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THE EFFECTS OF AGING AND DIETARY EXCESS ON MOUSE LIVER POLYPLOIDIZATION

Irene Pavai

A Thesis

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The Department

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ABSTRACT

THE EFFECTS OF AGING AND DIETARY EXCESS ON MOUSE LIVER POLYPLOIDIZATION

Irene Pavai

Polyploidization of mammalian liver cells increases with age. In aging mice, it has been reported that protein restriction decreases the level of polyploid liver cells. The questions posed in this study were as follows: 1) Could dietary excess influence ploidy levels in aging mouse liver? and 2) At what point in development does diet exert its greatest influence? Control mice received a balanced diet of rat chow and water while the tests diets included: rat chow/10% ethanol, 64% protein/water, 68% sucrose/water and 44% lipid/water.

Two parallel experiments were carried out. In the first experiment, female Swiss albino mice, 13 days pregnant, were placed on control and test diets. The first experimental group consisted of their male offspring which were maintained on the "maternal" diet and sacrificed at 3 weeks, 3, 6 10 and 12 months of age. The second group of mice consisted of adult male Swiss albino mice placed on the same diets at 3 months of age and sacrificed at 6, 10 and 12 months of age. Liver polyploidization in both groups was assessed in terms of changes in average nuclear diameter, percentage of nuclei of various ploidy classes and polyploidization index.

The liver weight, liver/body weight ratio, food and

liquid consumption, daily energy intake were all significantly affected by dietary composition as was liver polyploidization. Liver polyploidization was most affected by excesses in dietary protein causing significantly increased levels of liver cell polyploidization above that seen in controls when mice were first exposed to test diets in the early stages of development.

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I would like to dedicate this thesis to my loving parents, Nicholas and Irene Pavai, who inspired my love and appreciation of nature and respect for the earth and to my wonderful sisters, Anita, Marianne and Evelyne. I would also like to extend my appreciation to all my friends for their support and encouragement during my studies.

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INTRODUCTION

In this thesis, the phenomenon of liver polyploidization and how it is influenced by age and diet will be examined. As a background for this study, a review of cellular aging changes is required.

The possibility has been suggested that long-lived, non-mitotic tissues such as nerve and muscle, and to a lesser extent low mitotically active tissues such as the liver, act as the "pacemakers" of biological aging (Curtis, 1963; Lamb, 1977). Cumulative age-related damage that occur in the cells of these tissues cannot be eliminated through cell turnover. This damage could cause a decrease in the functional capacity of the organ, possibly resulting in the shortening of the lifespan of the organism (Curtis, 1963; Lamb, 1977, Uryvaeva, 1981). It is thought that aging may originate from spontaneous free-radical reactions which cause genetic damage to DNA or cause disruption of biological membranes. In addition, lipofuscin, an inert production of lipid peroxidation caused by free radicals, may accumulate in time and physically impair the cells' ability to function normally (Curtis, 1963; Gahan, 1977). Another age-dependent phenomenon occurring at the cellular level is polyploidization (Bohm and Noltemeyer, 1981a; Enesco and Samborsky, 1983; Evans, 1976; Shima and Sugahara, 1976; Uryvaeva, 1981). Polyploidy is the phenomenon whereby a cell replicates its chromosomes

without cell division to produce cells with multiple sets of chromosomes above the diploid level. Although the biological significance is not fully understood as yet, polyploidization itself is not considered to be a deleterious change. However, cellular changes which accompany polyploidization may have potentially harmful consequences (Gaub et al. 1981; Harris, 1971).

Though some reports indicate that polyploid cells are found in lower vertebrates (Brasch, 1980), they are not as prevalent as in mammals. Polyploidy is particularly pronounced in those tissues that are long-lived, highly differentiated and geared towards large-scale synthesis and export of particular proteins (Brasch, 1982). Liver polyploidy, characteristic to many species of mammals, has been the subject of extensive study especially in rodents where ploidy levels are quite high (Brasch, 1980; Brodsky and Uryvaeva, 1977; Carriere, 1969).

In humans, polyploidization of the liver commences around the onset of puberty, at 11-14 years of age (Gahan and Middleton, 1984; Swartz, 1956). In mice, it starts at 14 to 21 days of age, concomitant with time of weaning (Evans, 1976; Shima and Sugahara, 1976). There is very little mitotic activity in the adult liver. When the liver is called upon to divide during the replacement of damaged or dead cells, it will do so at a rate of approximately 1 per 20,000 cells every 24 hours in rat and mouse

(Doljanski, 1960; Gahan, 1977). At the age of 3 to 4 weeks in mice, there is a shift from liver growth by normal mitoses to liver growth by polyploidization (Uryvaeva, 1981). At first, liver cells undergo acytokinetic mitoses where nuclear division occurs in the absence of cytoplasmic division giving rise to binuclear cells with two 2N nuclei. After this, the two nuclei of the binuclear cell enter a synchronous S-phase and prophase then their chromosomes unite in a common metaphase; the following stages of mitosis then proceed as usual. This second type of mitosis is referred to as "bimitosis". Regardless of whether cytokinesis follows the bimitosis or not, two nuclei with the next higher degree of polyploidy are formed (Brasch, 1982; Brodsky and Uryvaeva, 1977; Epstein, 1967; Gahan, 1982; James, 1977; Wilson and Leduc, 1948). The cell may undergo several normal replicative cycles giving rise to daughter cells with the same ploidy values, thereby enhancing the number of polyploid cells or undergo more polyploidizing mitoses to form another class of polyploid cells at a higher ploidy level.

The polyploid cells that are produced follow the geometrical progression of 2^n, that is with 2N, 4N, 8N ... nuclei, with no intermediate ploidy classes. This progression is accompanied by an increase in nuclear diameter by a factor of 1.26 with every move to the next higher ploidy class (Epstein, 1967). Thus, the adult

mammalian liver is a mixed population of hepatocytes with different number of nuclei and different DNA content (Brodsky and Uryvaeva, 1977; Shima and Sugahara, 1976, Uryvaeva, 1981).

It is believed that liver cell polyploidization is under endocrine control. It has been discovered that the nuclear class series present in normal mice are absent in hereditary pituitary dwarfs, but that a polyploid cell population can be restored by growth hormone treatment (Bass and Dunn, 1957; DiStefano et al., 1955, 1959; Nadal and Zajdela, 1966; Wheatley, 1972). Polyploidization of the liver can be arrested by hypophysectomy as well as by thyroidectomy (Bass and Dunn, 1957; Carriere, 1969; Distefano, 1959). There is also evidence which shows that thymus plays a role in controlling polyploidization by modulating the level of growth hormone in the blood. Newborn thymus grafted into old recipients was found to decrease the percentage of tetraploid cells in mice (Pieri, 1982). The formation of polyploid cells in the liver also appears to be altered by such factors as X-radiation, ethanol, and phenobarbital which increase the level of polyploidization expected for rodents of a given age group (Bohm and Noltemeyer 1981b, Gaub et al., 1981; Shima and Sugahara, 1985). Diet, more specifically, dietary protein restriction, decreases the rate of polyploidization of the liver in mice (Enesco and Samborsky, 1983).

There appear to be several schools of thought as to the biological significance of mammalian liver polyploidization. One school of thought is that a shift from lower to higher ploidy levels provides the cell with an increased work capacity (Evans, 1976; Gahan and Middleton, 1982). That is, multiple gene copies rendered by multiple sets of chromosomes may be called into action to produce gene dose-dependent proteins when required to deal with increased work loads. In mammals, the move to higher ploidy levels is accompanied by an increase in cell size and decrease in mitotic activity (Enesco and Sambørsky, 1986; Epstein, 1967; Gahan and Middleton, 1982). Since gene-activity is believed to be proportional to gene-dose (Evans, 1976; Gahan and Middleton, 1982), this gene amplification in association with the changes mentioned above has been suggested to increase the efficiency of the cell to deal with increased work loads without submitting the organ to high stress conditions and possible damage through wear and tear (Brasch, 1982; Evans, 1976; Gahan, 1977; Gahan and Middleton, 1982). Due to the liver's important role in metabolism and detoxification, it is important that the liver respond as efficiently as possible throughout the life of the organism. Possibly polyploidization allows a certain plasticity in the functional capacity of the liver throughout the lifespan of the organism.

The second hypothesis as to the significance of liver polyploidization is that a polyploid cell with multiple chromosome homologues is protected against different kinds of genetic damage (Uryvaeva, 1981). The liver is directly exposed to toxic substances and potentially mutagenic substances because its major blood supply is derived from the gut and because of the development of the liver microsomal mixed function oxidase system of enzymes capable of converting promutagens to mutagens (Evans, 1976; Uryvaeva, 1981). Also, within the course of metabolism, highly reactive free radicals are generated. All of these factors make hepatic DNA particularly susceptible to damage which is probably why the liver is known to have a high level of spontaneous chromosomal aberrations. Tissues with high rates of cell turnover are able to eliminate these defective cells from the population. In contrast, long-lived cells like hepatocytes which have a very low mitotic rate do not have the same opportunity to discard and replace mutant cells (Curtis, 1963; Uryvaeva, 1981). When these cells are stimulated to divide, chromosomal aberrations could lead to aberrant mitoses. The unequal distribution or loss of genetic material due to aberrant mitoses may result in cell death, malignant transformation or impairment of differentiated functions (Uryvaeva, 1981). It has been shown that short-lived species such as mice that have an increased capacity for chemical mutagen

production and for high levels of spontaneous chromosomal aberrations combined with a decreased ability for DNA-repair, also have the highest levels of liver cell polyploidy (Uryvaeva, 1981).

Could it possibly be that both of these mechanisms are operative simultaneously in the liver? It does not seem implausible that polyploidization of the liver confers short-term benefits upon the liver through a temporary increase in the functional capacity when induced by an increased work load through the introduction of toxic substances or increased dietary loads which must be eliminated. The benefit is short-term in this case because studies have shown that there is a decline in polyploidization back down to age-dependent levels shortly after the physiological stress has been lifted (Bohm and Noltemeyer, 1981b).

The age-dependent polyploidization which occurs in the absence of an increased work load may possibly confer long-term benefits to the liver through minimizing the severity of any mutagenic damage that may accumulate in the cell throughout its lifespan. Because of the functional importance of the liver, it is imperative that the liver retain its capacity for regeneration in response to damage and it is therefore essential that the regulatory genes responsible for such controlled growth remain intact and functional (Evans, 1976). Should such genes become

damaged, a polyploid cell with multiple gene copies which can compensate for the defective gene, will not be functionally impaired (Gahan, 1977). Thus, polyploidy provides the cells with a genetic integrity which is necessary to maintain the liver's proper functioning throughout the lifespan of the animal.

Because dietary protein restriction has already been shown to alter the rate of age-dependent polyploidization of mouse liver, the primary objective of this study was to determine whether or not the pattern or rate of polyploidization could be altered by dietary excess and to determine which dietary component: protein, lipid or carbohydrate, exerts the greatest influence, if any, on this process. In this study, the progression of polyploidization of the liver was followed in two different groups of mice. The first experimental group consisted of mice which were the offspring of females that were exposed to one of the test diets starting at day 13 of gestation through to parturition and during the lactation period. The offspring were weaned onto the same diet as their mothers and maintained on the diets up to 12 months of age. The second experimental group consisted of mice which were started on one of the diets as adults. Some of the questions attempted to be answered by this study are: 1) Do any of the experimental diets alter the pattern or rate of polyploidization, and how do the two experimental groups of

mice compare? 2). Can polyploidization be induced to occur earlier than it normally would in mice that have been subjected to the diet as early as 1 week prior to birth? If not, what is the degree of response when the mechanism of polyploidization is finally turned on? 3) Since ethanol has already been found to increase polyploidization of liver, on which of the two groups does it exert its greatest effect?

MATERIALS AND METHODS

Animals used

Swiss albino mice used in this study were obtained from Charles River Laboratories, St. Constant, Quebec. Pregnant female mice were individually housed in polypropylene cages 28 x 18 x 12.5 cm with a wire cover. Groups of young males were housed 2 per cage. Throughout the experiments, the animals were kept at 22°C and received a 12h light/12h dark light cycle on a uniform schedule. Food and water were available ad libitum.

Diets used

The diets used in this study were as follows: 1. rat chow/water; 2. rat chow/10% ethanol; 3. 64% protein/water; 4. 68% sucrose/water; 5. 44% lipid/water. Diet 1, the control diet, consisted of Prolab Rat Chow R-M-H 4020, a commercially prepared diet by Agway Inc.,

distributed by Charles River Inc. Diet 2 consisted of the same rat chow used in Diet 1, however, instead of water the mice were provided with a 10% ethanol solution for drinking (Chaughan et al., 1969; Ewart et al., 1979; Fuchs et al., 1967). Test Diets 3 and 4 were obtained from ICN Biomedicals Inc., catalogue no. 904669 and 901943 respectively. Diet 5, the high lipid diet, consisted of shelled, raw, unsalted peanuts obtained from Awatto House Health Foods Inc., Montreal, Quebec. The lipid content of the peanuts was verified through a crude extraction of lipid from 3 different batches of peanuts and was found to be within the range listed in food composition guides (Church, C.F. and Church, H.N., 1975). The 44% lipid diet was also supplemented with 2-3 drops of Hagen Vitamin Supplement-Conditioner in the drinking water daily. Appendix I provides the food composition of the diets. All diets were sufficient for reproduction and growth.

Experiment 1: Early exposure to diets

In experiment 1, mice were first exposed to the test diets in utero by placing pregnant female mice on the special diets, and then raising the male offspring on the same "maternal" diet.

Fifteen pregnant females at the thirteenth day of gestation were divided into 5 groups of 3 mice each and placed on the diets mentioned earlier. The females were

housed separately and maintained on the designated diet throughout their pregnancy and lactation period. At age of weaning (3-4 weeks), 5 male offspring were chosen from each mother for a total of 15 mice of a particular dietary group. Each group of 15 mice continued to be supplied ad libitum with the same diet as their mothers. These young male offspring constituted Group 1 mice. These mice were housed 2 per cage under the same environmental conditions described earlier. Three mice from each dietary group were sacrificed at 3 weeks, 3, 6, 10 and 12 months of age.

Experiment 2: Late exposure to diets

Forty-five adult male Swiss albino mice were randomly divided into 5 groups. Each group of 9 mice were placed on one of the five diets described in experiment 1 and maintained under the same conditions. Prior to placement on test diets, mice were fed a uniform diet of rat chow and water from the time they were obtained at 3 weeks of age to the time they were placed on the test diets at 3 months of age. These animals constituted Group 2 mice which were sacrificed at 6, 10 and 12 months of age.

Food and Liquid Consumption Studies:

These studies were performed at 1, 3, 6, 10 and 12 months of age on Group 1 mice and at 3, 6, 10 and 12 months of age on Group 2 mice.

Five mice were randomly selected from each dietary group and were housed singly for these experiments, however, in the 12 month age group the remaining 3 mice were used. Food was weighed to the nearest 0.01 gram using a Sartorius electronic balance, at the start and finish of each test period which lasted 2-3 days. A known volume of liquid was measured out at the beginning of the test period using a graduated cylinder and approximated to the nearest 0.01 milliliter. The amount remaining at the end of the test period was measured by the same means. Care was taken to weigh food and measure liquid at the same time of day throughout the experimental period. Food and liquid consumption measurements were averaged over the 3-day test period to determine the average daily consumption.

Preparation of Tissue for Microscopic Observation

After mice were sacrificed via chloroform asphyxiation, whole liver was dissected out and weighed. Samples of liver 5 mm^3 were taken and placed in glass vials containing 20 ml of Lillie's Neutral Buffered Formaldehyde solution (Appendix III) for 24-48 hours for fixation (Lillie, 1965). After fixation, the fixative was replaced with 70% ethanol for temporary storage until embedding.

Embedding

Tissue samples were placed in appropriately labelled

tissue baskets and dehydrated according to the schedule listed in Appendix II. After dehydration, the tissues were placed in the first preparation of Tissue Prep paraffin (Fisher Scientific Co.) at 58°C with 5 ml xylene added to it to aid in permeation of the tissues. The tissues were kept in the tissue overnight at a reduced temperature of 55°C. The tissues were then transferred to a second preparation of Tissue Prep (no xylene) for an additional 6 hours. Next, the tissues were embedded in Tissue Prep to produce solid 1" tissue blocks.

Sectioning

Tissue blocks were sectioned on a microtome (American Optical Co.) set for 10 microns. To remove any wrinkles from the sections, the sections were floated on the surface of a water bath set at 38°C until the sections appeared smooth. Special attention was paid so that air bubbles would not accumulate on the bottom surface of the tissue which might have interfered with the adhesion of the tissue sections to the microscope slide. Smooth tissue sections were floated onto clean glass microscope slides and allowed to dry overnight on a warming tray set at 40°C.

Staining

The tissues were stained with Gill's hematoxylin no. 1 (Fisher Scientific Co.) and eosin (BDH Chemicals Ltd.).

The preparation of the staining solutions is described in Appendix III. The procedure for staining the tissue sections is given in Appendix IV. The procedure listed is a modification of the regressive method of the Delafield hematoxylin-eosin staining method (Humason, 1975).

Polyploidy Studies

The effect of age and diet on mouse liver polyploidization was assessed by the following means: 1) By examining changes in the average nuclear diameter of a sample of liver cells from mice of various ages and diets; 2) by examining changes in the percentage of nuclei distributed among the various ploidy classes; and 3) through the determination of the polyploidization indices for the different dietary groups being tested at the aforementioned ages.

1. Changes in Average Nuclear Diameter

Liver cell polyploidization, characterized by a doubling of chromosome number, is accompanied by various changes in nuclear size. Each doubling of chromosome number which occurs with a shift to the next higher degree of nuclear polyploidy results in an approximate doubling of nuclear volume and an increase in nuclear diameter by a factor of 1.26. Thus, the average nuclear diameter of a sample of liver cell nuclei may be used as an index of changes in polyploidization occurring in the liver.

A calibrated ocular micrometer placed in the eyepiece of a Leitz light microscope was used to measure the nuclear diameter of mononuclear hepatocytes at 1000X magnification. Because nuclei of different ploidies were not distributed homogeneously throughout the tissue sections, i.e. nuclei of a certain size were sometimes found clustered together, measurements of approximately 25 nuclei in 12 different fields of view were pooled for a total of 300 liver cell nuclei measured per animal. The average nuclear diameter for a particular age and dietary group was determined from the nuclear diameter measurements of 3 mice for a total sample size of 900 nuclei.

2. Distribution of nuclei among the various ploidy classes

The average nuclear diameter of a sample of liver cell nuclei may indicate trends towards increased or decreased levels of polyploidization in the liver. However, it does not describe changes occurring in the composition of the liver parenchyma in terms of the number or percentage of nuclei distributed among the various ploidy classes for a particular age and diet. In order to determine the percentage of nuclei in each ploidy class for a given sample of liver cell nuclei, the nuclear measurements of the sample must first be evaluated to see whether the nuclei form regular groups which characterize a polyploid series defined by the ratio 1:2:4:8 (Collin, 1978;

Epstein, 1967; Shima and Sugahara, 1976; Swartz, 1956).

Nuclear size frequency distributions were constructed for each of the 900 nuclei measured at each of the ages studied for all dietary groups. Visual examination of these histograms allowed the determination of the size limits for each ploidy class (Carriere and Patterson, 1962). Marked decreases in the number of nuclei between successive peaks of these polymodal size distributions marked the approximate limits between different nuclear populations. The limits of the ploidy classes were verified by the calculation of the average nuclear diameter of each ploidy class. If the limits were correct, the average nuclear diameters of two successive ploidy classes differed by a factor of 1.26, the theoretical difference expected for a two-fold increase in nuclear volume that accompanies a doubling of chromosome number (Epstein, 1967). Once the size limits of the ploidy classes were defined, the percentage of nuclei within each of the ploidy classes were determined from the summation of the frequencies of the nuclear sizes occurring within the limits.

3. Polyploidization Index

The term polyploidization index (P.I.) first introduced by Shima and Sugahara (1976) is a means of evaluating the progression of liver cell polyploidization throughout the lifespan as a continuum. In this study the

term was used to assess the polyploidization occurring during the experimental period. The polyploidization index was calculated by dividing the percentage of polyploid nuclei by the percentage of diploid nuclei at a given age. The polyploidization of the liver cell nuclei having occurred during the experimental period may be illustrated graphically by plotting the polyploidization index (P.I.) against the age in months. The slopes of the regression lines, represented by b in the multiple regression equation P.I. = a + b Age, indicates the rate of polyploidization occurring in the livers of mice of different dietary regimes as they age. The multiple correlation coefficient, R, expresses the strength of the association of the P.I. with age and diet.

Statistical Analysis

Data were analyzed by 2 and 3-way nested analysis of variance (ANOVA) comparing the effect of time started on the diets, age and diet simultaneously (Sokal and Rolf, 1981). When a significant value was obtained, a posteriori Dunnett's test was used to determine which dietary regimes yielded results significantly different from the controls (Zar, 1984). Where percentages were involved, an arcsine transformation was performed followed by ANOVA analysis and Dunnett's testing (Schefler, 1979). Where ratios were involved, a Kruskal-Wallis test or analysis of variance by

rank was employed (Zar, 1984). The level of significance was taken to be $p \leq 0.05$ throughout.

RESULTS

Whole Body Weight

Figures 1 and 2 represent the whole body weight profiles as it changes with age for Group 1 and Group 2 mice respectively.

Figure 1 shows the mean whole body weight plotted against age for Group 1 mice. As described in the Material and Methods section, Group 1 mice had early exposure to the various diets *in utero* 1 week prior to birth, neonatally through maternal lactation and after weaning as they were maintained on the special diets throughout their adult life. Group 1 mice were weighed weekly up to 1 month of age and thereafter weighed on a monthly basis. It can be observed in Figure 1 that the control mice were heavier than any of the mice on the special diets, even at 1 week of age, possibly reflecting a more balanced maternal nutrition. This initial difference in mean body weight was maintained throughout the lifespan. This presentation demonstrates that growth had slowed or plateaued by 3 months of age for all dietary groups.

Figure 2 represents the whole body weight plotted against age for mice of Group 2 which had been placed on

Figure 1 The whole body weight profile of control mice on a rat chow/H_2O diet is compared with that of the experimental mice of Group 1 on the following dietary regimes: rat chow/10% ethanol, 64% protein/H_2O; 68% sucrose/H_2O and 44% lipid/H_2O. (N = 3).

Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water

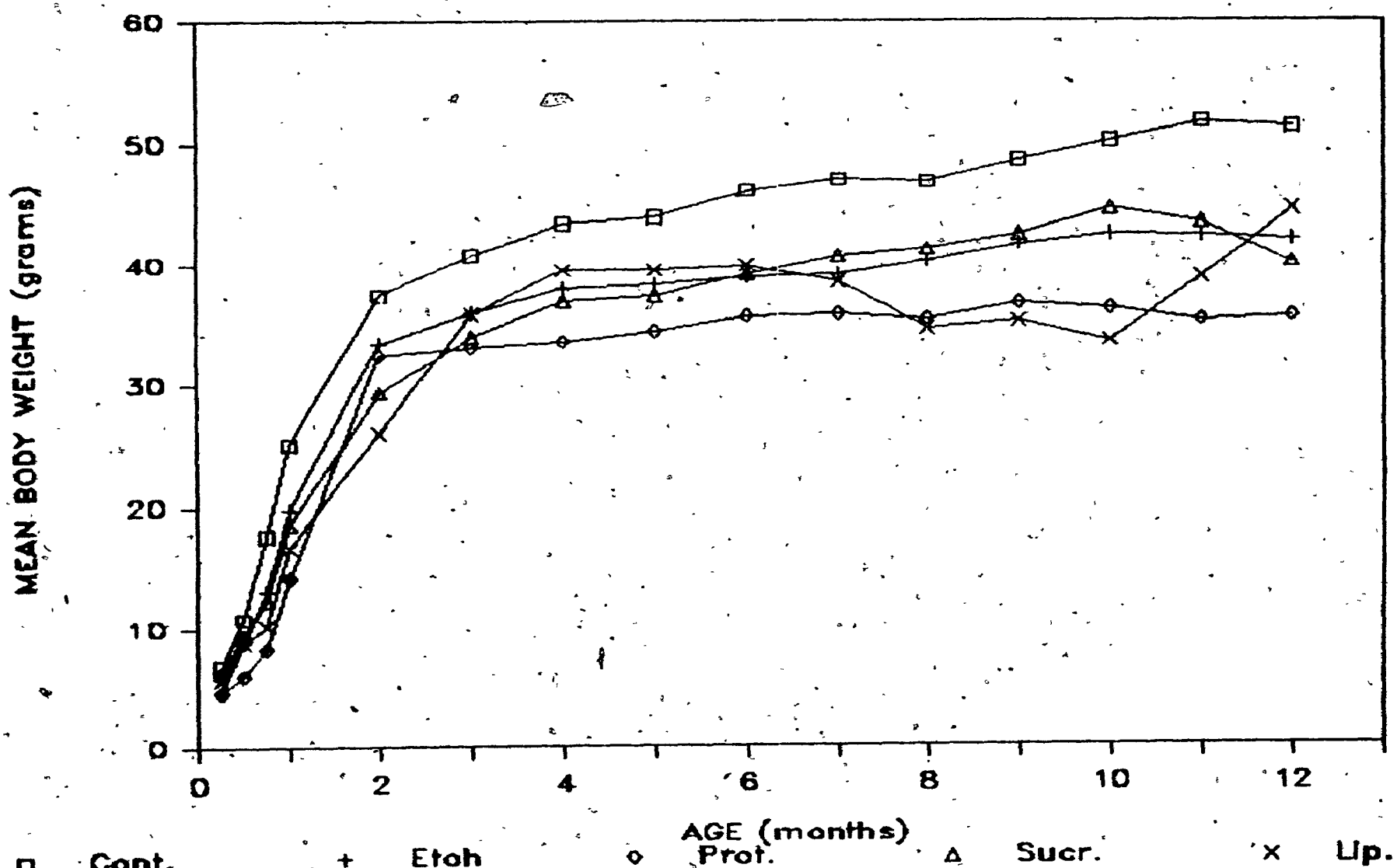

MEAN BODY WEIGHT (grams)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
AGE (months)
Cont.
Etoh
Prot.
Sucr.
Lip.

Figure 2 The whole body weight profile of control mice on a rat chow/H_2O diet is compared with that of experimental mice of Group 2 on the following dietary regimes: rat chow/10% ethanol, 64% protein/H_2O, 68% sucrose/H_2O and 44% lipid/H_2O. (N = 3)

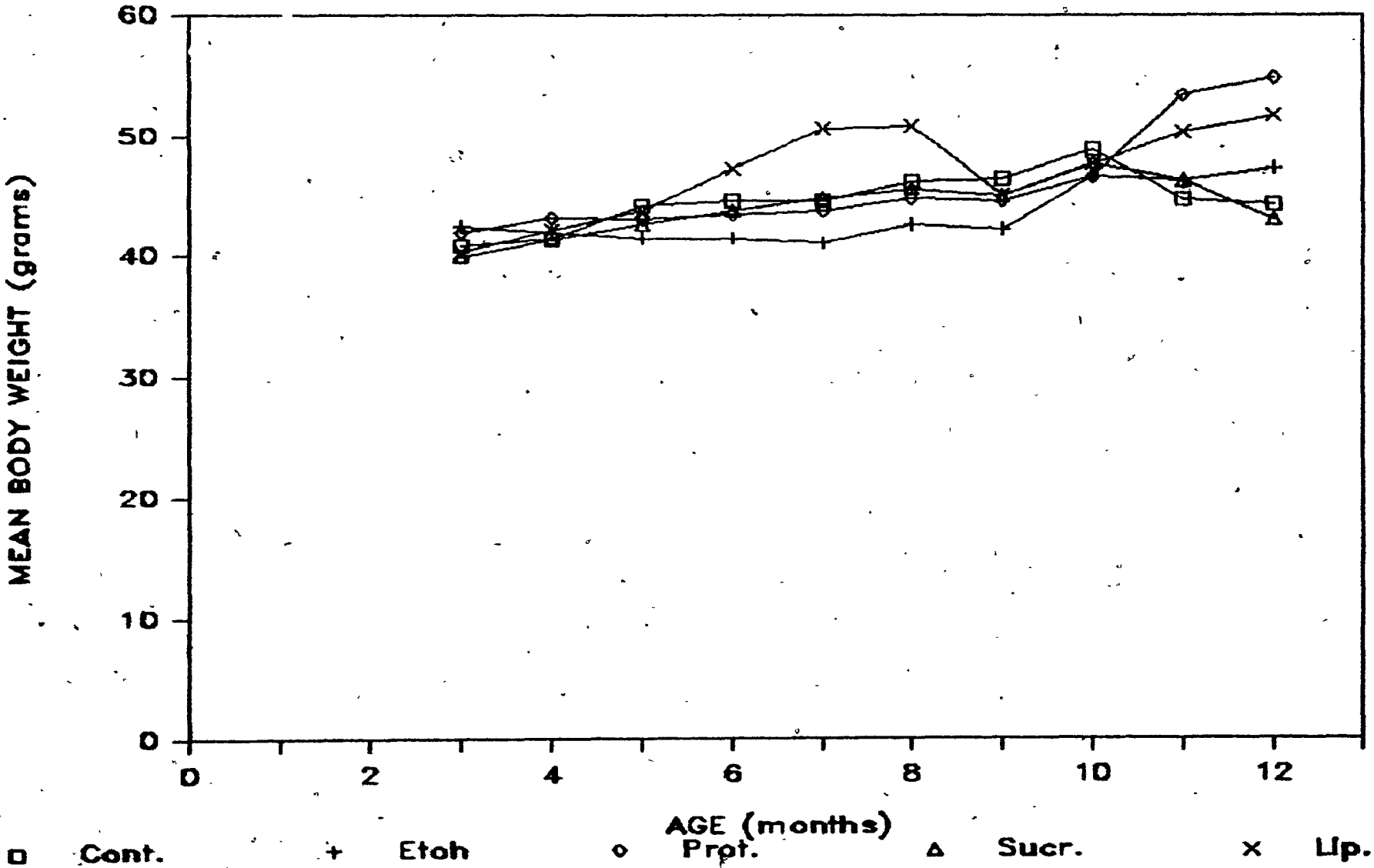
Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water


MEAN BODY WEIGHT (grams)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
AGE (months)
Cont.
Etoh
Prot.
Sucr.
Lip.

the diets as adults of 3 months of age. From 3 weeks to 3 months of age they had been maintained on a uniform diet of rat chow and water. These mice were weighed on a monthly basis from 3 months of age onwards. All groups were at nearly constant weight at 3 months of age. One may observe that weight is very little influenced when the special diets are given late in development, following the early phase of rapid growth. Figure 2 emphasizes that the weight of mice of all dietary groups is quite uniform up to 10 months of age after which the curves diverge.

Data from 4-10 months of age from mice of all diets in Groups 1 and 2 were analyzed simultaneously using a 3-way nested ANOVA. Since Group 2 mice were only placed on the diets at 3 months of age, data was not available for these mice from 1 week to 3 months of age, therefore, these ages could not be included in the analysis. This statistical analysis thus applies to adult mice whose growth has plateaued. Data at 11 and 12 months of age was not analyzed because only 3 mice were available at these ages for each diet.

The analysis showed that the stage of development at which the mice first started on the diets, i.e. early developmental vs. post-developmental, did have a significant effect on the weight profiles of the animals as they aged ($F = 31.10$, $p \leq 0.01$). Group 1 mice whose entire development has taken place on the special test diets,

weighed less than Group 2 mice for the same age and diet. Once the adult body weight had been attained by the mice in Groups 1 and 2, age had little effect on body weight. Surprisingly, diet had no significant effect on the weight profiles of these animals between the ages of 4 months and 10 months.

Group 1 data was also analyzed separately from Group 2 so that the weights of the mice at the younger ages of 1 week to 3 months could be analyzed. A 2-way nested ANOVA of the whole body weights of Group 1 animals between the ages of 1 week to 10 months showed that there was a significant effect of age ($F = 20.38$, $p \leq 0.01$) but not of diet on the body weight profiles of these animals. The effect of age on the body weight is particularly evident for mice of all dietary groups between the ages of 1 week to 4 months. With all diets, there was weight gain during this period after which the weights became more or less constant. From Figure 1, it can be seen that the weights of the control group of mice were consistently above those of the mice on the special test diets. With increasing age this divergence of mean body weight between control mice and mice on the test diets became more pronounced although statistically, this difference was not found to be significant. In Figure 2, the weight profiles of Group 2 mice started on the tests diets only as adults appear to be more erratic than those of Group 1 suggesting that the

adult mice may be less capable of adjusting to dietary excess than animals which have been exposed to the same type of diet for all of their life.

Mean Liver Weight

In Tables 1 and 2, the mean liver weight and liver weight expressed as a percentage of the whole body weight are shown for Groups 1 and 2 respectively. Liver weights were measured at 3 weeks, 3, 6, 10 and 12 months for Group 1 and at 6, 10, and 12 months of age for Group 2 mice.

A 3-way nested ANOVA was performed to determine whether there were significant differences in liver weight at 6, 10, and 12 months as a function of age, diet or time at which the diet was started. This analysis showed that there were no significant differences between Group 1 and Group 2 mice in terms of mean liver weight. That is to say, the stage of development at which the mice were first placed on the diets did not significantly influence the mean liver weight of the adult animals of the various diets as they aged. The analysis also showed that there were no significant differences in mean liver weight between ages of 6, 10 and 12 months of age for mice on a particular diet. Therefore, age did not play a significant role in determining the mean liver weight of adult animals. There was, however, a significant effect of diet on the mean liver weight of the mice at 6, 10 and 12 months of age (F =

Table 1

Comparison of mean liver weight and liver weight as percentage of total body weight for control and test diets at the various ages studied for Group 1 mice. N = 3 for each determination.

Diet	Age	Mean Liver Weight g ± S.D.	Mean Liver Weight as % Total Body Weight
Rat Chow/H_2O	3 wks	1.66 ± 0.41	9.39
	3 mo	2.36 ± 0.10	5.80
	6 mo	2.77 ± 0.05	6.02
	10 mo	2.73 ± 0.42	5.44
	12 mo	2.98 ± 0.13	5.81
Rat Chow/10% Etoh	3 wks	1.06 ± 0.14**	8.17
	3 mo	2.56 ± 0.36	7.14*
	6 mo	2.79 ± 0.09	7.19*
	10 mo	2.67 ± 0.30	6.30
	12 mo	2.73 ± 0.24	6.50
64% Protein/H_2O	3 wks	0.97 ± 0.57**	11.51**
	3 mo	2.26 ± 0.14	6.83**
	6 mo	2.60 ± 0.02	7.30*
	10 mo	2.44 ± 0.15	6.74*
	12 mo	2.36 ± 0.43*	6.64
68% Sucrose/H2O	3 wks	0.83 ± 0.17**	6.69**
	3 mo	2.68 ± 0.34	7.88**
	6 mo	2.30 ± 0.21*	5.90
	10 mo	2.76 ± 0.44	6.23
	12 mo	2.60 ± 0.29	6.50
44% Lipid/H_2O	3 wks	0.48 ± 0.09**	4.70**
	3 mo	1.84 ± 0.06*	5.14
	6 mo	1.86 ± 0.18*	4.67*
	10 mo	1.94 ± 0.49**	5.77
	12 mo	2.45 ± 0.31*	5.52

* Significantly different from control ($p \leq 0.05$).

** Significantly different from control ($p \leq 0.01$).

Figure 3 The mean liver weight profile of control mice on a rat chow/H_2O diet is compared with that of experimental mice of Group 1 on the following dietary regimes: rat chow/10% ethanol, 64% protein/H_2O, 68% sucrose/H_2O and 44% lipid/H_2O. (N = 3)

Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water

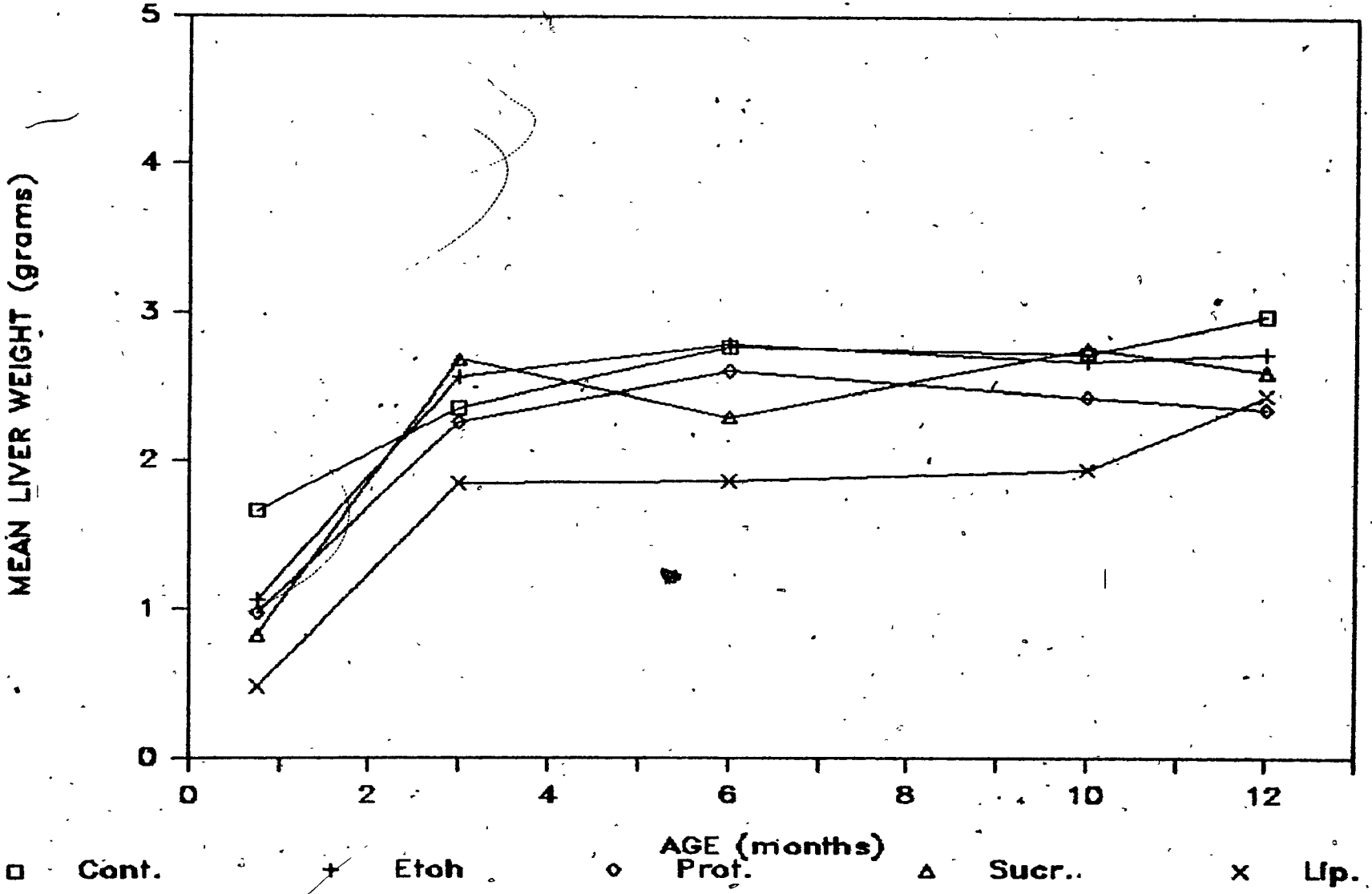

MEAN LIVER WEIGHT (grams)
0
1
2
3
4
5
0
2
4
6
8
10
12
AGE (months)
Cont.
Etoh
Prot.
Sucr..
Lip.

Table 2

Comparison of mean liver weight and liver weight as percentage of total body weight for control and test diets at the various ages studied for Group 2 mice. N = 3 for each determination.

Diet	Age	Mean Liver Weight g ± S.D.	Mean Liver Weight as % Total Body Weight
Rat Chow/H_2O	6 mo	2.26 ± 0.29	5.07
	10 mo	2.71 ± 0.41	5.55
	12 mo	2.25 ± 0.20	5.08
Rat Chow/10% Etoh	6 mo	2.59 ± 0.17	6.26
	10 mo	2.73 ± 0.36	5.86
	12 mo	2.78 ± 0.10*	5.88
64% Protein/H_2O	6 mo	2.56 ± 0.26	5.90
	10 mo	2.87 ± 0.41	6.61
	12 mo	3.29 ± 0.11**	6.01
68% Sucrose/H_2O	6 mo	2.89 ± 0.13*	6.60*
	10 mo	2.82 ± 0.18	5.92
	12 mo	2.41 ± 0.34	5.60
44% Lipid/H_2O	6 mo	2.14 ± 0.13	4.53
	10 mo	2.37 ± 0.64	4.98
	12 mo	2.49 ± 0.06	4.82

* Significantly different from control ($p \leq 0.05$).

** Significantly different from control ($p \leq 0.01$).

Figure 4 The mean liver weight profile of control mice on a rat chow/H_2O diet is compared with that of experimental mice of Group 2 on the following dietary regimes: rat chow/10% ethanol, 64% protein/H_2O, 68% sucrose/H_2O and 44% lipid/H_2O. (N = 3)

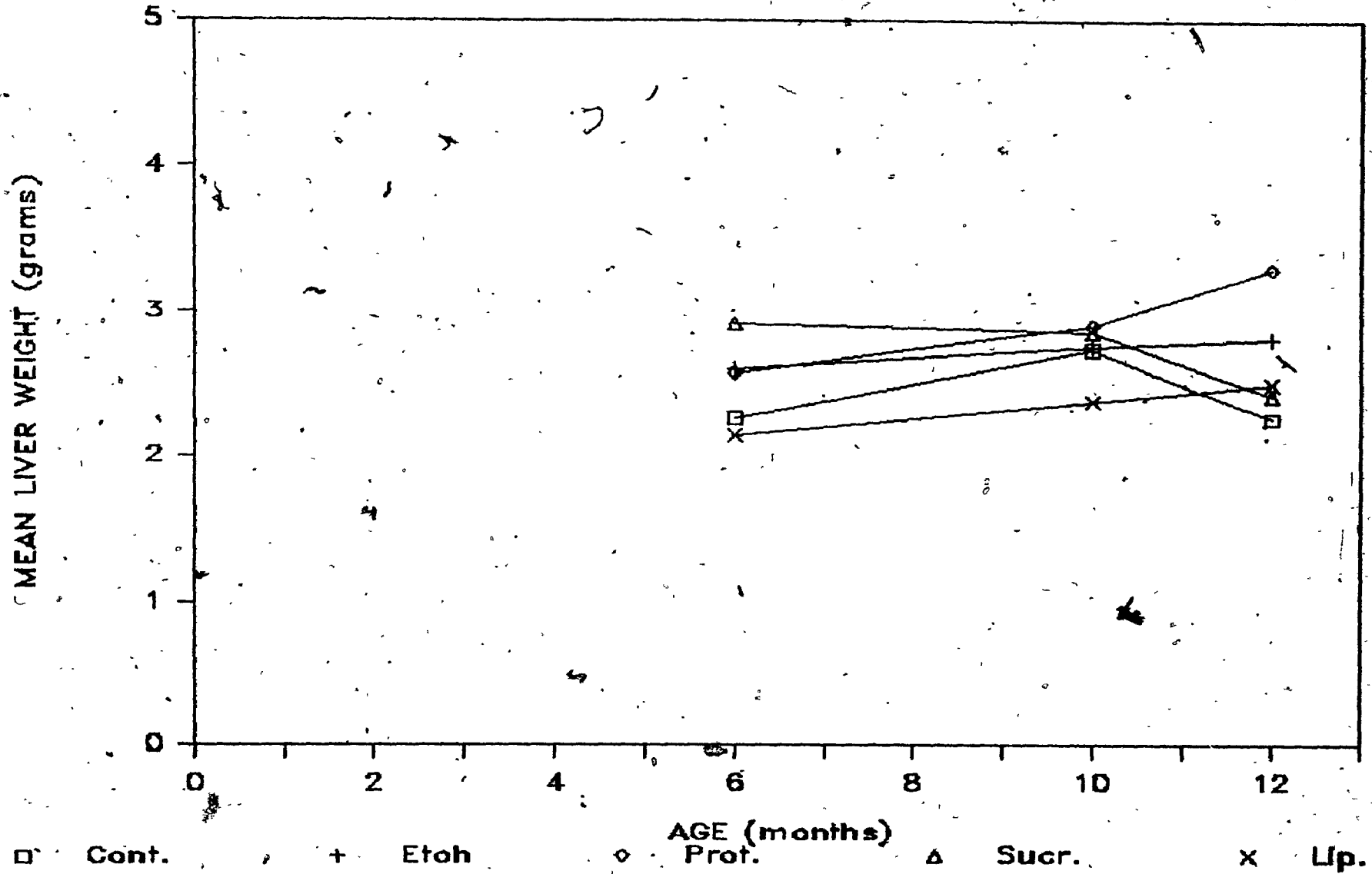
Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water


MEAN LIVER WEIGHT (grams)
5
4
3
2
1
0
0
2
4
6
8
10
12
AGE (months)
□ Cont.
+ Etoh
◇ Prot.
△ Sucr.
× Lip.

2.21, $p \leq 0.05$). Dunnett testing for Group 1 mice showed that mice on most of the test diets had mean liver weights similar to the controls at 6, 10 and 12 months of age. The one exception was that mice on the 44% lipid diet had mean liver weights significantly below those of the controls for all ages in Group 1 and at 6 and 10 months in Group 2. When a separate second ANOVA was performed on Group 1 mice to include the 3 week and 3 month age groups, it was found that age had a significant effect on the mean liver weight ($F = 18.14$, $p \leq 0.01$). This is primarily due to the fact that the mouse liver was still growing between the ages of 3 weeks and 3 months. This is evident at 3 weeks of age where mice of all the test diets show mean liver weights significantly below ($p \leq 0.01$) that of the control indicating the importance of a balanced diet during this period of rapid growth.

In Group 2, the mean liver weights of mice of the different test diets were similar for a particular age with few exceptions. As a double check, Group 2 results were also analyzed separately from Group 1 for the ages of 6, 10 and 12 months. The analysis showed that there was no significant effect of age on the mean liver weight of the mice at these ages and that diet had a significant effect on the mean liver weight at 6, 10 and 12 months of age ($F = 2.45$, $p \leq 0.05$). These results confirm what was previously found in the initial analysis, that is, there is a

significant effect of age on the mean liver weight during the early stages of growth. However, once the liver attains its adult weight, age has little or no effect on the mean liver weight. Diet, on the other hand, has a significant effect on the mean liver weight at all ages studied.

Figure 3, where mean liver weight was plotted against age for Group 1 mice, illustrates the dramatic increase in mean liver weight during the early stage of growth between 3 weeks and 3 months. After 3 months of age, the liver weight varies little during adulthood with little variation between the dietary groups with the exception of those mice on the 44% lipid diet.

Figure 4 illustrates the mean liver weight as it changes with age for Group 2 at 6, 10 and 12 months of age. In Group 2 mice there appears to be a greater degree of variation in mean liver weight within a particular dietary group. This, much like what was observed with Group 2 body weights, may be an indication of adults having difficulty in adjusting to a drastic change in diet.

Ratio of Liver Weight/Whole Body Weight

The results presented in Tables 1 and 2 also represent liver weight expressed as a percentage of the total body weight of Group 1 and Group 2 mice respectively.

From Table 1, it may be observed that the liver

comprises approximately 5-8% of the total body weight of mice. This value is slightly elevated at 3 weeks of age since the growth rate of the liver is greater than the growth rate of the body at this stage of development. By 3 months of age the mice have reached maturity, resulting in a fairly constant liver/body weight thereafter for both Group 1 and Group 2 mice.

Since the values shown in Tables 1 and 2 express liver weight as a percentage of the total body weight, an arcsine transformation was required prior to ANOVA analysis. ANOVA analysis comparing Group 1 and Group 2 mice at 6, 10 and 12 months of age revealed that the stage of development at which the mice were first exposed to the test diets had no significant effect on determining the liver/body weight ratio later in life since by 6 months of age the mice of both Groups 1 and 2 were fully matured. The ANOVA analysis showed age also had no significant effect on altering the liver/body weight ratio at 6, 10 and 12 months of age. Diet was the only factor studied that had a significant effect on the liver/body weight ratio once the post-developmental stage had been reached ($F = 4.10$, $p \leq 0.01$). In Group 1, both mice on the rat chow/10% ethanol or the 64% protein diets maintained liver/body weight ratios consistently above the controls for most of the other ages studied. In Group 2, once again mice on the rat chow/10% ethanol, 64% protein diets as well as the 68% sucrose diet

showed liver/body weight ratios elevated above the controls but significant differences were not as consistent. Therefore, it appears that once the mice have matured and reached their adult size, only diet appeared to have a significant effect on changing the liver to body weight ratio.

Food Consumption

In this study, food consumption was measured in two ways. First, mean food intake was measured as g/day. Secondly, food intake was expressed in relation to the mean body weight of the animal, as g/day/g body weight. Tables 3 and 4 show the results for Group 1 and Group 2 mice respectively, for each of the diets and ages studied. Food intake as g/day will first be considered.

Table 3 shows that in Group 1, food intake as g/day remains fairly constant within a particular dietary group, changing little with age. ANOVA analysis showed there were no significant differences in food consumption (g/day) between Group 1 and Group 2 mice of the same age and diet. Analysis of Group 1 and 2 mice from the ages of 3 months to 12 months showed that there was no significant effect of age on total food intake during this period. When a second analysis was performed on Group 1 separately so that the 1 month age group may be analyzed, age still did not have a significant effect. Diet did have a significant effect on

Table 7

Food consumption (Group 1). Mean ± S.D. N = 5 for each determination except at 12 months where N = 3.

Diet	Age	Food Consumption g/day ± S.D.	Food Consumption g/day/g body wt. ± S.D.
Rat Chow/H_2O	1 mo	9.59 ± 2.23	0.38 ± 0.09
	3 mo	7.79 ± 0.70	0.19 ± 0.02
	6 mo	6.66 ± 1.20	0.14 ± 0.03
	10 mo	7.71 ± 0.96	0.15 ± 0.03
	12 mo	8.83 ± 2.06	0.17 ± 0.02
Rat Chow/10% Etoh	1 mo	5.45 ± 0.70**	0.27 ± 0.09
	3 mo	6.73 ± 0.66	0.19 ± 0.02
	6 mo	4.88 ± 0.72	0.12 ± 0.02
	10 mo	6.06 ± 0.31	0.14 ± 0.02
	12 mo	6.36 ± 0.68*	0.15 ± 0.01
64% Protein/H_2O	1 mo	6.98 ± 0.27*	0.50 ± 0.02
	3 mo	5.61 ± 1.04	0.17 ± 0.03
	6 mo	3.76 ± 0.12*	0.10 ± 0.01
	10 mo	4.81 ± 0.22*	0.13 ± 0.01
	12 mo	4.01 ± 0.04**	0.11 ± 0.01
68% Sucrose/H2O	1 mo	5.86 ± 0.78**	0.32 ± 0.04
	3 mo	3.78 ± 0.53**	0.11 ± 0.02
	6 mo	3.16 ± 0.24**	0.08 ± 0.01
	10 mo	5.16 ± 0.48*	0.12 ± 0.02
	12 mo	5.54 ± 0.69**	0.14 ± 0.03
44% Lipid/H_2O	1 mo	6.04 ± 1.39**	0.36 ± 0.09
	3 mo	4.22 ± 1.16**	0.12 ± 0.09
	6 mo	4.55 ± 2.21	0.11 ± 0.06
	10 mo	3.16 ± 0.66**	0.09 ± 0.02
	12 mo	4.22 ± 1.49**	0.10 ± 0.02

* Significantly different from control ($p \leq 0.05$).

** Significantly different from control ($p \leq 0.01$).

Figure 5 Food consumption (g/day) of Group 1 mice on control (rat chow/H_2O) and test diets.

(N = 23 total)

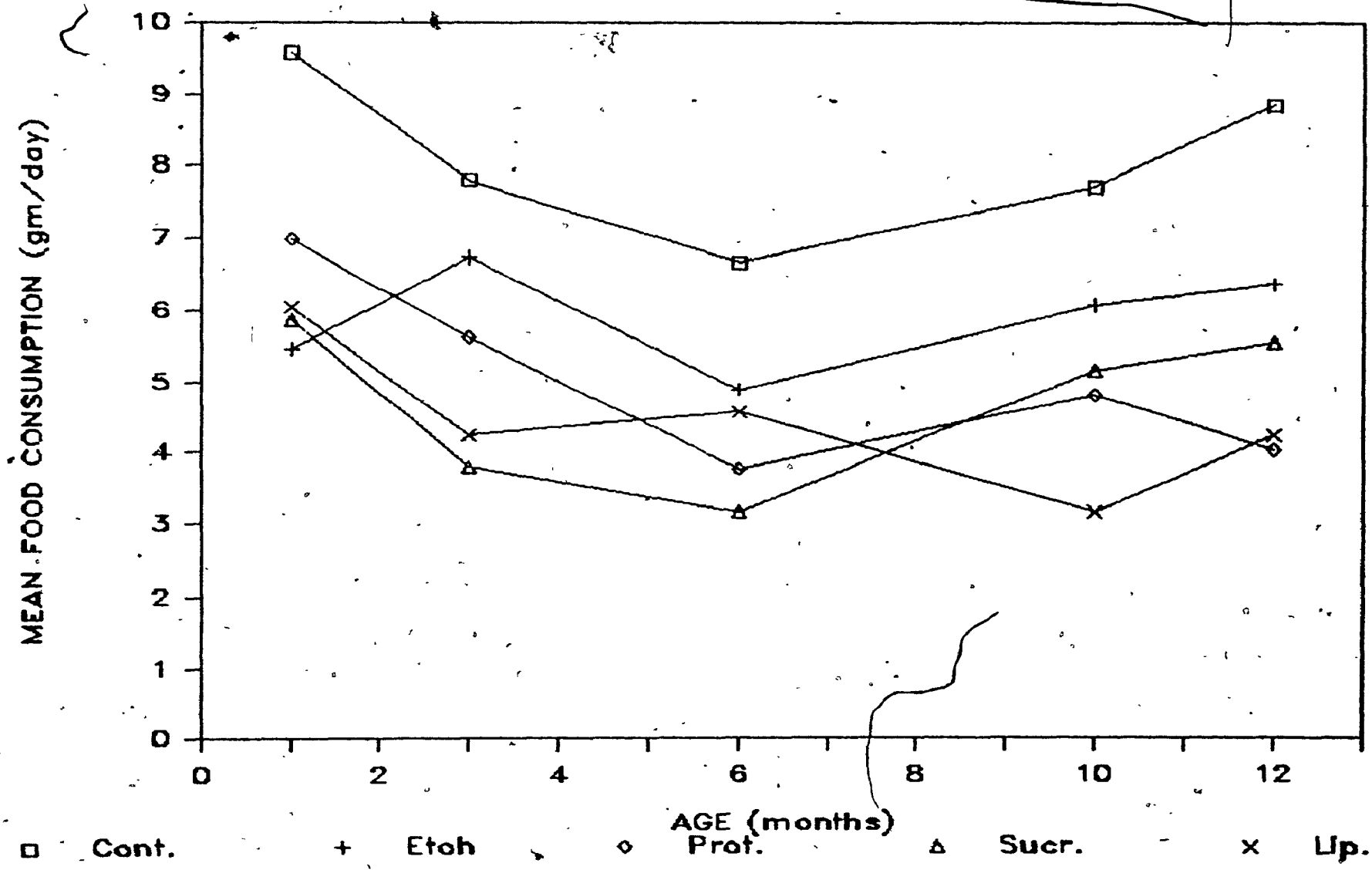
Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water


MEAN FOOD CONSUMPTION (gm/day)
10
9
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7
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3
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1
0
0
2
4
6
8
10
12
AGE (months)
Cont.
Etoh
Prot.
Sucr.
Lip.

food intake for both Groups 1 and 2 ($F = 131.78$, $p \leq 0.01$).

Dunnett testing performed separately on Group 1 and 2 revealed which diets resulted in significant deviations in food consumption from control values. In Group 1, mice on the control diet of rat chow/H_2O had the highest mean food intake values for all the ages studied. Food intake for mice on the rat chow/10% ethanol diet was the most like the control although intake was reduced. Food intake values for 64% protein, 68% sucrose and 44% lipid fed mice were all well below control values at all the ages studied.

Figure 5 graphically illustrates mean food intake patterns for mice of all diets and ages in Group 1. Mice of all diets, except those on rat chow/10% ethanol, exhibited a decrease in total food intake as g/day between the ages of 1 to 3 months. From 3 to 6 months, the food intake decreased with the exception of mice on the 44% lipid diet whom experienced a slight increase in food intake during this period. Mice on all diets, again with the exception of those on 44% lipid, displayed an increase in food intake which continued to 12 months of age. Only mice on the 64% protein diet experienced a decreases in food intake between the ages of 10 and 12 months. Throughout the test period, mean food intake values of the controls remained well above those of the mice on the test diets.

In Table 4, Group 2 food intake values (g/day)

Table 4

Food consumption (Group 2). Mean ± S.D. N = 5 for each determination except at 12 months where N = 3.

Diet	Age	Food Consumption g/day ± S.D.	Food Consumption g/day/g body weight ± S.D.
Rat Chow/H_2O	3 mo	7.41 ± 0.87	0.18 ± 0.02
	6 mo	8.21 ± 0.65	0.18 ± 0.02
	10 mo	7.71 ± 0.96	0.16 ± 0.02
	12 mo	6.26 ± 2.69	0.14 ± 0.01
Rat Chow/10% Etoh	3 mo	8.03 ± 0.88	0.19 ± 0.02
	6 mo	6.63 ± 0.66	0.16 ± 0.03
	10 mo	7.04 ± 0.31	0.15 ± 0.02
	12 mo	8.32 ± 0.68	0.18 ± 0.02
64% Protein/H_2O	3 mo	4.60 ± 0.84*	0.11 ± 0.03
	6 mo	5.62 ± 0.50*	0.13 ± 0.02
	10 mo	5.37 ± 0.27	0.12 ± 0.01
	12 mo	7.38 ± 0.19	0.13 ± 0.01
68% Sucrose/H2O	3 mo	4.22 ± 0.97**	0.10 ± 0.03
	6 mo	5.89 ± 0.32	0.13 ± 0.02
	10 mo	5.57 ± 0.48	0.12 ± 0.03
	12 mo	5.64 ± 1.49	0.13 ± 0.01
44% Lipid/H_2O	3 mo	5.19 ± 1.77*	0.13 ± 0.03
	6 mo	6.29 ± 0.62	0.13 ± 0.01
	10 mo	3.88 ± 0.66**	0.08 ± 0.01
	12 mo	6.73 ± 0.40	0.13 ± 0.01

* Significantly different from control ($p \leq 0.05$).

** Significantly different from control ($p \leq 0.01$).

Figure 6 Food consumption (g/day) of Group 2 mice on control (rat chow/H_2O) and test diets. (N = 18 total)

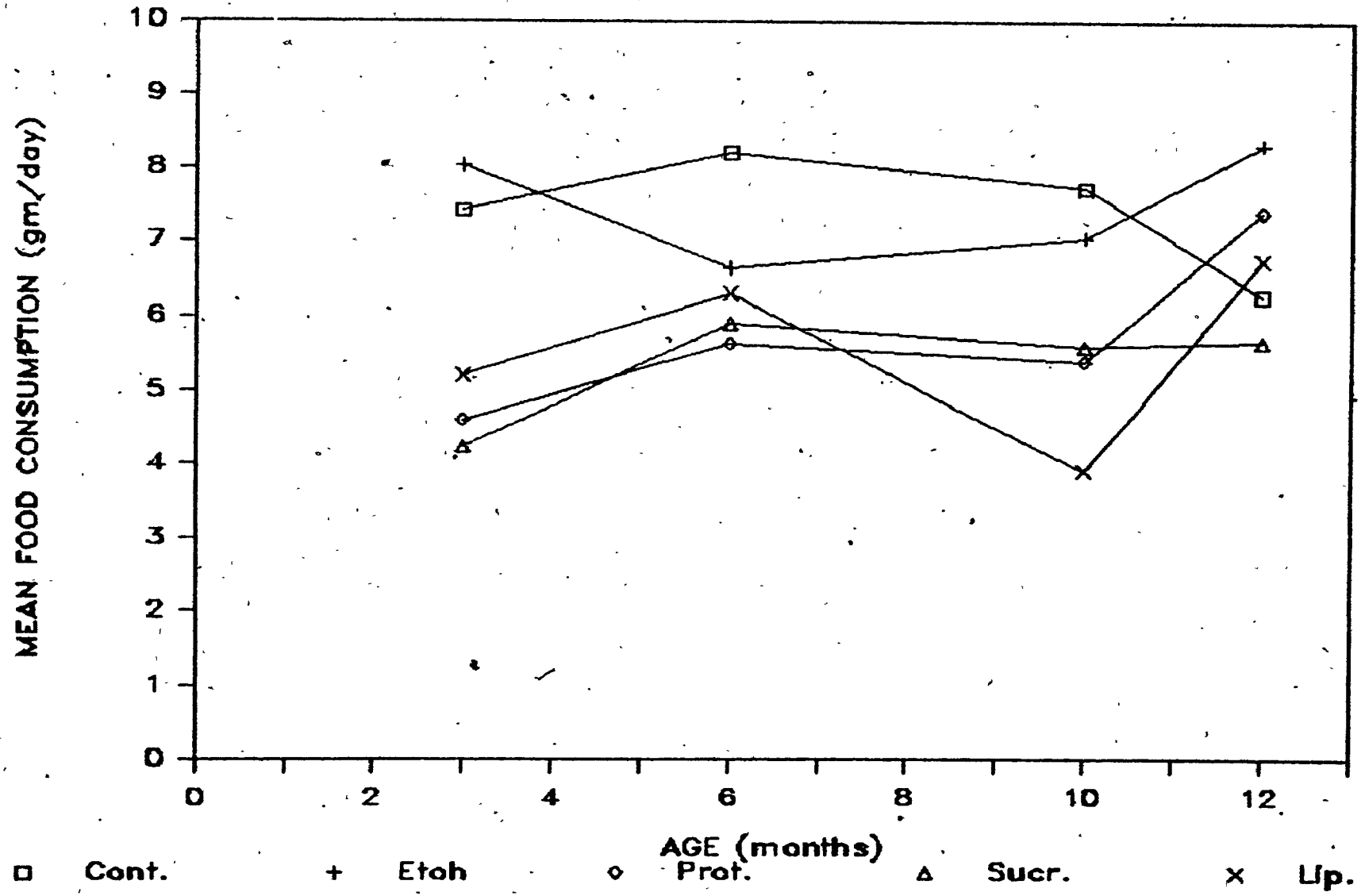
Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water


MEAN FOOD CONSUMPTION (gm/day)
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
AGE (months)
Cont.
Etoh
Prot.
Sucr.
Lip.

appeared to be similar to those seen in Group 1. Statistical analysis showed that there was no significant difference between Group 1 and Group 2 for those ages studied. Within Group 2, significant differences between the control and the test diets were not as consistent as in Group 1. No statistically significant differences could be detected between the controls and ethanol treated mice. Although the overall intake of mice on the 64% protein diet appeared to be less than the controls, significant differences were only found at 3 and 6 months of age ($p \leq 0.05$). As in Group 1, mice on the 68% sucrose diet were always found to have intake values consistently lower than the control values but significant only at 3 months of age. Such was also the case with mice on the 44% lipid diet with significant differences found at 3 months ($p \leq 0.05$) and 10 months ($p \leq 0.01$).

Figure 6 illustrates mean food intake patterns (g/day) for mice of all diets and ages studied for Group 2. The overall pattern of food intake (g/day) for controls showed a slight decrease in food intake with increasing age. Mice fed the rat chow 10% ethanol diet appeared to have an intake pattern almost the mirror image of that of the controls, first decreasing between the ages of 3 to 6 months then increasing from 6 to 12 months. Despite the striking appearance of this curve, no statistically significant differences were found between these mice and

the controls at any age, as stated earlier. Food intake for mice on the 64% protein appeared to increase with increasing age. Mice on the 68% sucrose diet also showed an increase in food intake but this increase was restricted to the earlier ages. By 6 months of age, food intake had stabilized and remained fairly constant from thereon. The food intake pattern for the 44% lipid fed mice appears to be the most erratic of all the test diets, showing a sharp decrease between the ages of 6 to 10 months and a subsequent increase from 10 to 12 months.

When food consumption is viewed in terms of mean food intake in relation to body weight (g/day/g body weight), a slightly different picture is painted. The statistical analysis of this data consisted of a Kruskal-Wallis test also known as analysis of variance by ranks. It was found that there was no significant difference in food consumption between Group 1 and Group 2 mice. That is, the stage of development at which the mice were first placed on the diets did not have a significant influence on the food consumption of the mice at later ages. It was also found that age did have a significant effect on food consumption ($H = 18.37$, $p \leq 0.001$). Young, actively growing and developing mice have greater nutritional demands than do adult mice, where the nutritional requirements are restricted to maintenance rather than growth. This is clearly evident in the Group 1 mice where the food

consumption in g/day/g body weight at 1 month of age was 2-3 times higher than at 3 to 12 months of age for all dietary regimes. The analysis also showed that diet had a significant influence on food consumption ($H = 37.94$, $p \leq 0.001$). At 1 month of age, food consumption was greatest in mice fed the 64% protein diet and the lowest in the ethanol treated mice. The controls, 68% sucrose and 44% lipid fed animals were found to have similar values. By the age of 3 months the mice were fully grown and food consumption remained fairly constant from 3 to 12 months of age. During this period, food consumption was greatest in the controls followed closely by the mice fed rat chow/10% ethanol, 64% protein, 68% sucrose and lastly 44% lipid fed mice. The similar trends were observed in Group 2 mice.

Liquid Consumption

As with food consumption, liquid consumption was measured both in terms of mean liquid intake (ml/day) and expressed in relation to the mean body weight (ml/day/g body weight). Tables 5 and 6 show these results for Group 1 and Group 2 mice respectively, for each of the diets and ages studied.

Statistical analysis compared mean liquid intake (ml/day) values of Group 1 and Group 2 mice of all diets and ages 3 to 12 months simultaneously. The analysis showed that there were no significant differences in mean

Table 5

Liquid consumption (Group 1). Mean ± S.D. N = 5 for each determination except at 12 months where N = 3.

Diet	Age	Liquid Consumption ml/day ± S.D.	ml/day/g body weight ± S.D.
Rat Chow/H_2O	1 mo	10.20 ± 0.80	0.40 ± 0.03
	3 mo	7.27 ± 1.18	0.18 ± 0.03
	6 mo	5.00 ± 1.52	0.11 ± 0.03
	10 mo	6.97 ± 2.83	0.14 ± 0.06
	12 mo	9.00 ± 0.56	0.18 ± 0.03
Rat Chow/10% Etoh	1 mo	5.53 ± 0.89**	0.29 ± 0.05
	3 mo	5.47 ± 1.07	0.15 ± 0.03
	6 mo	4.80 ± 1.65	0.11 ± 0.04
	10 mo	3.97 ± 0.69	0.16 ± 0.05
	12 mo	6.00 ± 2.13	0.26 ± 0.03
64% Protein/H_2O	1 mo	9.73 ± 1.06	0.69 ± 0.09
	3 mo	8.60 ± 0.93	0.26 ± 0.02
	6 mo	9.33 ± 1.26**	0.26 ± 0.05
	10 mo	6.00 ± 1.41	0.16 ± 0.05
	12 mo	8.88 ± 0.04	0.26 ± 0.03
68% Sucrose/H2O	1 mo	5.07 ± 0.84**	0.27 ± 0.04
	3 mo	5.53 ± 1.09	0.16 ± 0.02
	6 mo	4.67 ± 2.17	0.14 ± 0.06
	10 mo	4.80 ± 1.34	0.11 ± 0.03
	12 mo	7.45 ± 1.97	0.19 ± 0.04
44% Lipid/H_2O	1 mo	10.00 ± 1.28	0.60 ± 0.08
	3 mo	9.33 ± 2.14	0.26 ± 0.06
	6 mo	9.93 ± 4.32**	0.25 ± 0.08
	10 mo	4.00 ± 1.41	0.12 ± 0.05
	12 mo	7.33 ± 1.63	0.16 ± 0.02

* Significantly different from control ($p \leq 0.05$).

** Significantly different from control ($p \leq 0.01$).

Figure 7 Liquid consumption (ml/day) of Group 1 mice on control (rat chow/H_2O) and test diets. (N = 23 total)

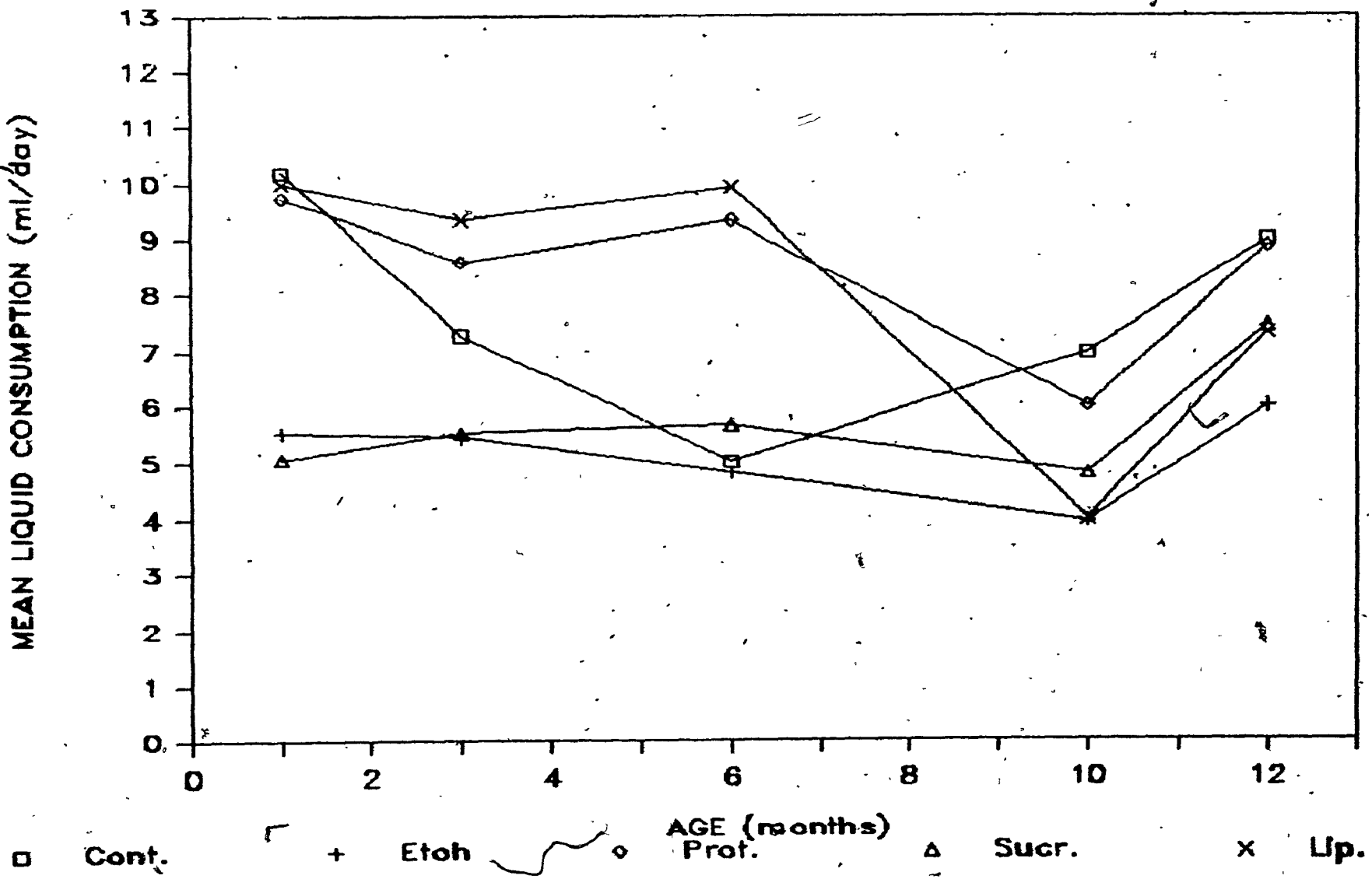
Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water


MEAN LIQUID CONSUMPTION (ml/day)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
AGE (months)
□ Cont.
+ Etoh
◇ Prot.
△ Sucr.
× Lip.

liquid intake between Group 1 and Group 2 mice. As with food consumption, the stage at which the mice were first exposed to the diets did not influence liquid intake in either group. Neither was there an effect of age on liquid intake in terms of ml/day. There was, however, a significant effect of diet on the mean liquid intake of Group 1 and Group 2 mice ($F = 6.40$, $p \leq 0.01$). Since diet was found to have a significant effect, Dunnett testing followed to determine which of the diets resulted in liquid intake values different from the controls. Group 1 and Group 2 data were analyzed separately.

In Group 1 mice, the liquid intake level of the control mice started out being slightly higher than that of mice on the tests diets at 1 month of age. The mean intake of the controls began to decrease between the ages of 1 month and 6 months. This dramatic decrease in mean liquid intake in the control mice was reversed by an equally dramatic increase in liquid intake between the ages of 6 to 12 months resulting in a mean liquid intake level above all the test diet levels at 12 months of age. Mean liquid intake levels in the ethanol treated mice were consistently below those of the controls but only being significantly lower at 1 month of age where intake was almost half of that of the controls. Figure 7 shows that the ethanol treated mice experienced a moderate decline in liquid intake between the ages of 1 month and 10 months. At 10

months of age, ethanol treated mice had the lowest intake levels of all the mice. Between the ages of 10 and 12 months, the mice showed an increase in liquid intake as did all the other mice, however, intake levels remained below those of the other test diets and well below that of the control. Mice on 64% protein diet had mean liquid intake levels similar to controls at all of the ages studied with the exception of 6 months of age where the mice drank almost twice as much as the controls. If one observes Figure 7 for mice on 64% protein diet, one can see an overall decline in mean liquid intake between the ages of 1 to 10 months of age. The increase in liquid intake from 10 months to 12 months of age for mice on all the diets including those on 64% protein resulted in liquid intake levels similar to control level at 12 months of age. Mice on the 68% sucrose diet appear to have mean liquid intake levels consistently below those of the controls throughout the test period, however, this difference was only found to be statistically significant at 1 month of age. In Figure 7, we may observe that the mean liquid intake of the mice on this high sucrose diet remained fairly constant between the ages of 1 to 10 months with only a slight increase in intake from 3 to 6 months of age. The subsequent increase in liquid intake from 10 to 12 months still resulted in a liquid intake level below that of the control. The liquid intake pattern of the mice on 44% lipid diet is quite

similar to that of the mice on the high protein diet as well as the controls. The intake values of these mice are similar to those of the controls except at 6 months of age where intake levels are significantly higher than the control level as was also the case of the mice on the high protein diet. Although mice on all the test diets experienced a decline in liquid intake from 6 to 10 months of age, none was as striking as the decline for those mice on the high lipid diet as displayed by Figure 7. As with all the other test diets, at 12 months of age the mean liquid intake of mice on the high lipid diet was below the control level.

In Group 2 mice, liquid intake values appear not to fluctuate as erratically as in Group 1, although statistically, there are no significant differences between the two groups of animals. With the exception of the slight initial decrease in liquid intake, the intake values of the controls was fairly constant between the ages of 6 to 12 months. Ethanol-treated mice in Group 2 show a linear increase in liquid intake from 3 to 10 months of age whereby at 10 months, liquid intake was significantly higher than that of the controls. The period of 10 to 12 months resulted in a decline in liquid intake for these mice returning intake levels back to control level by 12 months of age. Mice on the 64% protein diet had liquid intake levels consistently above those of the controls at

Table 6

Liquid consumption (Group 2). Mean ± S.D. N = 5 for each determination except at 12 months where N = 3.

Diet	Age	Liquid Consumption ml/day ± S.D.	ml/day/g body weight ± S.D.
Rat Chow/H_2O	3 mo	7.87 ± 1.54	0.19 ± 0.04
	6 mo	6.93 ± 0.93	0.16 ± 0.02
	10 mo	6.70 ± 0.86	0.14 ± 0.02
	12 mo	6.78 ± 2.83	0.15 ± 0.03
Rat Chow/10% Etoh	3 mo	5.47 ± 0.88	0.13 ± 0.03
	6 mo	7.47 ± 0.80	0.18 ± 0.03
	10 mo	10.50 ± 3.65**	0.24 ± 0.05
	12 mo	8.11 ± 0.57	0.22 ± 0.04
64% Protein/H_2O	3 mo	8.93 ± 0.89	0.21 ± 0.03
	6 mo	11.13 ± 1.75**	0.26 ± 0.03
	10 mo	11.10 ± 2.33**	0.24 ± 0.05
	12 mo	12.11 ± 2.00**	0.22 ± 0.04
68% Sucrose/H2O	3 mo	5.33 ± 0.56	0.13 ± 0.03
	6 mo	6.07 ± 0.65	0.14 ± 0.03
	10 mo	6.50 ± 1.96	0.14 ± 0.06
	12 mo	6.88 ± 1.40	0.16 ± 0.04
44% Lipid/H_2O	3 mo	8.87 ± 1.05	0.22 ± 0.03
	6 mo	8.53 ± 0.96	0.18 ± 0.01
	10 mo	10.20 ± 3.61**	0.21 ± 0.08
	12 mo	8.22 ± 1.13	0.16 ± 0.03

* Significantly different from control ($p \leq 0.05$).

** Significantly different from control ($p \leq 0.01$).

Figure 8 Liquid consumption (ml/day) of Group 2 mice on control (rat chow/H_2O) and test diets. (N = 18 total)

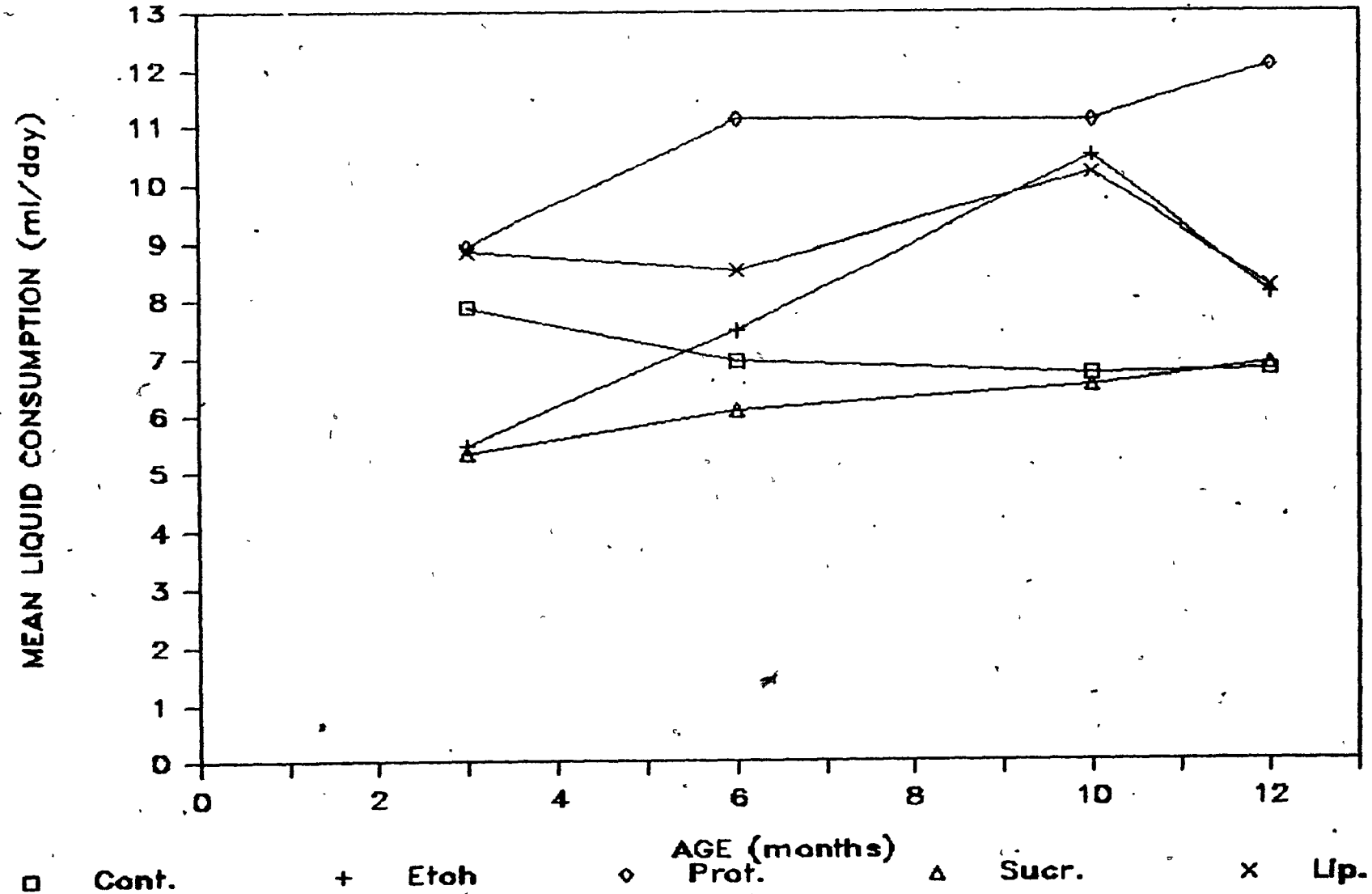
Cont = control, rat chow/water

Etoh = rat chow/10% ethanol

Prot = 64% protein/water

Sucr = 68% sucrose/water

Lip = 44% lipid/water


MEAN LIQUID CONSUMPTION (ml/day)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
AGE (months)
Cont.
Etoh
Prot.
Sucr.
Lip.

all the ages studied. The differences between the control and high protein group were highly significant ($p \leq 0.01$) at 6, 10 and 12 months of age. Mice on the 64% protein diet had the highest mean liquid intake values of all the mice in Group 2 at those ages. Figure 8 depicts a stepwise increase in liquid intake for mice on the 64% protein diet during the experimental period. Mice on the 68% sucrose diet had intake values below those of the controls throughout most of the experimental period, however, no statistical differences were found between these two groups of mice at any of the ages studied. Figure 8 shows us that mice on the 68% sucrose diet experienced a slow, gradual increase in mean liquid intake throughout the test period. For mice on the 44% lipid diet, liquid intake values in Table 6 appear to be slightly higher than those of the controls, however, the liquid intake is significantly higher at 10 months of age only. In Figure 8, one may observe that as in Group 1, the liquid intake pattern of the mice on the high lipid diet is erratic and therefore it is difficult to see a definite trend in liquid intake.

Liquid consumption was also viewed in terms of ml/day/g body weight, taking into account the size of the animal in relation to its liquid intake. As with the food consumption study, statistical analysis failed to show a significant difference in liquid consumption between those mice with a very early exposure to the test diets (Group 1)

and those started on the diets as adults (Group 2). It was found that age did have an effect on liquid consumption ($H = 24.20$, $p \leq 0.001$) as did the diet ($H = 28.39$, $p \leq 0.001$). As adults, Group 1 mice on 64% protein had the highest overall liquid consumption followed by mice on the 44% lipid diet, ethanol treated mice, control mice and lastly mice on the 68% sucrose diet, the latter three being very similar.

In viewing the results of Group 2 mice, one may observe that they are similar to those of Group 1 mice. The trends which occurred in Group 1 when considering liquid consumption (ml/day/g body weight) also occur in Group 2 mice when observing the effect of diet on liquid consumption. Mice on the 64% protein diet had the highest liquid consumption values followed by mice on 44% lipid diet. Again control, sucrose and ethanol fed mice displayed similar values for liquid consumption.

Average Daily Energy Intake

The average daily energy intake values shown in Tables 7 and 8 were determined from the average daily food consumption (g/day) by multiplying those values by the corresponding energy equivalents for 1 gram of foodstuff listed in Appendix I. In the case of the mice fed rat chow and a 10% ethanol solution instead of water, the average

Table 7

Average Daily Energy Intake Values for Group 1 Mice.
Mean ± S.D.

Diet	Average Daily Energy Intake (cal/day) 1 mo	3 mo	6 mo	10 mo	12 mo
Rat Chow/H_2O	39.89 ± 9.28	32.41 ± 2.91	27.91 ± 4.49	32.07 ± 3.99	36.73 ± 8.51
Rat Chow/10% Etoh	25.71 ± 3.40	31.03 ± 3.34	22.94 ± 3.91	27.39 ± 2.76	29.86 ± 4.00
64% Protein/H_2O	31.48 ± 3.33	25.30 ± 4.69	16.96 ± 0.54	21.69 ± 0.99	18.09 ± 0.18**
68% Sucrose/H_2O	24.85 ± 1.33	16.03 ± 2.25*	13.40 ± 1.02*	21.88 ± 2.04	23.49 ± 2.93
44% Lipid/H_2O	34.56 ± 7.51	22.79 ± 6.26	24.57 ± 11.93	17.06 ± 3.56*	22.79 ± 9.67*

* Significantly different from controls ($p \leq 0.05$).

** Significantly different from controls ($p \leq 0.01$).

Table 8

Average Daily Energy Intake Values for Group 2 Mice.
Mean ± S.D.

Diet	Average Daily Energy Intake (cal/day) 3 mo	6 mo	10 mo	12 mo
Rat Chow/H_2O	30.83 ± 3.62	34.15 ± 2.70	32.07 ± 3.99	26.04 ±11.19
Rat Chow/10% Etoh	36.41 ± 4.13	31.69 ± 3.19	35.07 ± 3.19	39.07 ± 3.14
64% Protein/H_2O	20.75 ± 3.79	25.35 ± 2.26	24.22 ± 1.22	33.28 ± 0.86
68% Sucrose/H_2O	17.89 ± 4.10	24.97 ± 1.36	23.62 ± 2.04	23.91 ± 6.32
44% Lipid/H_2O	28.03 ± 9.56	33.97 ± 3.35	20.95 ± 3.56	36.24 ± 2.16

daily energy intake value was calculated from the combined calories derived from solid food and from the daily consumption of the 10% ethanol solution.

Simultaneous comparisons of the average daily energy intake values of Group 1 and Group 2 mice of all diets of ages 3 months to 12 months were performed by 3-way ANOVA analysis. The analysis failed to show any significant differences in daily caloric intake between Group 1 and Group 2 mice. That is to say, the stage of development at which the mice were when first started on the test diets (early-developmental vs. post-developmental) did not greatly effect the average daily energy intake. Within a particular dietary group, daily energy intakes did not vary significantly between the ages of 3 months to 12 months. Diet did have a significant effect on the daily energy intakes of the mice ($F = 11.23$, $p \leq 0.01$). A second analysis performed on Group 1 mice alone, to include the 1 month age group, confirmed the results of the first statistical analysis in that only diet had a significant effect on altering daily energy intakes ($F = 8.44$, $p \leq 0.01$).

In Group 1, the controls consistently had the highest average daily energy intake values. The energy intake of mice fed fat chow supplemented by 10% ethanol instead of drinking water did not show any significant deviations from the control values, although, intake was

reduced in this group of mice. Daily energy intakes were particularly affected by the 64% protein, 68% sucrose and 44% lipid diets. Mice on the 64% protein diet showed reduced energy intakes; at 12 months of age the daily energy intake was reduced by as much as 50.7% of the control value for the same age. Mice on the 68% sucrose diets had the lowest daily intake overall. Energy intake reduction ranged from 31.7% to 50.5% of the control values. Energy intake for the 44% lipid fed mice was particularly reduced at 10 and 12 months of age.

In Group 2, the average daily energy intake was highest in the ethanol-supplemented group. The overall energy intake values for mice fed on 64% protein, 68% sucrose and 44% lipid diets was reduced below controls but not to the same extent first observed in Group 1 mice. It appears that the average daily energy intake of mice in Group 2 was not as affected by diet as in Group 1 since no significant deviations from control values were detected in Group 2 mice.

Average Nuclear Diameter

Increased polyploidization of the liver is reflected in the average nuclear diameter of a distribution of liver cell nuclei of varying sizes. An increase in the number of polyploid nuclei within a sample of liver cell nuclei results in an increase in the average nuclear diameter of

Table 9

Average nuclear diameter/300 nuclei measured for control and test diets at the various ages studied for Group 1 mice. Mean ± S.D. N = 3 for each determination.

Diet	Average Nuclear Diameter (μm)				
	3 wks	3 mo	6 mo	10 mo	12 mo
Rat Chow/H_2O	7.16 ± 0.02	8.10 ± 0.22	9.14 ± 0.55	10.43 ± 0.43	10.43 ± 0.24
Rat Chow/10% Etoh	7.59 ± 0.38	9.39 ± 0.46**	9.05 ± 0.21	9.63 ± 0.17**	9.85 ± 0.18
64% Protein/H_2O	7.16 ± 0.70	9.27 ± 0.29**	9.74 ± 0.09*	10.16 ± 0.12	10.98 ± 0.38*
68% Sucrose/H_2O	6.95 ± 0.97	8.56 ± 0.49*	8.86 ± 0.37	10.31 ± 0.16	10.34 ± 0.25
44% Lipid/H_2O	6.76 ± 1.08	8.97 ± 0.33**	9.41 ± 0.16	9.52 ± 0.06**	10.34 ± 0.14

* Significantly different from controls ($p \leq 0.05$).

** Significantly different from controls ($p \leq 0.01$).

Table 10

Average nuclear diameter/300 nuclei measured for control and test diets at the various ages studied for Group 2 mice. Mean ± S.D. N = 3 mice for each determination.

Diet	Average Nuclear Diameter (μm) 6 mo	10 mo	12 mo
Rat Chow/H_2O	8.75 ± 0.13	8.89 ± 0.14	8.94 ± 0.43
Rat Chow/10% Etoh	7.41 ± 0.39**	9.11 ± 0.17	9.60 ± 0.13*
64% Protein/H_2O	8.01 ± 0.11**	8.60 ± 0.22	9.59 ± 0.91*
68% Sucrose/H_2O	7.92 ± 0.12**	7.99 ± 0.16**	9.15 ± 0.41
44% Lipid/H_2O	8.09 ± 0.11**	8.60 ± 0.30	8.88 ± 0.22

* Significantly different from controls ($p \leq 0.05$).

** Significantly different from controls ($p \leq 0.01$).

the sample since it has been found that a two-fold increase in polyploidy e.g. 2N to 4N, is accompanied by an increase in nuclear diameter of 1.26. In this part of the study, the effect of age and diet on liver polyploidization was evaluated by comparing the average nuclear diameter of a sample of 900 liver cell nuclei for mice of a particular diet and age.

Tables 9 and 10 show the average nuclear diameter for 300 nuclei measured per mouse at each of the ages studied for each dietary regime for Group 1 and Group 2 mice respectively. Statistical analysis consisted of a 3-way analysis of variance (ANOVA) comparing the average nuclear diameters of liver cells of mice in Groups 1 and 2, for all diets at 6, 10 and 12 months simultaneously. The 3 week and 3 month age groups were excluded from this analysis because Group 2 mice had just been placed on the test diets at 3 months of age and diameter measurements were only started at 6 months of age in Group 2 mice. To compensate for this, a second analysis was performed, a 2-way ANOVA, on Group 1 mice so that the 3 week and 3 month age groups could be included in the analysis. When the analysis showed that differences did exist between the different treatments, a multiple comparison Dunnett's test was performed to determine the significance of the departures from control values.

Results from the 3-way ANOVA suggest that there were

no significant differences in average nuclear diameter between mice of Group 1 and Group 2 on the same diet, at the same age. That is, the stage of development that the mice had reached when initially exposed to the test diets produced no statistically significant effect on the progression of polyploidization of the liver cell nuclei. Age, on the other hand, did have a highly significant ($F = 18.31$, $p \leq 0.01$) effect on the average nuclear diameter of both Group 1 and 2. Mice of all diets showed an increase in the average nuclear diameter with increasing age. From this, it can be said that with increasing age, there is an increase in the polyploidization of liver cell nuclei as reflected by an increase in average nuclear diameter. Even more interesting is the finding that diet also had a highly significant effect on the average nuclear diameter of liver cell nuclei ($F = 3.33$, $p \leq 0.01$). To determine which of the diets caused significant deviations in average nuclear diameter from control values, the Dunnett's test was employed at each age studied within a particular dietary regime.

In Group 1 at 3 weeks and 3 months, ethanol-treated mice had average nuclear diameters above those of the controls although the average nuclear diameter was only significant than the control at 3 months. At 6 months, there appeared to be a "slowing down" of polyploidization in the ethanol-treated animals since at 10 and 12 months of

age, the average nuclear of these mice were below those of the controls for the same age. Mice on the 64% protein diet show average nuclear diameters similar or higher than those of the controls, being significantly higher at 3, 6 and 12 months of age. An increase in the average nuclear diameter above the control value suggests that mice on the 64% protein diet have more polyploid cells or cells of higher ploidy classes than the controls of the same age. Mice on the 68% sucrose diet showed average nuclear diameters similar to those of the controls of the same age with one exception, that being at 3 months of age where these mice had an average nuclear diameter significantly higher ($p \leq 0.05$) than the control. Mice on the 44% lipid diet also appear to have average nuclear diameters similar to the controls at the same ages. No clear trend above or below control values can be established within this dietary group.

In Group 2 mice, controls show only a slight increase in the average nuclear diameter between 6 and 12 months of age. This may be interpreted as a "slowing down" of polyploidization of liver cell nuclei, a sort of stabilization or plateau reached during adulthood. This phenomenon was also observed in Group 1 mice of all diets except 44% lipid where it was not yet evident. For the ethanol-treated mice, the average nuclear diameter did increase with increasing age. The fairly large increase in

nuclear diameter from 7.41 μm at 6 months to 9.11 μm at 10 months suggests a large shift in the cell population to more numerous polyploid cells with a possible shift towards the higher ploidy classes. At 12 months of age, the ethanol-treated mice had an average nuclear diameter significantly higher ($p \leq 0.01$) than the control. A similar pattern of increase was found in the mice on the 64% protein diet. The average nuclear diameter started out being significantly lower than the control value at 6 months, however, it exceeds the control value by 12 months of age. In the 68% sucrose fed mice, the average nuclear diameter remains significantly below the control values at 6 and 10 months with a rather large increase at 12 months, comparable to the control. Mice on the 44% lipid diet showed an average nuclear diameter similar to that of the mice on the 64% protein fed mice at 6 and 10 months of age, the average nuclear diameter of these mice still remained slightly but not significantly below the control value.

Distribution of Nuclei Among the Ploidy Classes

Tables 11 and 12 show the distribution of nuclei in each ploidy class expressed as a percentage of the 900 nuclei measured for a particular age and diet for Group 1 and Group 2 mice respectively.

In experimental Group 1, one may observe that at 3 weeks of age less than 50% of the total number of nuclei

Table 11

Percentage of polyploid nuclei per ploidy class for control and test diets at the various ages studied (Group 1).

Diet	Age	Ploidy Class 2N	4N	8N	16N	32N	64N
Rat Chow/H_2O	3 wks	38.4	58.8	2.9	-	-	-
	3 mo	10.7	78.4	9.8	1.1	-	-
	6 mo	2.8	52.7	35.9	8.2	0.4	-
	10 mo	1.3	26.1	43.8	27.4	1.2	0.1
	12 mo	1.0	24.8	41.9	29.2	2.8	0.3
Rat Chow/10% Etoh	3 wks	22.6	71.2	5.7	0.6	-	-
	3 mo	7.6	47.0	33.3	11.6	0.6	-
	6 mo	3.8	52.6	28.6	14.3	0.7	-
	10 mo	2.0	46.2	35.9	15.2	0.7	-
	12 mo	1.1	37.3	43.7	16.9	0.9	-
64% Protein/H_2O	3 wks	36.6	60.0	3.3	0.1	-	-
	3 mo	4.7	49.9	36.2	9.2	-	-
	6 mo	1.9	41.3	37.6	18.6	0.7	-
	10 mo	1.1	35.3	36.2	24.8	2.3	0.2
	12 mo	0.8	18.6	38.9	34.7	6.4	0.7
68% Sucrose/H_2O	3 wks	52.0	41.6	5.8	0.7	-	-
	3 mo	10.8	59.2	25.7	4.2	0.1	-
	6 mo	4.0	43.3	46.8	5.6	0.3	-
	10 mo	1.4	30.6	44.7	19.2	4.0	0.1
	12 mo	0.9	27.9	40.3	25.7	5.0	0.1
44% Lipid/H_2O	3 wks	63.1	32.1	4.3	0.4	-	-
	3 mo	5.9	56.2	30.4	7.3	0.1	-
	6 mo	3.2	46.4	39.1	10.8	0.4	-
	10 mo	1.8	40.9	42.2	13.8	1.3	-
	12 mo	0.9	27.0	44.0	26.0	2.1	-

Table 12

Percentage of polyploid nuclei per ploidy class for control and test diets at the various ages studied (Group 2).

Diet	Age	Ploidy Class 2N	4N	8N	16N	32N	64N
Rat Chow/H_2O	6 mo	3.2	62.1	29.8	5.4	-	-
	10 mo	2.0	61.2	30.6	6.1	0.1	-
	12 mo	1.4	61.0	31.0	6.2	0.3	-
Rat Chow/10% Etoh	6 mo	20.4	75.7	3.6	0.3	-	-
	10 mo	2.8	49.7	37.2	9.3	0.9	0.1
	12 mo	1.8	45.2	41.7	10.2	1.0	0.1
64% Protein/H_2O	6 mo	12.0	70.9	15.6	1.6	-	-
	10 mo	5.8	63.2	25.1	5.8	0.1	-
	12 mo	1.1	50.1	31.2	14.2	2.9	0.4
68% Sucrose/H_2O	6 mo	8.2	79.7	11.6	0.6	-	-
	10 mo	5.7	80.0	10.8	4.0	-	-
	12 mo	4.4	53.6	30.0	11.6	0.4	-
44% Lipid/H_2O	6 mo	9.6	73.4	13.9	3.1	-	-
	10 mo	4.6	69.9	21.1	4.4	-	-
	12 mo	2.1	60.9	29.3	7.6	0.1	-

observed were diploid (2N) in mice fed the control diet of rat chow/H_2O or test diets of rat chow/10% ethanol and 64% protein/H_2O. In these dietary groups, the majority of nuclei fell in the tetraploid (4N) ploidy class. In mice fed the 44% lipid and 68% sucrose diets, over 50% of the nuclei observed were still 2N with the 4N ploidy class well established. It may be of interest to note that the mice of all the test diets show the appearance of nuclei in the 16N ploidy class while no such nuclei were observed in the control group at this age.

At 3 months of age, the percentage of diploid liver cell nuclei observed for mice of all diets decreased dramatically. The largest decrease in the percentage of diploid nuclei occurred in the mice fed 44% lipid diet followed in decreasing order by mice on 64% protein, 68% sucrose, control and rat chow/10% ethanol diets. This decrease in the percentage of diploid nuclei was, as expected, accompanied by an increase in polyploid nuclei. At 3 months of age, 78.4% of the nuclei observed in the controls were 4N and 10.9% of the nuclei were in ploidy classes above the 4N level. For mice on the test diets, the majority of the nuclei were also 4N but unlike the controls, they also had 3 to 4 times as many nuclei in ploidy classes above the 4N level.

At 6 months of age, the percentage of diploid nuclei for mice of all dietary groups were reduced from previous

levels. With the exception of 68% sucrose fed mice, the majority of the nuclei observed for the controls and remaining test diets were still 4N although reduced from the levels seen at 3 months. Also at this age, the 8N ploidy class had become well established and comprised over 30% of the sample for mice of all dietary groups. When observing the distribution of nuclei among the upper ploidy classes above the 8N level, the controls had over 8.6% of their nuclei distributed among the 16N and 32N ploidy classes while mice of all the test dietary groups showed values above this level. Mice on the 64% protein diet had twice as many nuclei in the 16N and 32N classes than the controls of the same age.

At 10 months of age, the liver cell sample was primarily made up of 4N, 8N and 16N ploidy classes in the controls with the majority of nuclei in the 8N class and almost equal numbers of 4N and 16N nuclei. In the test groups, one may observe that the bulk of the nuclei also fall into these three ploidy classes, however, fewer nuclei were found in the 16N class with the exception of 64% protein fed mice. The 64% protein-fed mice had a similar level of 16 N nuclei as controls but also had twice as many nuclei in the 32N and 64N ploidy classes.

At 12 months of age less than 2% of the nuclei observed for mice of all diets were diploid. Over 90% of the liver cell nuclei measured fell into the 4N, 8N and 16N

ploidy classes. While the distribution of liver cell nuclei of mice fed control, 68% sucrose and 44% lipid diets approached a normal distribution such was not the case with mice fed 64% protein or rat chow/10% ethanol diets. The distribution of nuclei of the mice fed the rat chow/10% ethanol was skewed with most of the nuclei in the 4N and 8N classes and only 17.8% of the nuclei fell into the 16N, 32N and 64N classes. Quite the opposite situation was found in the group fed the high protein diet. In this group, the distribution of liver cell nuclei was skewed with most of the nuclei in the 8N and 16N ploidy classes and 41.8% of the nuclei were distributed among the 16N, 32N and 64N ploidy classes which was 1.3 times higher than the controls.

In the experimental Group 2, where mice were started on the test diets as adults of 3 months, one may observe that at 6 months of age the majority of nuclei were 4N but at higher percentages than those seen in the Group 1 mice. Also, one may note that the percentage of nuclei in ploidy classes 8N and over is reduced in all the test groups as compared to the values seen in Group 1 for this age. At 6 months, it was the control group that had the most polyploid nuclei as well as having a higher percentage of nuclei in the higher ploidy classes. At 10 months, the majority of the nuclei (over 50%) for mice of all dietary groups were 4N, however, there was an increase in the

percentage of 8N nuclei from values seen at 6 months. If one recalls, for Group 1 mice the majority of the nuclei were 8N at 10 months of age with higher percentages of nuclei in the 16N and 32N classes.

At 12 months the distribution of nuclei in the controls of Group 2 was skewed indicating that over 60% of the nuclei remained in the lower ploidy classes of 2N and 4N unlike the controls of the same age in Group 1 where only 25.8% of the nuclei were in the 2N and 4N classes. Also, the amount of nuclei seen in the 16N, 32N and 64N classes is greatly reduced from that seen in Group 1 controls. Both of these control groups were treated in the exact manner, thus, the differences observed here between the controls of Group 1 and 2, are possibly due to genetic variations among the individual mice. When comparing the effect of the different test diets on polyploidization in Group 2 mice, it may be seen that most of the nuclei remained in the lower ploidy classes and that mice on the high protein diet had the greatest percentage of cells in the upper ploidy classes of 16N, 32N and 64N.

In summary, the mice on the 64% protein diet showed a premature shift to higher ploidies when compared to controls of the same age. The shift towards higher ploidy values appears to lag in Group 2 mice although previous statistical analysis of average nuclear diameter as a

representative of increased polyploidization showed no statistically significant differences in the polyploidization between the two experimental groups.

Polyploidization Index

The polyploidization index is the percentage of polyploid nuclei divided by the percentage of diploid nuclei. It is used as a way of quantitatively evaluating the progression of polyploidization throughout the experimental period as a continuum. The level of polyploidy of liver cell nuclei is expressed in terms of polyploidization index (P.I.) (Tables 13 and 14). An increase in the number of polyploid cells is reflected by an increase in the polyploidization index.

Table 13 shows the polyploidization indices of Group 1 mice for all diets. One may observe that mice on all diets demonstrate an increase in the polyploidization index with increasing age. At the early age of 3 weeks, animals on the rat chow/10% ethanol diet showed a P.I. above the control as well as the other diets. This trend, however, was not maintained as the animals aged in that by 6 months of age, these mice had a P.I. below that of the controls. This may be an indication of a "slowing down" or stabilization of polyploidy in the liver cell population during adulthood in these ethanol-treated mice.

Mice fed the 64% protein diet had a P.I. similar to

Table 13

Polyploidization Index for Group 1 mice. N = 3 for each determination.

Diet	Polyploidization Index 3 wks	3 mo	6 mo	% polyploid nuclei / % diploid nuclei 10 mo	12 mo
Rat Chow/H_2O	1.61	8.34	34.71	75.85	99.00
Rat Chow/10% Etoh	3.43	12.17	25.34	49.00	89.90
64% Protein/H_2O	1.74	20.28	51.68	89.82	124.13
68% Sucrose/H_2O	0.93	8.26	24.00	70.43	110.11
44% Lipid/H_2O	0.58	15.92	30.22	54.56	110.11

Figure 9 - Polyploidization index of Group 1 mice on control (rat chow/H_2O) and test diets at the various ages studied. (N = 3)

Cont = control, rat chow/water

= -12.927 + 8.949 Age, R = 0.990

Etoh = rat chow/10% ethanol

= -8.647 ± 7.026 Age, R = 0.949

Prot = 64% protein/water

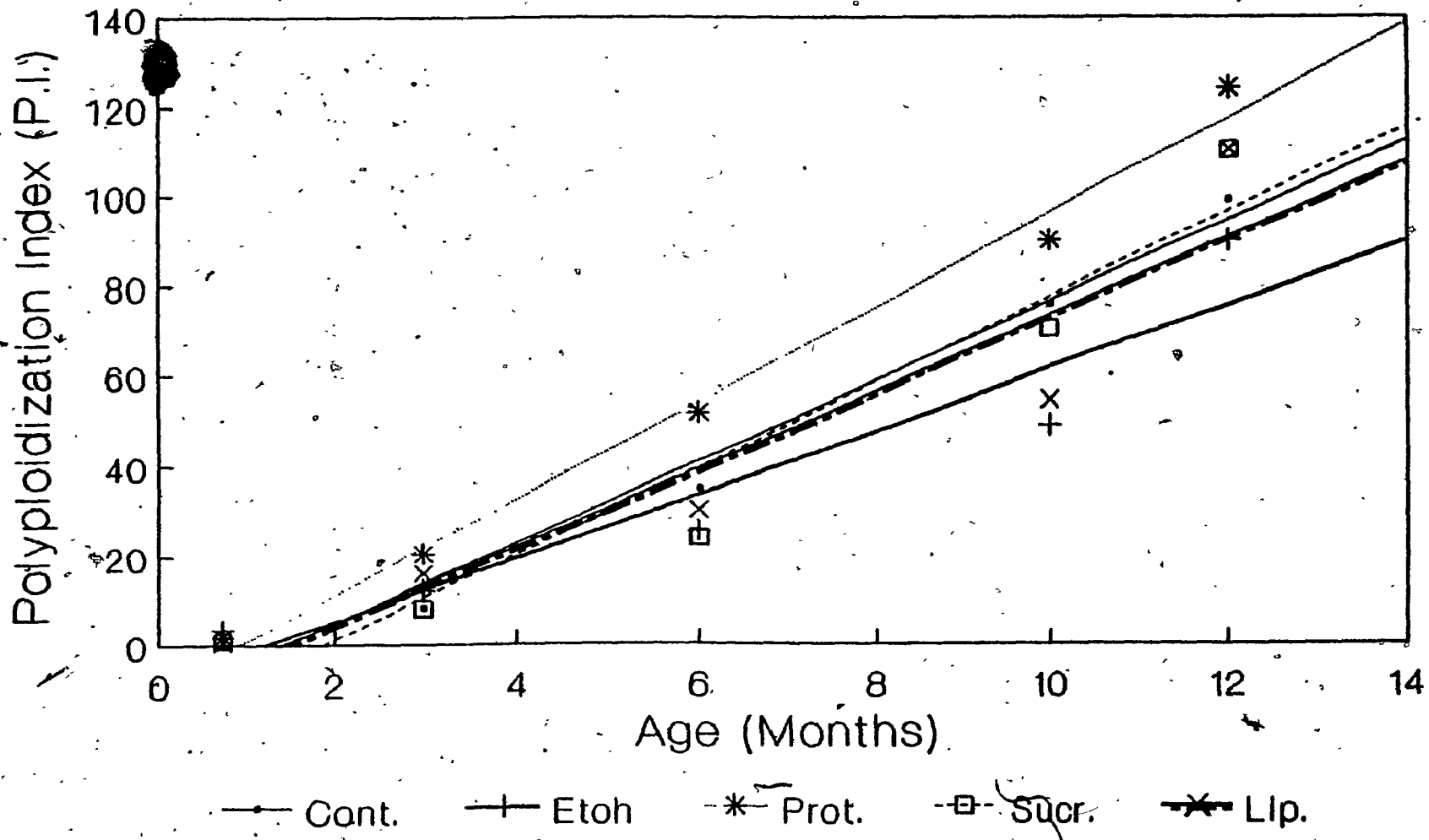
= -9.826 + 10.613 Age, R = 0.995

Sucr = 68% sucrose/water

= -17.751 + 9.527 Age, R = 0.937

Lip = 44% lipid/water

= -12.167 + 8.574 Age, R = 0.939


Polyploidization Index (P.I.)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Age (Months)
Cont.
Etoh
Prot.
Sucr.
Lip.

the controls at 3 weeks of age. However, there appeared to be a rapid accumulation of polyploid nuclei after this time so that by 3 months of age, the P.I. of mice on the 64% protein diet was more than double that of the control for the same age. The P.I. of these mice remained well above those of the controls through to 12 months of age. For mice on the 68% sucrose diet, at 3 weeks the P.I. is below control level and only at 12 months of age did the P.I. of these animals exceed that of the control. Mice on the 44% lipid diet had the lowest P.I. at 3 weeks of age. It was only a third of the value for the controls of the same age. After 3 weeks of age, there was a sharp increase in polyploid nuclei up to 3 months. After 3 months of age, the P.I. continued to increase at a slower rate to 10 months of age, subsequently followed by another "surge" of polyploidization to 12 months of age.

In Figure 9, polyploidization index (P.I.) was plotted against the animal's age in months. By calculating a regression line, the relationships shown in Figure 9 were obtained. Correlation analysis revealed a positive correlation between age and polyploidization index as indicated by the correlation coefficient, R, for each of the diets. As the mice aged, differences in the rate of polyploidization in the mice of the different dietary groups were noticed and reflected by the differences in the slopes of the lines in Figure 9. One may observe that the slopes of

the lines representing polyploidization of the hepatocytes of mice on the 68% sucrose and 44% lipid diets are similar to that of the control during the experimental period while the rate of polyploidization appeared to be increased above the controls in those mice fed the 64% protein diet. The rate of polyploidization in mice treated with 10% ethanol fell below that of the control, especially after 6 months of age.

Table 14 shows the polyploidization indices for Group 2 mice. As with Group 1 mice, the polyploidization index increased with age for all diets. However, the polyploidization indices seen here were below those values seen for Group 1 mice for the same age. Two possible explanations may account for these results. The first could be that polyploidization occurs at a slower rate in adult mice, which were exposed to the special diets only as adults. Alternatively, it is possible that the time spent on the diets (rather than the stage of development that the mice had reached when first started on these diets) was the factor which determined the levels and degree of polyploidization of the liver cell nuclei. Despite the "lag" in polyploidization in the Group 2 mice, some of the same trends may be observed in these mice that were seen in Group 1 mice. Firstly, polyploidization does increase with advancing age for mice of all dietary regimes. Also, it was the mice on the 64% protein which ultimately achieved

Table 14

Polyploidization Index for Group 2 mice. N = 3 for each determination.

Diet	Polyploidization Index: % polyploid nuclei / % diploid nuclei		
	6 mo	10 mo	12 mo
Rat Chow/H_2O	30.22	48.95	70.36
Rat Chow/10% Etoh	3.90	34.71	54.56
64% Protein/H_2O	7.34	16.24	89.82
68% Sucrose/H_2O	11.21	16.54	21.73
44% Lipid/H_2O	9.42	20.74	46.62

Figure 10 Polyploidization Index of Group 2 mice on control (rat chow/H_2O) and test diets at the various ages studied. (N = 3)

Cont = control, rat chow/water

= -9.920 + 6.403 Age, R = 0.974

Etoh = rat chow/10% ethanol

= -46.760 + 8.338 Age, R = 0.999

Prot = 64% protein/water

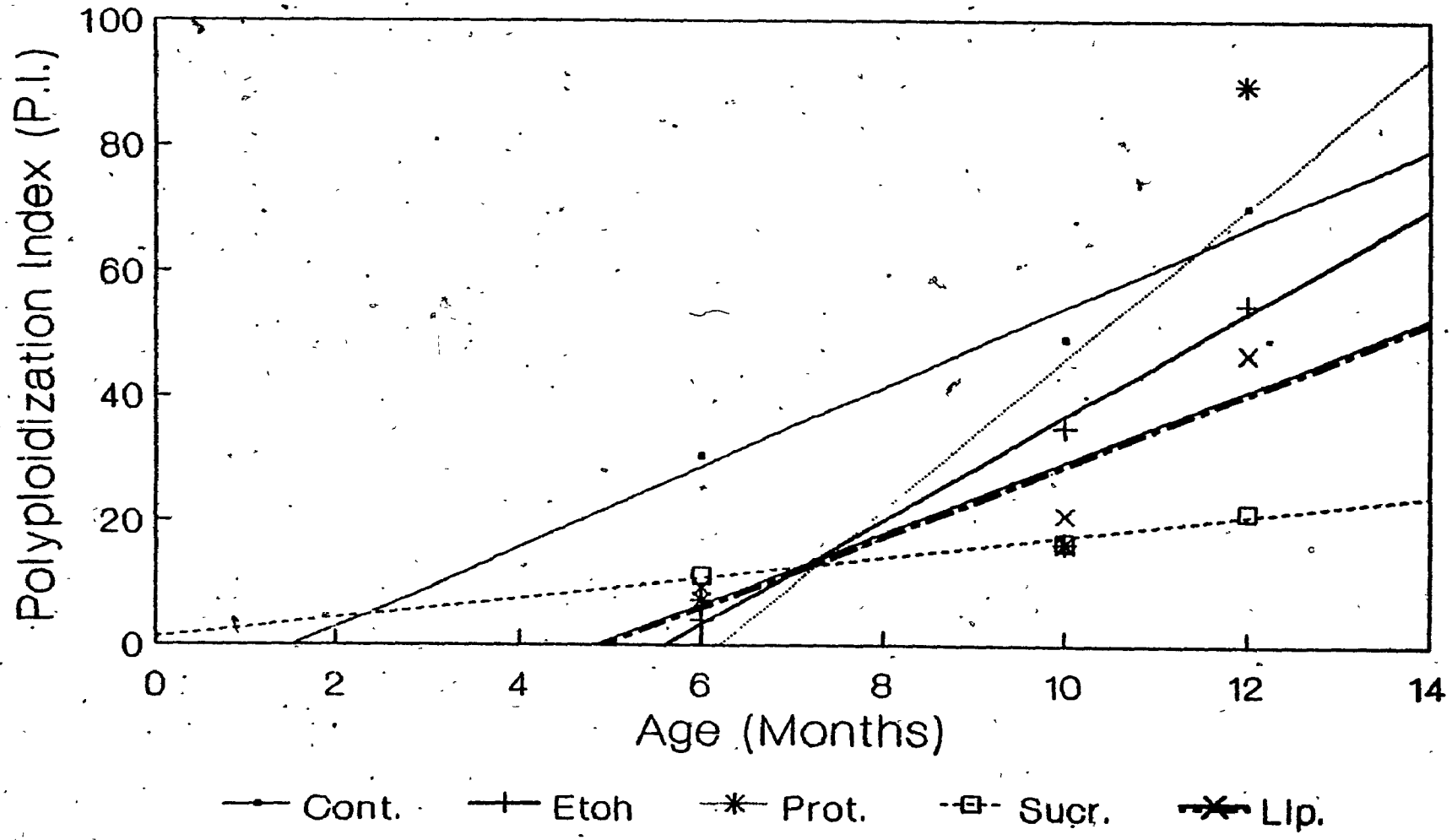
= -75.140 + 12.101 Age, R = 0.812

Sucr = 68% sucrose/water

= -1.120 + 1.632 Age, R = 0.988

Lip = 44% lipid/water

= -27.78 + 5.719 Age, R = 0.916


Polyploidization Index (P.I.)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Age (Months)
Cont.
Etoh
Prot.
Sucr.
Lip.

the most polyploid nuclei for a given sample of liver cells exceeding that of the controls by 12 months of age. It is noteworthy that this "overshoot" experienced by the mice on the high protein diet only occurs late during the experimental period, between 10 and 12 months, possibly indicating that it took longer for these mice to adapt to the dietary protein excess through increased polyploidization above control levels.

Figure 10 illustrates the P.I. plotted against age for Group 2 mice. Increased polyploidization with increased age may be observed for all dietary groups. The lines representing the polyploidization of liver of mice on the high protein, and ethanol-treated animals have steeper slopes than that of the control diet. This indicates that the hepatocytes of these mice were becoming polyploid at a faster rate than those of the controls after first being exposed to the special diets at 3 months of age. Based on the values presented in Table 14, the polyploidization index of the controls was higher than the other diets during the experimental period. However, if one extrapolates, on the basis of the slope of the regression lines, it would appear in time, that the polyploidization of the liver of mice on the 64% protein, 44% lipid and ethanol-supplemented dietary groups would surpass that of the control. This already appeared to be the case for mice on the 64% protein diet at 12 months of age. Mice on the

68% sucrose diet had the lowest slope of all the diets showing that polyploidy increased at a slower rate than in the controls.

DISCUSSION

Body and Liver Growth

The results indicate that the growth rate of mice, measured as gain in body weight, can be significantly affected by the stage of development at which the mice are first exposed to the supplemented diets used in this study. The adult body weight eventually attained by the mice was particularly affected by dietary manipulations during the period of rapid growth and development.

The National Research Council's report on the nutrition of the laboratory mouse suggests that a diet consisting of 20-25% protein, 45-55% carbohydrate and 10-12% fats together with the suggested compliment of vitamins and minerals is sufficient for the general growth and maintenance of mice under laboratory conditions (Simmons and Brick, 1970). Commercial diets, such as the one used for the controls in this study, are in compliance with the standards outlined in the NRC's report. The mice raised on this diet achieved the maximal growth rate observed in this study. In Group 1, it was the mice on the control diet which achieved the highest body weights, whereas, the

mice on the supplemented diets all had body weights below the controls beginning as early as 1 week of age. An earlier short-term study by Muramatsu and Ashida (1962) showed that body growth was proportional to the protein content of the diet in growing rats. When weanling rats were placed on diets varying in the amount of protein from 0-60% for 15 days, their weight gain was proportional to the dietary protein content up to 25% protein after which no further increase in weight was noted. The 60% protein diet produced a lower weight gain. Similar results were attained with the mice on the 64% protein diet in this experiment which showed the lowest growth rate of any dietary group.

In Group 2, all mice were weaned on the commercial diet and only switched to the supplemented diets once maturity was reached. Because they had passed through the period of rapid growth on a nutritionally balanced diet, they had similar body weights at 3 months. There was little difference in body weights between the dietary groups thereafter. The divergence of the growth curves from 10 to 12 months of age may be the result of an age-dependent loss in their ability to cope with the dietary excesses imposed on them at these later ages (Ross, 1976).

Unlike body weight, liver weight does not appear to be significantly affected by the stage of development at which the mice were first placed on the supplemented test diets.

Age only had an effect on liver weight while the liver was still growing. After maturity was reached, the liver weight remained fairly constant within a dietary group regardless of the age of the animals. Diet was the only factor tested which continued to affect liver weight into adulthood. The effect of dietary excess on growing mice was most noticeable in mice of Group 1 at 3 weeks. Mice on all of the supplemented test diets had liver weights significantly below the controls. After maturity had been reached at 3 months, only the mice on the high lipid diet continued to show liver weights below control values. Farid and associates (1978) reported that an increase in dietary fat from 0 to 20% resulted in a decrease in liver weight in rats fed diets varying in fat content for a period of 12 days. When considering liver/body weight ratios of the Group 1 mice, only mice on the high protein diet had consistently higher ratios than the controls for all ages studied. Muramatsu and Ashida (1962) have reported that liver weight as well as body weight of young rats is directly proportional to the protein content of the diet. When rats were fed diets ranging in protein content from 0 to 25%, the highest liver weights were attained by the rats fed the 60% protein diet.

In Group 2, liver growth had taken place primarily on the nutritionally balanced commercial diet. The switch to the supplemented test diets only took place after most of

the liver's growth was completed. Thus, neither liver weight nor the liver/body weight ratio were radically affected by switching the mice to the supplemented diets at maturity. The influence of diet was most pronounced in Group 1 mice especially at the early age of 3 weeks, when the liver was still actively growing.

It has been suggested that increase in liver weight during its growth phase is due primarily to cell proliferation via normal mitoses whereas post-natal liver growth is caused by polyploidizing mitoses. By the reduction of normal mitoses, there is a decrease in the risk of accumulating deleterious changes in the genome resulting from aberrant mitoses in a cell population that rarely divides (Brodsky and Uryvaeva, 1977). This hypothesis will be examined in a later section dealing with liver polyploidization.

The body weight differences seen in Group 1 mice may be attributed to differences in food consumption of the mice of the different dietary groups. The results showed that there were no significant differences in the daily food intake (g/day) between Group 1 and Group 2 mice nor were these values significantly altered as the mice aged. However, if food consumption is viewed in relation to the body weight of the animal, the results showed that the nutritional demands of the young mice of 1 month of age were 2 to 3 times higher than the adults on the same diet

owing to the greater energy requirements of these mice due to growth rather than maintenance alone.

Whether considering food consumption in terms of daily intake (g/day) or in relation to body weight (g/day/g body weight), diet significantly altered food consumption patterns. Food consumption values among the different dietary groups show similar trends for Groups 1 and 2. In both groups of mice, controls had the highest food consumption values. The amount of food consumed daily by normal mice on a commercial stock diet is 3-5 g (Simmons and Brick, 1970), thus, the controls in this experiment ate slightly in excess of this amount. Mice on the chow/10% ethanol diet consumed less than the controls in Group 1 but were comparable to controls in Group 2. In Group 1, the mice on the 64% protein diet had daily food intake values well below the controls for all ages studied, but in relation to body weight, at 1 month of age food consumption as above that of the control and when maturity was reached, only slightly below controls. Food consumption of Group 2 mice on 64% protein diet was also reduced from control levels but not as dramatically as seen in Group 1. Although the amount of food consumed by the mice on the 64% protein diet was reduced for both Groups 1 and 2, the excess of protein and the necessity to eliminate the excess urea, the toxic end product of protein metabolism, had a profound effect on the amount of liquid consumed by

these mice (Dougherty, 1978). In both Groups 1 and 2, mice on the 64% protein diet had the highest liquid consumption values. The daily water consumption of normal mice fed a commercial stock diet is 4.2-6.9 ml/day (Brick and Simmons, 1970). Food consumption for mice on either a 68% sucrose or 44% lipid diet was reduced below control values in Group 1 and Group 2.

The differences in body weights observed between the mice of the different dietary groups may also be due to differences in daily energy intake rather than solely food intake. From Appendix I, it may be seen that while the caloric content of the diets were similar, they were not identical, thus, the daily caloric intake varied for each dietary group. In Group 1, it was the controls which had the highest daily caloric intake followed secondly by the mice fed a rat chow diet supplemented with a 10% ethanol drinking solution. In Group 2, the situation was reversed. It is interesting to note that it was the controls that attained the highest mean body weight in both Groups 1 and 2. Despite having consumed less solid food than the controls, one might expect that the extra calories provided by the ethanol would compensate in the ethanol supplemented group, however, the calories provided by ethanol may not be fully utilized by the body.

Ethanol is metabolized primarily in the liver by two pathways. In the hepatocyte cytosol, ethanol is

metabolized by the aldehydehydrogenase (ADH) pathway where ethanol is oxidized to produce NADH which is subsequently oxidized the electron transport chain of the mitochondria to produce ATP. Approximately 50% of the ethanol is also oxidized by the microsomal ethanol oxidizing system (MEOS) of the smooth endoplasmic reticulum without yielding any high energy compounds. The chemical energy lost is believed to be dissipated as heat and, if not required for thermoregulation, is wasted. In conjunction, it has been found that ethanol increased the body's energy requirements (Sandler, 1980), thus, if food intake is inadequate or reduced below what is considered normal, a decrease in body weight may result. This may explain why the body weights of the ethanol supplemented mice were below the controls despite having an excess of calories provided by ethanol. Of course, other factors such as physical activity, not measured in this experiment, has an important effect on body weight.

Energy intake was reduced for mice of the remaining 3 test diets in both Groups 1 and 2, with the lowest intakes having occurred in the 68% sucrose-fed group. It is interesting to note that the reductions in food and energy intake were self-imposed by mice fed diets having an energy content greater than the control diet (i.e. 64% protein, 68% sucrose, 44% lipid diets) and could possibly reflect an internal sensitivity to the richness of the diets. This

self-imposed dietary restriction diminished the availability of energy required for maximum growth during the period of rapid growth and development spanning the period shortly after birth up to 3 months of age. The inhibitory effect of reduced energy intake on RNA synthesis and protein synthesis resulting in decreased mitotic activity (DiStefano et al., 1959) may be the reason why animals in Group 1 fed diets of 64% protein, 68% sucrose or 44% lipid during the rapid growth phase never attained body weights comparable to controls. Animals fed their diets only after maturity was reached (Group 2) were not as greatly affected by reductions in energy intake.

Liver Polyploidization: Effect of Age and Diet

Among the age-associated changes which take place in hepatocytes, the most notable and most constant changes occur in the nuclei. It has been well documented that liver cell polyploidization increases with age (Alfert and Geshwind, 1958; Brodsky and Uryvaeva, 1977; Enesco and Samborsky, 1983; Shima and Sugahara, 1976; Swartz, 1956). Comprehensive studies on liver polyploidization have been conducted in rodents and in man, using both whole liver sections as well as isolated hepatocytes (Bohm and Noltemeyer, 1981; Engelmann, et al., 1981; Steele et al., 1981). In rodents, hepatocyte polyploidization commences in the early postnatal period around 3 to 5 weeks of age

(Evans, 1976; Shima and Sughara, 1976). In humans, a definite tetraploid cell population is established by 11 to 14 years of age (Swartz, 1956).

It is generally accepted that polyploid cells arise from the fusion of the two nuclei of binuclear liver cells which originate from mononucleate cells which have undergone acytokinetic mitosis (Himes et al., 1957; James, 1977; Nadal and Zajdela, 1966; Wilson and Leduc, 1948). Binucleate cells, although polyploid themselves in terms of total chromosome number, act as cellular intermediates in the progression of polyploidization of mononucleate liver cells (James, 1977). Nuclear fusion in binuclear cells has been found to occur during prometaphase resulting in a single metaphase plate. Cells in late metaphase are found exclusively with a single chromosomal mass which have been identified cytophotometrically as having multiple sets of chromosomes (James, 1977). After nuclear fusion, if the cytoplasm divides, two mononucleate cells of the next higher order of ploidy result. If nuclear fusion does not take place, a binucleate cell of the next higher order of ploidy remains. Regardless of cytoplasmic division, a polyploid cell of the next magnitude of ploidy results (Wilson and Leduc, 1948). Thus, the mature liver of man, rodents and a few other vertebrate species is a mixed population of mononucleate and binucleate cells of varying degrees of polyploidy (Brodsky and Uryvaeva, 1977;

Uryvaeva, 1981). The present study, however, was restricted to the mononucleate cell population.

Common indices of polyploidy have been measurements of the mean DNA content of hepatocytes by biochemical (Gaub, et al., 1981) and cytophotometric methods (Bohm and Noltemeyer, 1981a,b; DiStefano et al., 1959; Enesco, 1967; Epstein, 1967; Falzone, et al., 1959; Shima and Sugahara, 1976). These measurements have shown the adult liver nuclei of rodents and man contain different amounts of DNA related to each other by a ratio of approximately 1:2:4 (Collins, 1978; Epstein, 1967; Shima and Sugahara, 1976; Steele et al., 1981; Swartz, 1956). Nuclear size has also been used as an index of polyploidy by several investigators working with hepatic tissue (Andrew et al,. 1943; Carriere et al,. 1962; Enesco et al., 1983; Epstein, 1967; Falzone et al., 1959; Helweg-Larsen, 1952; Hildebrand, 1980; Medda et al., 1984; Swartz, 1956; Tauchi and Sato, 1962). They have found that a doubling of DNA content is accompanied by an almost equal increase in nuclear volume and an increase in nuclear diameter by a factor of 1.26 (Epstein, 1967). Andrew and his coworkers (1943) showed an age-dependent increase in the average nuclear diameter of mouse hepatocytes during senescence. Thus, the average nuclear diameter of a sample of hepatocytes may be used to estimate changes in polyploidy within the liver with age, where an increase in average

nuclear diameter may be regarded as a shift towards increased polyploidy in the liver.

The results of this study are consistent with the findings cited previously in that polyploidization (expressed here as an increase in average nuclear diameter and polyploidization index) increased with age in mice of all dietary regimes tested. The occurrence of nuclei with very high ploidies (16N, 32N, 64N) increased with the age of the mice. Such high ploidies are rarely observed in rats and humans. Evidence from past studies indicates that liver polyploidization commences in rodents around the time of weaning (21 days) (Epstein, 1967; Evans, 1976; Doljanski, 1960; Shima and Sugahara, 1976; Wheatly, 1972). In this study, a substantial polyploid population already had been established by the 3 week old mice in Group 1. It is known that liver polyploidization is more extensive in mice than rats (Carriere, 1969) and that the degree of polyploidy occurring in mice varies with mouse strain (Steele et al., 1981). From the results presented in this study, polyploidization of the liver in Swiss albino mice begins prior to the estimated age of 3 weeks, regardless of diet.

There is reason to believe that the polyploidization of liver cells is dependent on the presence of growth hormone. When growth hormone is absent, as in mice with anterior pituitary hypoplasia (Helweg-Larsen, 1952), liver

polyploidization fails to develop. Also, after hypophysectomy, further polyploidization is arrested (Bass and Dunn, 1957; DiStefano et al., 1955, 1959; Nadal and Zajdela, 1966; Wheatley, 1972). It was also discovered that growth hormone treatment, in either case, restores liver polyploidy to normal age-dependent levels. It does appear, however, that the effect of growth hormone on the development of liver polyploidy is mediated by dietary factors (Doljanski, 1960; DiStefano et al., 1959; Enesco and Samborsky, 1983).

Prior to 3 weeks of age, the nutritional demands of the young mice are dependent upon the maternal milk supply. Thus, the effect of diet on liver polyploidization in pre-weanling mice is the direct result of the composition and availability of the maternal milk supply. The composition of mouse milk, based on average values, is as follows: 69.5% of the calories are derived from fat; 24.6% are derived from protein and; 5.9% are derived from carbohydrates (Cox and Mueller, 1937). Studies of the effect of maternal diet on the composition of milk in mice and rats show that manipulations in the proportions of macronutrients in the maternal diet does not significantly alter the amounts of these substances in the milk produced by these animals (Williamson et al., 1984; Grigor et al., 1987; Roberts and Coward, 1985). Lactating female rats fed diets with an excess of protein (Mueller and Cox, 1946;

Williamson et al, 1984), lipid (Farid et al., 1978; Grigor and Warren, 1980) or carbohydrate (Carrick and Kuhn, 1978) did not significantly alter the total amounts of these macronutrients in the milk. However, dietary supplementation was found to increase milk yield (Mueller and Cox, 1946; Roberts and Coward, 1985; Williamson et al., 1984). Ethanol feeding in lactating animals and humans was found to affect the availability of nutrients to the offspring due to its inhibitory effect on the release of oxytocin (Fuchs et al., 1967; Fuchs and Fuchs, 1969). This hormone is released by the neurohypophysis and is responsible for the milk let-down response ellicited by the sucking stimulus upon the nipple (Guyton, 1981). Thus, the availability of milk to the pups whose mothers drank 10% ethanol in Group 1 of this experiment, may have been reduced. At 3 weeks of age, Group 1 mice on differing dietary regimes displayed slight differences in the distribution of polyploid nuclei among the various ploidy classes. While 4N nuclei predominated in the mice fed the control, ethanol supplemented or 64% protein diets, in mice fed the 68% sucrose or 44% lipid diets, the 2N ploidy class remained predominant. Whether the slight differences in ploidy distribution observed were a response to the small but insignificant differences that occur in milk composition with changes in maternal diet or rather a response to changes in milk yield is unclear since neither

of these parameters were measured. These variations in distribution of polyploid nuclei however were not of sufficient magnitude to manifest themselves as a significant deviation in average nuclear diameter from controls at 3 weeks of age.

Brodsky and Uryvaeva (1977) have shown that the sharpest change in liver parenchyma composition occurs in baby and young rats during the period of rapid growth encompassing the time of weaning until the attainment of adult body weight at about 3 to 4 months of age. In this study, the largest decrease in the amount of diploid cells and largest increase in average nuclear diameter was also observed between the ages of 3 weeks and 3 months for all dietary groups in Group 1. Around 3 weeks of age, the mitotic index in the liver falls to almost zero (Doljanski, 1960; Epstein, 1967) and liver growth by cellular multiplication almost comes to a halt (Brasch, 1982). From this point on, it is believed that liver growth occurs at a slower rate by cell polyploidization (Brodsky and Uryvaeva, 1977; Doljanski, 1960) and the increase in cell size which accompanies it (Bohm and Noltemeyer, 1981; Epstein, 1967; Tongiani and Puccinelli, 1970; Van Bezooijen, 1972-73). Because this drastic increase in polyploidization is concurrent with an increased rate of body growth, Naora and Naora (1964) proposed that the large increase in polyploidization at this period of development is a means

by which an essentially non-dividing tissue is able to meet the functional demands placed upon the liver by the increased number of cells proliferating in the body. Thus, as the number of cells increases in the body, so does the polyploidy in the liver. This claim was substantiated by showing that when one increased the number of cells proliferating in a young mouse by means of transplanting sarcoma tissue subcutaneously, the amount of DNA in the liver cells increased at a faster rate in these mice than in the controls. Relating Naora's theory to the present study, one would expect to observe the highest degree of polyploidization in the largest, and therefore, heaviest animals. In Group 1, the controls were the heaviest animals at all times. However, polyploidization was highest in mice on the 64% protein diet as shown by an average nuclear diameter which was significantly higher than the controls during the period of 3 to 12 months of age although they had the lowest mean body weight during this period. The mice on the 64% protein diet also had the highest percentage of nuclei in the 16N, 32N and 64N ploidy classes which usually only accompany senescence (Shima and Sugahara, 1976). The mice fed the 64% protein diet also had reduced food energy intakes when compared to controls indicating that the content of the food, especially dietary protein, rather than the amount of food or energy consumed had a significant role in the development of liver

polyploidy in these mice. Rats show a stabilization of polyploidization with stabilization of body weight (Brodsky and Uryvaeva, 1977; Naora, 1957). In contrast, polyploidization in mice continues throughout the lifespan. The accumulation of cells with high degrees of ploidy occurs in the later stages of life when both body and liver weight are on the decline (Bourne, 1961; Medvedev and Medvedeva, 1985). In Group 2, body weights for mice from the different dietary groups were similar from 6 to 10 months of age (Fig. 2). After 10 months of age, the weight curves diverged. By 12 months of age mice on the 64% protein diet had the highest mean body weight while the controls had the lowest observed body weight during this period. It was only at 12 months of age that significant increases in polyploidization were observed in the high protein group. A significant increase in average nuclear diameter was also noted in the ethanol-supplemented mice in Group 2 at 12 months of age. Ethanol has been found to increase the level of polyploidization occurring in the liver of adult rats (Gaub et al., 1981). Because the rate of increase in polyploidization seen in Group 1 mice between the ages of 3 weeks and 3 months failed to be maintained throughout adulthood, it is difficult to draw any definite conclusions on the effect of ethanol on liver polyploidization. The drop in the rate of liver polyploidization observed in Group 1, ethanol-supplemented

mice, after the age of 3 months may represent an adaptation developed early in these mice to small amounts of alcohol present in their diet.

The rate of polyploidization of liver cells slows once maturity is reached. This has been demonstrated by Brodsky and Uryvaeva (1977) and Shima and Sugahara (1976) as well as in this study. Because of this phenomenon, the effect of diet on polyploidization may take longer to manifest itself in animals exposed to changes in diet composition once maturity had been attained. Although no significant differences in average nuclear diameter were detected between mice of Group 1 and Group 2 for the same age and diet, differences were observed in the distribution of nuclei among the ploidy classes. In Group 1, at 6 months of age, the 4N ploidy class was predominant in all dietary groups except the 68% sucrose fed mice where the 8N class predominated. In all cases, the 8N class comprised a substantial portion (30-40%) of the nuclei observed at this age. Nuclei with ploidy values above the 8N level (16N, 32N and 64N) ranged between 5.7% and 19.3%. In Group 2, at 6 months of age, tetraploids also constituted the greater portion of the nuclei observed. However, the percentage of octaploids observed in Group 2 mice were reduced from levels seen in Group 1 and nuclei above 8N only ranged between 0.3 and 5.4 %. By 12 months of age, octaploid nuclei constitute 40 to 45%, tetraploids 19 to 37% and

16N, 32N and 64N constitute 18 to 42% of the nuclei observed in Group 1 mice. In Group 2, the tetraploid ploidy class continued to remain the predominant ploidy class (45-61%) and octaploids comprised 30 to 42% of the nuclei observed with nuclei of 16N, 32N and 64N classes amounting to only 7.7 to 17.5%.

Mature hepatocytes are cells in which tissue-specific programs and the ability for proliferation coexist. In differentiated tissues, secretion of tissue-specific products only commences once their growth has been completed. Withdrawal from the mitotic cycle is irreversible. Hepatocytes retain the ability to divide despite having become specialized. It has been suggested that in hepatocytes, tissue-specific and proliferative functions are in a state of mutual balance. Because the metabolic resources of hepatocytes are limited, the intensification of one function results in a weakening of the function of the other (Brodsky and Uryvaeva, 1977). For example, in regenerating liver, metabolic resources are diverted away from tissue-specific functions towards increased proliferative syntheses. In the course of liver regeneration following partial hepatectomy, the activities of several drug-metabolizing enzymes are reduced (Fouts et al., 1961) and results in a temporary resistance of the regenerating liver to such hepatotoxins as carbon tetrachloride and paracetamol (Brodsky and Uryvaeva,

1977). Examples of the reverse effect, where retardation of the regeneratory response occurs upon intensification of tissue specific functions, is also known to occur (Takata, 19[illegible]). Since hepatocytes must provide tissue-specific products to some degree even in the event that cellular division is required, the result is a reduced or incomplete mitotic cycle, the end result of which is polyploidization (Brodsky and Uryvaeva, 1977). Thus, it is logical that when an increase in tissue-specific proteins is required, for example, to deal with increased metabolic demands, polyploidization is the response or "trade off" by which metabolic energy is conserved. Brodsky and Uryvaeva (1977) suggest that stimulating processes predominant at a certain stage of development results in the displacement of the competing one. Schneyer (1973) showed that the injection isoproterenol (a chemical which causes proliferation and hypertrophy of parotid gland cells of the adult rat) during the first postnatal weeks actually speeds up the differentiation of the gland cells. In the same light, increasing the metabolic demands of the liver displaces growth by cellular multiplication, causes acceleration of liver cell differentiation and the polyploidization which accompanies it (Brodsky and Uryvaeva, 1977; Uryvaeva, 1981). Kennedy and Pearce (1956) showed that suckling rats undergoing rapid growth due to increased milk supply and associated macronutrients, underwent a polyploidization

shift at an earlier age than unsupplemented controls. Therefore, the higher degree of polyploidy seen in Group 1 mice may, in fact, represent an early shift from growth by multiplication to growth by polyploidization. This premature shift in polyploidization was most sensitive to dietary protein.

The Biological Significance of Liver Polyploidization:

While the functions of the liver are numerous and varied, it is sufficient to say that its primary functions lie 1) in the transformation and storage of nutritive substances in such physical and biochemical forms that can be utilized by the rest of the body and 2) in the conversion of waste metabolites into excretable substances (Doljanski, 1960). Because of its role in metabolism and detoxification, the liver comes into direct contact with numerous toxic, carcinogenic and mutagenic substances whether it be from exogenous sources absorbed into the blood from the gut or produced endogenously during the normal course of metabolism. As such, liver cells have been shown to be more susceptible to genetic damage than other cell types (Carriere, 1969; Brodsky and Uryvaeva, 1977). Therefore, it has been suggested that polyploidization of liver cells plays a protective role. Redundant information and reiteration of vital genes could reduce the deleterious consequences of chromosomal

aberrations. These occur with a high frequency due to the detoxification functions of the liver (Uryvaeva, 1981).

Another hypothesis suggests that polyploidization provides a safe alternative means of growth and repair for the liver. By reducing the length and occurrence of mitosis in the liver cell, considered to be the most sensitive stage of the cell cycle, the accumulation of aberrations could be reduced (Brodsky and Uryvaeva, 1977). The cellular hypertrophy which accompanies polyploidization makes it possible to maintain the normal size and function of the liver without the risk of accumulating aberrant cells resulting from abnormal mitoses due to the harsh chemical environment of the liver.

It has been suggested that because of the increase in the number of templates available for the transcription provided by increased amounts of DNA as well as increased nucleo/cytoplasmic ratios (Engelmann et al., 1981; Gahan and Middleton, 1982; Swartz, 1956; Tauchi and Sato, 1962), polyploidization increases the cell's functional efficiency. This theory is disputable since several investigators (Brasch, 1980; Brodsky and Uryvaeva, 1977; Epstein, 1967) found that cell volume increases almost in proportion to the nuclear volume during polyploidization. Thus, the nucleo/cytoplasmic ratio remains nearly constant at all ploidy levels, resulting in cells which are metabolically comparable despite differences in ploidy.

Studies comparing the relative amounts of RNA synthesized by cells of differing ploidies reveal that the relative rate of RNA synthesis is almost equal for a particular age group but that the absolute amounts of RNA synthesized within a particular ploidy class decreases with age (Collins, 1978). Other studies have shown that increased polyploidization of a cell may actually decrease its efficiency because the surface area/volume decreases considerably with increased polyploidization (Harris, 1971; Van Bezooijen, 1972-73). Because surface area is proportional to volume$^{2/3}$, the replacement of diploid cells by an equal volume of tetraploid cells will result in a decrease in total surface area by 20.5%. If these diploid cells are replaced by an equal volume of octaploid cells, total surface area decreases by 36.7% (Epstein, 1967). Gaub (1981) and co-workers showed that increased polyploidization of rat liver cells induced by ethanol-feeding resulted in protein accumulation in the cell. This may represent an impairment in liver functions associated with the surface membrane of the cell. Van Bezooijen (1972-73) demonstrated that the liver's ability to excrete bromsulphalein (BSP), a physiological indicator of the liver's functional ability, decreased with increased liver polyploidization.

Medvedev (1986) has suggested that polyploidization of the liver evolved as a special type of self-protection

against the toxic effect of ammonia and urea, the end products of normal protein/amino acid metabolism. This theory may explain the prevalence of liver polyploidization among mammals more than any other vertebrate group. To corroborate this hypothesis, Medda (1984) has shown that the polyploidization which occurs in the liver of chick embryos is concomitant with a period of urea production in the chick's liver during embryonic development. Towards the end of chick embryonic development, urea formation is replaced by the final adult uric acid-producing means of ammonia detoxification; this is accompanied by a decline in the number of polyploid cells. This theory implicates dietary protein and subsequent concentrations of its metabolites as a major contributor to the development of polyploidy in the liver of mammals. When the body has an excess of protein, the excess amino acids may be degraded by deamination in the liver and used as energy or stored as fat. The ammonia resulting from deamination is extremely toxic and if it is allowed to accumulate in the blood by insufficient transformation to the less toxic urea by the liver, death is imminent (Guyton, 1981). The conversion of ammonia to urea allows the end products of protein metabolism to be stored temporarily and disposed of at random intervals, thus, conserving the body's water which is essential for a terrestrial existence. Thus, the urea cycle of ammonia detoxification is considered to be the

most important function of the liver (Guyton, 1981; Medvedev, 1986).

Evidence best supports the hypothesis that polyploidization of the liver had, indeed, evolved primarily as a type of self-protective response of the liver to a urea-producing mechanism of ammonia detoxification that accompanied a terrestrial mode of existence as suggested by Medvedev (1986). Therefore, liver polyploidization is influenced by levels of dietary protein and the resulting metabolites produced by the liver. It is probable that liver polyploidization evolved secondarily, as a safe means to respond to the increased metabolic stress placed on the liver by toxic, exogenous substances such as phenobarbital (Bohm and Noltemeyer, 1981) or carbon tetrachloride (Himes et al., 1957) without having to increase the cell number by undergoing a complete mitotic cycle which may have deleterious effects in the presence of these substances or their metabolites.

The benefits derived from liver cell polyploidization most likely outweighs the limitations imposed on polyploid cells of the 4N and 8N classes predominant during most of the lifespan in mice, however, it is not prudent to overlook the severe limitations, such as, dramatic decreases in surface area imposed on cells of very high ploidies (16N, 32N, 64N) that accumulate at the later stages of the lifespan in mice, and their contribution to

the decline of the liver's function during this period. Factors which accelerate the appearance and preponderance of such cells, may be, in essence, contributing to the acceleration of the senescence of the organ itself.

SUMMARY

The effect of dietary excess on liver cell polyploidization was examined in both developing and post-developmental mice fed either a balanced diet (control) or one of the following diets: 1) rat chow/10% ethanol 2) 64% protein/H2O; 3) 68% sucrose/H2O or 4) 44% lipid/H2O.

It was found that liver polyploidization in Swiss albino mice increased with age, regardless of dietary composition or the stage of development the mice were at when first exposed to the test diets, however, dietary composition, especially protein content, was found to significantly alter the rate at which polyploidization had occurred.

In Swiss albino mice, liver cell polyploidization commences before weaning (21 days of age) since a tetraploid class of nuclei was found to be well established at 3 weeks of age for mice from all dietary groups. Prior to weaning, the effect of diet on liver cell polyploidization was the direct result of maternal

nutrition (in utero) and composition maternal milk immediately after birth. At 3 weeks of age no significant differences were found in average nuclear diameter, an index of polyploidization, of liver cells of suckling mice whose mothers were fed diets varying in composition. This indicated that the changes in maternal nutrition or in milk composition, if any, which occurred as a result of the composition of maternal diet were insufficient to illicit major changes in the rate of liver polyploidization in their offspring.

The greatest shift towards increased levels of polyploidy occurred in rapidly growing mice between the ages of 3 weeks and 3 months. It was also during this period of development that liver polyploidization was most sensitive to dietary composition. Mice from all test diets showed a significant increase in polyploidy above the control level at 3 months of age, however, it was only mice fed the 64% protein diet which continued to show increased levels of polyploidization well into adulthood. Also, the increased polyploidization expected for ethanol-treated mice failed to be maintained after 3 months of age. Once maturity was reached, liver polyploidization was not as dramatically affected by age or diet and as such, changes in the levels of polyploidization took longer to manifest themselves as significant changes from the control levels.

In this study, polyploidization was affected to a

greater extent by the composition of diet, particularly dietary protein, rather than the actual amount of food or calories consumed, two factors found instead to play a greater role in body growth.

The sensitivity of liver polyploidization to dietary protein suggests that it may be linked to the protein metabolism of the liver and further supports the theory that polyploidization of mammalian liver cells initially evolved as a type of self-protective mechanism from the toxic products of protein metabolism generated in the liver, namely, ammonia and urea.

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APPENDICES

APPENDIX I

Food Composition of Diets Used in Study.

Diet	Protein* %	Carbohydrate* %	Fat* %	Energy Value cal/g
Rat Chow/H_2O	22.5	58.5	4.5	4.16
Rat Chow/10% Etoh	22.5	58.5	4.5	4.16 and 0.56 cal/ml
64% Protein/H_2O	64.0	22.0	8.0	4.51
68% Sucrose/H_2O	18.0	68.0	8.0	4.24
44% Lipid/H_2O	25.5	21.3	44.0	5.40

* Nutrients expressed as a percentage of the ration.

APPENDIX II

Dehydration Schedule of Liver Tissues

From temporary storage of liver tissues in 70% ethanol, tissues are run through the following solutions.

1. 95% Ethanol..... 2 hr
2. 100% Ethanol..... 2 hr
3. 100% Ethanol..... 2 hr
4. Xylene/100% Ethanol, 3:1..... 2 hr
5. Xylene..... 2 hr
6. Tissues are now carried over to the first preparation of paraffin.

APPENDIX III

Preparation of Solutions for Fixation and Staining

Fixative: Lillie's Neutral Buffered Formaldehyde Solution

37-40% formaldehyde solution	100 ml
Water	900 ml
Acid sodium phosphate, monohydrate	4.0 g
Anhydrous disodium phosphate	6.5 g

Staining Solutions:

1) Acid-Alcohol

70% ethanol	100 ml
Hydrochloric acid, conc	1.0 ml

2) Gill's Hematoxylin

Hematoxylin	4.0 g
Sodium iodate	0.4 g
Aluminum sulfate	35.2 g
Distilled water	710.0 ml
Ethylene glycol	250.0 ml
Glacial acetic acid	40.0 ml

3) Eosin

Spirit soluble eosin	1.0 g
95% Ethanol	100 ml

APPENDIX IV

Hematoxylin and Eosin Staining Schedule

1.	Xylene	2 min
2.	Xylene	2 min
3.	Absolute Alcohol	2 min
4.	Absolute Ethanol	2 min
5.	95% Ethanol	2 min
6.	95% Ethanol	2 min
7.	70% Ethanol	2 min
8.	50% Ethanol	2 min
9.	Distilled water	2 min
10.	Gill's Hematoxylin	3-4 min
11.	Running tap water	5 min
12.	Acid Alcohol (destain)	rinse
13.	Running tap water	5 min
14.	Eosin (counterstain)	5 min
15.	Running tap water	2 min
16.	50% Ethanol	2 min
17.	70% Ethanol	2 min
18.	95% Ethanol	2 min
19.	Absolute Ethanol	2 min
20.	Absolute Ethanol	2 min
21.	Xylene	5 min
22.	Xylene	5 min
23.	Mount coverslip	