.

Animals fed the Tween 20 diets at the five and 10 percent levels were very similar to the control animals in this phenomenon.

The high frequency of Tween 60 - 10 percent diet animals exhibiting pericentral infiltration appears to be indicative of an effect by Tween 60.

Animals fed the Tween 60 diet at the five percent level were quite similar to the control animals in pericentral infiltration.

Andrew (1941) considered these cell masses as compensatory growths of splenic pulp tissue brought about when increased activity of splenic tissue is needed, such as in infections. He postulated that splenic cells migrate through the portal circulation to the liver where colonies of cells are set up which grow in size by arrival of new cells and by cell multiplication. The growth in size of the cell mass leads to further infiltration with destruction of numerous hepatic cells.

The cytoplasmic vacuolation observed in the hepatic cells among the mice strongly indicates the presence of fat vacuoles. This is based upon the following indirect evidence. Fat is commonly stored in the liver, and the hepatic cells in the central region of the lobules generally store fat before other regions within the lobule. In the present study if vacuolation was found at all in a section of liver, it was always observed within hepatic cells in the central part of the lobules. In examination of hepatic cells with large fat vacuoles, the cytoplasm and nucleus of the hepatic cells are collected at the periphery of the cell. Further, if histological techniques are used which remove the fat within the vacuoles of hepatic cell cytoplasm, the areas formerly occupied by fat in the hepatic cell are revealed as white, round areas. Hepatic cells with cytoplasmic vacuolation observed in the present study presented the same description as given above.

Also, the close topographical location of hepatic cell mitochondria to the vacuoles indicated the presence of fat. According to Palade

(1958) there is a close morphologic relationship between fat droplets and mitochondria.

Since the cytoplasm of a hepatic cell in normal mice may present a variable appearance that reflects the functional state of the cells, the relationship between Tween effects and presence of cytoplasmic vacuolation should be evaluated accordingly. Animals fed Tween 20 at the five and 10 percent levels possessed cytoplasmic vacuolation of hepatic cells at younger ages than among the control animals. Tween 20 may be responsible for the earlier appearance of cytoplasmic vacuolation.

Cytoplasmic vacuolation of hepatic cells appeared to be a phenomenon which occurred normally in the C57 BL/6 Jax mouse and tended to be more pronounced among older animals.

As indicated in "results" the presence of granulation of glycogen within liver sections complicated the estimation of the relative liver glycogen concentration. A further shortcoming of the method used for estimation of the relative concentration of liver glycogen was the lack of data to show that observations can be made with sufficient consistency. While it was possible for the author to re-examine liver sections and reproduce results, it is another matter for two investigators to agree in their estimations. It is probable that with experienced observers there would be close agreement but the implication is that where two investigators wish to use this method they should check carefully to see to what extent they agree in their estimations, and wherever possible, all estimations for an investigation should be made by the same person. It is also evident that the values obtained for glycogen concentration by this

method are approximations which are useful in showing relative changes rather than absolute changes. While this method may not detect small differences in glycogen concentration among liver sections of different animals, it should be noted that the method is useful for demonstration of differences in glycogen concentration when the magnitude of the differences are large.

Comparison of male and female control animals for relative liver glycogen concentration revealed the following. Both male and female control animals had the highest relative concentration at 10 weeks of feeding time (18 weeks of age). Among the male animals there was a tendency for the relative liver glycogen concentration to be within a similar range from 34 through 93 weeks of feeding time with the exception of 54 weeks which was above the range. The female control animals presented an interval from 29 through 54 weeks of feeding time in which the values of relative glycogen concentration were similar. However, the mean values for relative glycogen concentration recorded for the female animals in this period of similarity were higher than those for the period of similarity among the male animals.

Male and female animals fed the Tween 20 diet at the five percent level for 10, 29, and 52 weeks possessed mean relative liver glycogen concentration values below control animal ranges. Female animals fed the Tween 20 diet at the 10 percent level for 54 weeks, and male animals fed the same diet for 29, 52, and 54 weeks were below control animal ranges of mean relative liver glycogen concentration. (See Figures 20 and 21.)

Male animals fed the Tween 60 diet at the five percent level for 29 weeks and female animals fed the same diet for 52 and 104 weeks were below control animal ranges for mean relative glycogen concentration. Among the animals fed the Tween 60 diet at the 10 percent level, male animals fed for 10 and 52 weeks, and female animals fed for 10 and 54 weeks were below control animal ranges. (See Figures 22 and 23.)

It is noteworthy that with the exception of male animals fed Tween 60 - five percent, below control animal values for mean relative glycogen concentration were obtained for all other Tween-fed animals fed for either 52 or 54 weeks. At 52 and 54 weeks of feeding time the cytoplasmic vacuolation of hepatic cells among Tween-fed animals occurred generally more frequently within the liver sections.

With the exception of male animals fed the Tween 20 - five percent diet for 18 weeks, all other Tween-fed animals with above control animal ranges for mean relative glycogen concentration were fed 34 weeks or more.

SUMMARY AND CONCLUSIONS

1. A study of the effects upon the histology and histochemistry of the liver of Tween-fed C57 BL/6 Jax mice was undertaken as part of a series of investigations in progress in the Department of Zoology and Entomology at Iowa State University.

2. Male and female animals were placed on each of five diets at the age of eight weeks. Food and water were supplied ad libitum.

3. Control animals were fed Purina Mouse Breeder Chow in meal form. Experimental diets of Purina Mouse Breeder Chow in meal form containing, in terms of percentage weight, five percent Tween 20 or Tween 60, or 10 percent Tween 20 or Tween 60 were fed to other animals.

4. Generally five male and five female animals were fed each of the diets for the following number of weeks: 10, 18, 29, 34, 52, 54, 79, 93, and 104. In addition, 10 control animals (five males and five females) were killed at the initial age (eight weeks) of all other animals utilized in the investigation.

5. Carnoy-fixed liver tissues cut at four and seven microns were stained with PAS, with or without prior treatment with malt diastase to remove glycogen.

6. Livers were examined for histological changes and glycogen content.

7. Liver glycogen content was determined by estimation of the relative concentration of glycogen in situ.

8. Gross observations of livers in all animals revealed no pathological changes.

9. Pericentral infiltration by nonhepatic tissue and some irregular-shaped hepatic nuclei were observed in older mice regardless of diet. The high frequency of Tween 60 - 10 percent diet animals exhibiting pericentral infiltration by nonhepatic tissue was indicative of a Tween effect.

10. Focal necrosis of hepatic cells was the most apparent effect of Tween 20 and Tween 60 at either five or 10 percent levels on the liver of male and female C57 BL/6 Jax mice. Tween 60 diets were more effective in inducing this phenomenon among younger mice than Tween 20 diets.

11. Intranuclear glycogen bodies in hepatic cells appearing at a frequency of less than 0.1 percent in each liver affected were observed. The high frequency of Tween-fed animals 18 to 42 weeks of age exhibiting these intranuclear bodies indicated that this senile change in normal liver tissue was initiated earlier among animals fed either Tween at each level.

12. Cytoplasmic vacuolation of hepatic cells, suggestive of fat vacuoles, was observed more frequently among younger mice (18 through 37 weeks) fed Tween diets than among control animals. This condition was initiated earlier among Tween-fed mice. Cytoplasmic vacuolation of hepatic cells was a normal phenomenon among older male and female C57 BL/6 Jax mice.

13. Male and female control animals possessed the highest relative liver glycogen concentration at 10 weeks of feeding time.

14. Male and female mice fed the Tween 20 - five percent diet were below control animal ranges for mean relative liver glycogen concentration

at 10, 29 and 52 weeks of feeding time.

15. Male mice fed the Tween 20 - 10 percent diet were below control animal ranges for mean relative liver glycogen concentration at 29 and 52 weeks of feeding time; for female mice on the same diet it was 54 weeks of feeding time.

16. Male mice fed the Tween 60 - five percent diet were below control animal ranges for mean relative liver glycogen concentration at 29 weeks of feeding time; for female mice on the same diet it was 52 weeks of feeding time.

17. At 10 and 52 weeks of feeding time male mice fed the Tween 60 - 10 percent diet, and at 10 and 54 weeks of feeding time female mice fed the same diet were below control animal ranges for mean relative liver glycogen concentration.

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