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Hematological effects in C57BL/6 mice fed selected Tweens

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HEMATOLOGICAL EFFECTS IN C_{57}BL/6 MICE FED SELECTED TWEENS

by

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TABLE OF CONTENTS

INTRODUCTION

Widespread use of nonionic emulsifiers in common foodstuffs, in cosmetics, and in the pharmaceutical industries has focused attention toward the possible effect of these emulsifiers upon the human being. Nonionic sorbitan derivative emulsifiers may be found in some bakery goods, ice cream and other dairy products, confections, soft drinks, and vitamin preparations. Several investigations (Schweigert et al., 1950; Wang et al., 1950; Harris et al., 1951a, 1951b; Wissler et al., 1954; Eagle and Poling, 1956; and Poling et al., 1956) have shown deleterious effects in laboratory animals fed various nonionic emulsifiers. Other publications (Krantz et al., 1952; Krehl et al., 1955; Oser and Oser, 1956a, 1956b, 1957a, 1957b, and 1957c; Orten and Dajani, 1957; and Brush et al., 1957) indicate the innocuousness of some of the same nonionic emulsifiers to laboratory animals.

Vorhes (1959) defines a food additive as a substance intentionally put into a food to serve a useful purpose, or a substance that may be expected to enter food incidental to production, processing, and packaging. Technological functions of nonionic emulsifiers include smoother texture, prolonged palatability, improved flavor, more attractive appearance, improved uniformity, better keeping qualities, and improved eating quality (Pratt, 1952). Consequently, nonionic emulsifiers have been classified as food additives.

A study of the effects of chronic feeding of certain nonionic sorbitan derivative emulsifiers on the hematological picture of the C_{57}BL/6 Jax mouse was initiated as one phase of an investigation currently in

progress in the Department of Zoology and Entomology at Iowa State University.

Other aspects of the total program with C_{57}BL/6 Jax mice include: (1) an investigation of the effect of chronic feeding of food additives on reproductive ability; (2) a histological and histochemical examination of the liver after chronic feeding of food additives; (3) a study on the effect of chronic ingestion of food additives in relation to anesthesia time; (4) a survey of biochemical blood assays of mice maintained on high percentage food additive supplemented diets; and (5) a quantitative measurement of liver glycogen deposition in the mouse maintained on food additive supplemented diets.

The present research was initiated for four reasons: (1) because of the particular paucity of normal hematological information relative to the C_{57}BL/6 Jax mouse; (2) because of the lack of pertinent data on age effect on the blood picture in laboratory mice; (3) because of the scarcity of literature relative to the effect of food additives on the hematology of laboratory animals in general, and mice in particular; and (4) because of the conflicting appraisals of effect of nonionic surface active additives on various structures and functions in laboratory animals.

The objectives of the present study were: (1) to determine the "normal" hematological pattern of the C_{57}BL/6 Jax mouse in relation to age; (2) to determine the effect of chronic feeding of certain nonionic emulsifier additives on the hematological picture of the C_{57}BL/6 Jax mouse; and (3) to demonstrate the practicability of a specific longitudi-

nal experimental design in collection and analysis of data for the described type of investigation.

REVIEW OF LITERATURE

Early accounts of the hematological picture of laboratory mice suggested an age effect, although few investigators endeavored to verify the validity of this apparent trend. Results of DeKock's (1931) work with Swiss and Rockefeller strains utilizing different mice at various ages suggest a decline in hemoglobin content with increased age. The Swiss strain exhibited a hemoglobin average of 19.4 g/100 ml blood for 58 mice at ages two, three, and four months; an average of 18.4 g/100 ml blood for 12 mice at one year of age; and an average of 17.1 g/100 blood for ten mice at two years of age. The data for Rockefeller strain mice showed an average hemoglobin content of 20.5 g/100 ml blood for eight mice at three months of age; of 19.5 g/100 ml blood for 35 mice at seven months of age; and 17.1 g/100 ml blood for 30 mice at one year.

Kalabukhov and Rodinov (1934) demonstrated an age effect in hemoglobin content and erythrocyte values in young albino mice (Mus musculus L.). For eight mice, one to three days old, the hemoglobin value was 13.5 g/100 ml blood; for ten different mice at 31 to 50 days of age, the average hemoglobin value was 9.4 g/100 ml blood. Erythrocyte counts increased from 3.215 million corpuscles/mm^3 blood for eight mice at one to three days of age to 6.390 million corpuscles/mm^3 blood for ten other mice at 31 to 50 days of age.

Francis and Strong (1938) reported a gradual loss of blood pigment associated with age of CBA strain mice. Their results for breeder mice showed an average hemoglobin value of 16.3 g/100 ml blood for 37 mice at 200 days of age; at 720 days of age the average for 12 different mice was

13.7 g/100 ml blood.

Strong and Francis (1940), employing different mice at various ages, showed a continuous decline in hemoglobin content in "A" strain (cancer-susceptible) breeder mice. The average hemoglobin value for 30 mice at 120 days of age was 17.1 g/100 ml blood; at 320 days of age the average was 14.2 g/100 ml blood for 52 mice; and at 520 days of age for 17 mice the average hemoglobin content was 12.3 g/100 ml blood. They also reported a slight, gradual decline in hemoglobin content of the blood in breeder mice of the CBA strain (cancer-resistant). The average hemoglobin value for 37 mice at 200 days of age was 16.3 g/100 ml blood; for 64 mice at 400 days of age the mean hemoglobin value was 15.3 g/100 ml blood; and for 29 mice at 640 days of age the mean hemoglobin value was 14.2 g/100 ml blood.

Few data have been recorded in the literature for the blood picture of the C_{57}BL/6 Jax mouse. No consideration of age effect on the hematological picture could be found in the literature.

Fekete (1941, p. 93) reported an average erythrocyte count of 9.292 million corpuscles/mm^3 blood for six C_{57}BL males between 77 and 86 days of age. Russell et al. (1951), using nembutal anesthetized C_{57}BL/6 Jax virgin males and females (five each), two to three months of age, reported an erythrocyte count of 9.66 million corpuscles/mm^3 blood; hematocrit, 44 percent; hemoglobin content, 13.3 g/100 ml blood; mean corpuscular volume, 45.5 μ^3; and mean corpuscular hemoglobin concentration, 30 percent. From their results, mean corpuscular hemoglobin would be 13.7 $\gamma\gamma$g (author's calculations).

Platt and Zeller (1951) reported values for three mice of the C_{57} strain. Age and sex of the mice were not reported. Their data were shown in bar graph form and were estimated by the present writer to be 14.3 g/100 ml blood for hemoglobin content; 54 percent for hematocrit; and erythrocyte count of 9.03 million corpuscles/mm^3 blood.

Gyllensten and Swanbeck (1959) reported hematological values for C_{57}BL/6 Jax mice at 30 days and three months of age. The sex and number of animals were not specified. At 30 days, the hemoglobin content was 10.69 g/100 ml blood; hematocrit, 39.8 percent; and average erythrocyte count, 8.04 million corpuscles/mm^3 blood. From these results, the mean corpuscular volume would be 49.5 μ^3; mean corpuscular hemoglobin, 13.3 $\gamma\gamma$g; and mean corpuscular hemoglobin concentration, 26.9 percent (author's calculations). For three month old mice, the average hemoglobin value was 12.1 g/100 ml blood; hematocrit, 40.4 percent; and erythrocyte count, 8.56 million corpuscles/mm^3 blood. The cell indices calculated from these data would be: mean corpuscular volume, 47.2 μ^3; mean corpuscular hemoglobin, 14.1 $\gamma\gamma$g; and mean corpuscular hemoglobin concentration, 29.9 percent (author's calculations).

No data could be found in the literature pertaining to the effect of nonionic emulsifiers on the blood picture of the C_{57}BL/6 Jax mouse. A survey of the literature reveals very little data concerned with the effect of nonionic emulsifiers on the hematology of laboratory animals in general.

Harris *et al.* (1951a) reported that rats fed sorbitan monolaurate at the 25 percent level showed a reduced hemoglobin content. Hamsters

fed either 5 percent sorbitan monolaurate, or 5 percent polyoxyethylene sorbitan monolaurate, or 5 percent polyoxyethylene monolaurate exhibited no differences from controls in blood hemoglobin content (Harris et al., 1951b). Oser and Oser (1957a) indicated no effect of a variety of nonionic emulsifiers on hemoglobin content and erythrocyte count in rats examined at 12, 52, 78 and 104 weeks on the diet. Normal values for hemoglobin content and erythrocyte counts in rats fed different nonionic emulsifiers ranging from two percent to 25 percent levels were reported by Fitzhugh et al. (1959).

EXPERIMENTAL PROCEDURE

Experimental Design

The present investigation consists of a long-term study of the effect of chronic ingestion of certain nonionic surface active agents on the hematology of an inbred strain of laboratory mouse.

Animals chosen for the present study were one of the Roscoe B. Jackson Memorial Laboratory stock, strain C_{57}BL/6. These animals were chosen because of their long average life-span (350-650 days for males; 400-650 days for females) and low incidence of tumor, especially in older ages.

Ten male C_{57}BL/6 Jax mice were placed on each of five diets at the approximate age of two months. Thereafter, these same animals were examined at the approximate ages of four, six, nine, 12, 16, 20, and 24 months.

Virgin males were selected for investigation in the present study for several reasons. Several possible sources of variation, e.g., sex, pregnancy, lactation, cyclic hormonal changes, and interactions among these factors were thus eliminated. Any influence of the female estrous cycle on the stability of the hematological picture would be eliminated, although Russell et al. (1951), using nembutal anesthetized mice of the C_{57}BL/6 Jax strain, reported no significant sex difference in erythrocyte count or hematocrit.

Non-anesthetized mice were used in the study for several reasons. Russell et al. (1951) reported that use of nembutal anesthetized mice

gave lower counts and lower relative variability. However, Budds et al. (1953) reported that "anesthesia contributes significantly to variations in granulocyte number." They also stated "the possible differing reaction of different strains to nembutal should also be of importance to the planning of experiments."

All blood samples were taken between 8:00 A.M. and 9:30 A.M. in order to eliminate as much as possible the effect of any diurnal variation in circulation of blood solids.

The present plan of study of age effect and chronic ingestion of food additives on the hematological picture of mice differs from any investigation found in the literature by the writer. The experimental design of most studies has been to use different animals at various ages. In one investigation (Francis and Strong, 1938), however, some mice were examined at two ages. In the current problem, groups of male C_{57}BL/6 Jax mice, each maintained on one of five diets, were examined at four, six, nine, 12, 16, 20, and 24 months of age. Several advantages of this experimental design are noteworthy: (1) elimination of a possible variable among different groups of the same strain; (2) need for a fewer number of animals for experimentation; (3) decreased maintenance cost (number of cages, cleaning, etc.); and, (4) emphasis of individual results. A possible disadvantage could be development of an anemia through repeated blood sampling of the individual. However, earlier work by the author (Ewing, 1962) indicated no development of a typical chronic hemorrhagic anemia (hypochromic microcytic) under the blood sampling conditions of the investigation.

Experimental Animals

The Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, supplied the C_{57}BL/6 Jax mice directly from their colony. Fifty-two male C_{57}BL/6 Jax mice were received from the supplier with the notation: "Age, 4 to 5 weeks." After about one month to become acclimated to new laboratory conditions, these mice were placed under experimental regimen.

Ten mice, approximately two months of age, were randomly selected for each diet group from the main stock.* Animals were housed five to a cage. Individual mice were earmarked for easy identification.

Mice were kept in clear plastic cages with perforated metal covers, and were provided with bedding of wood shavings. Direct natural light was partially excluded from the animal room so that mice were exposed to strong light only when being cared for, or when removed to the laboratory for examination. Cages, wood shavings, food containers, and water dispensers were routinely changed each week. Used cages, feeders, and water dispensers were washed in hot water with detergent, rinsed in distilled water, dipped in disinfectant (Hytron), and drained to dry. Food and tap water were replenished as needed during the week.

Experimental Diets

A series of nonionic water soluble surface active agents is produced by Atlas Powder Company, Wilmington, Delaware, under the trade name Tween.

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*This group of animals is referred to in our laboratory records as Series II. Mice in Series I were being used for a different study and are not included in the present report.

Tweens are fatty acid esters of anhydrosorbitols (sorbitan) which have been solubilized by etherification of free hydroxyl groups with ethylene oxide (Schwartz and Perry, 1949, p. 209). The polyol portion is a mixture of anhydrosorbitols and forms a linkage between the fatty acid and the polyethenoxy chain.

In the present problem two nonionic emulsifiers available commercially as Tween 20 and Tween 60 were added to the basal experimental diet (Purina Mouse Breeder Chow, mash form*). Tween 20 (polyoxyethylene sorbitan monolaurate) and Tween 60 (polyoxyethylene sorbitan monostearate) are prepared as direct reaction products of 20 mols of ethylene oxide with one mol of sorbitan monolaurate and one mol of sorbitan monostearate, respectively. These Tweens are fluid at room temperature, and thus are easily mixed with dry animal rations.

The basal control diet was Purina Mouse Breeder Chow (mash form). Experimental diets were mixed on a food additive-Purina Mouse Breeder Chow total weight-percentage basis. Four hundred fifty grams of Purina Mouse Breeder Chow were hand mixed with 50 grams of the particular food

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*Contents as stated on container:

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

Guaranteed analysis:

Crude protein not less than	17.0%
Crude fat not less than	11.0%
Crude fiber not more than	2.0%
Ash not more than	5.5%
N.F.E. (Nitrogen Free Extract: This item is currently omitted from the analysis shown on the feed container; it was included when the experiments were begun)	52.0%

additive to obtain the 10 percent level experimental diet. Four hundred seventy-five grams of Purina Mouse Breeder Chow were hand mixed with 25 grams of the specific food additive to obtain the five percent level experimental diet. After thorough hand mixing, the experimental diets were placed in a tumble-type motor driven mixer and allowed to mix for approximately 30 minutes per batch.

Polyoxyethylene sorbitan monolaurate (Tween 20) and polyoxyethylene sorbitan monostearate (Tween 60) were each fed, ad libitum, at five and 10 percent levels to respective experimental groups. The five diet groups are hereafter referred to as: (1) control, (2) Tween 20-5 percent, (3) Tween 20-10 percent, (4) Tween 60-5 percent, and (5) Tween 60-10 percent.

Procedure for Collection of Blood

Blood samples were collected from the tail. In order to obtain sufficient blood for the various determinations, without undue delay and dilution with tissue fluids, it was necessary to devise a rapid and simple method for taking blood samples. By means of a sharp scalpel, a quick, clean cut of the last one or two millimeters of the tail tip resulted in free blood flow. Bleeding was usually instantaneous, though in some cases a few seconds delay for free blood flow was noted. The delay of blood flow in some animals may have been caused by a temporary peripheral vascular reaction such as has been noted in some humans with the "stab" technique of blood sampling (Bryan et al., 1935).

Another problem associated with sampling was the maintenance of the animal in position during collection of blood so that one person could

perform the entire operation. A method was needed in which the animal could respire freely and would still be confined so the collector could utilize both hands for manipulations of collections and dilutions. After several attempts with various methods, such as placing the animal on a suspended wire screen, or placing the animal under an inverted glass beaker with the tail protruding from the pouring lip, it was suggested that an open glass container be utilized.*

A glass tube was tapered and constricted sufficiently at one end to prevent animal escape, but with the tapered end open to allow access to air (Figure 1). Movement of the mouse was restricted to a minimum, thus allowing the investigator use of both hands in collection and dilution of samples. Four glass tubes of different dimensions were employed in the present study to accommodate mice of various sizes.

A wooden rack was constructed to hold the various size glass tubes (Figure 1). A plywood gate was placed over the larger open end of the tube. It was held in place by means of an elastic strap connected at points 1 and 2 in Figure 1. A notch in the bottom of the gate allowed the tail to protrude outside the tube. Animals were guided into the tube and then were restricted by placement of the gate into position. This arrangement provided several advantages: (1) allowed the mouse adequate air during the collection of blood; (2) permitted the collector to utilize both hands in manipulations; (3) restricted animal movements to a minimum during the procedure; and, (4) eliminated the need for anesthesia during

*Suggested by Dr. Robert M. Melampy, Animal Science Department, Iowa State University, Ames, Iowa.

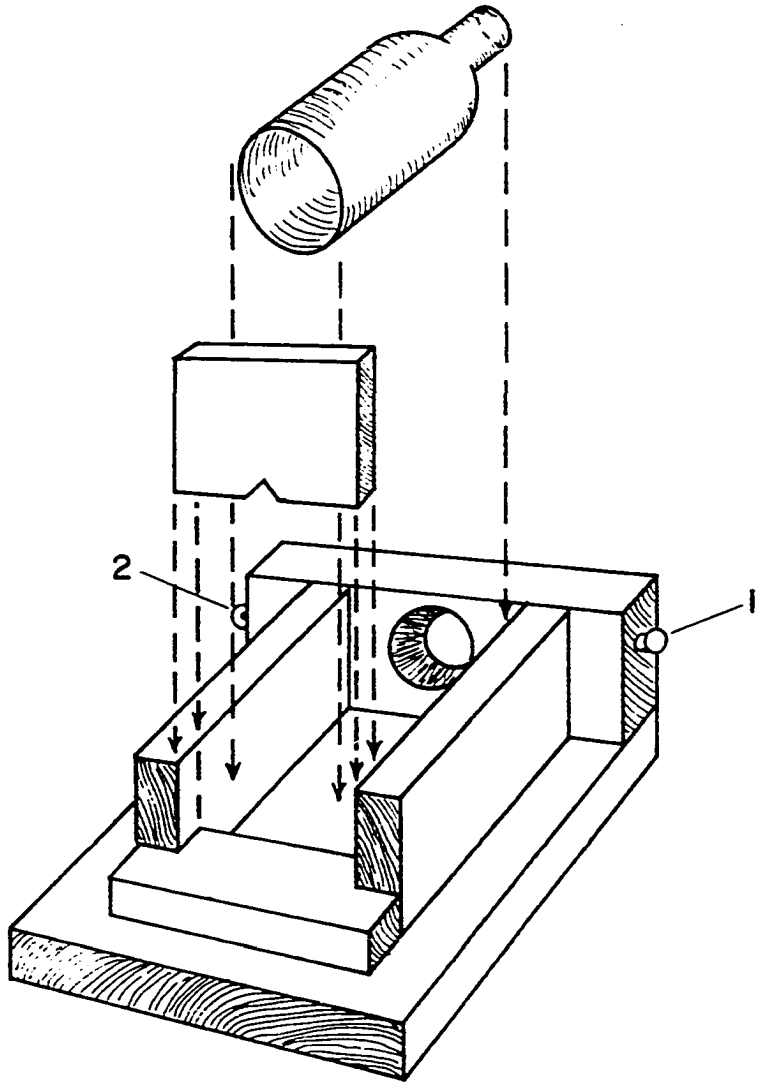

Figure 1. Equipment for restraining mouse during blood sampling

collection of blood samples.

After a short period of time to allow the mouse to become accustomed to, and settled in, the confines of the tube, a piece of transparent cellophane tape was placed lightly over the tail to further restrict movement. The tail was wiped gently, but thoroughly, with 70 percent alcohol, and was then dried and warmed for a short period with heat from a lamp. A clean, quick cut across the tip of the tail was made with a sterilized scalpel. The first two or three large drops of blood were discarded, and the tail wiped free of blood with a gauze sponge slightly moistened with 1.3 percent sodium oxalate to prevent clotting. Samples of blood were taken directly from the tail for the listed procedures in the following order: (1) initial hematocrit, (2) hemoglobin, (3) erythrocyte count, and (4) final hematocrit.

After collection of the last sample, the tail was cleansed thoroughly with 70 percent alcohol, and the wound cauterized with hot forceps. In over 600 collections of blood samples in the above described manner not one case of infection or obvious inflammation of the tail has been noted on any of the mice.

Hematological Determinations

The significance of the hematological values investigated in the present study is based on possible pathological conditions related to the erythron. The various measurements are especially important in morphological classification of the anemias. Fundamental morphological characteristics of anemias are based on changes in size and hemoglobin

content of the erythrocytes.

Measurements of erythrocyte count, hemoglobin content, and hematocrit in a unit volume are basic procedures. They indirectly indicate the amount of oxygen that can be carried by a quantity of blood. This appears to be the best measure of the functional efficiency of the blood, since in most pathological conditions involving the erythron, the total blood quantity remains nearly constant (Wintrobe, 1946, p. 285). Anemia is a reduction below "normal" in erythrocyte count, hemoglobin content, or hematocrit, i.e., a reduction in the functional efficiency of the blood.

The significance of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) is in classification of anemia type. A change in mean corpuscular volume is indicative of microcytic (decreased MCV) or macrocytic (increased MCV) anemia (Goodale, 1959, p. 25). Mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration are increased in macrocytic anemias and decreased in hypochromic anemias (Goodale, 1959, p. 33). In most anemias (macrocytic, normocytic, and simple microcytic) an increase or decrease in mean corpuscular volume is associated with corresponding increase or decrease in mean corpuscular hemoglobin, which means that the mean corpuscular hemoglobin concentration remains normal (Wintrobe, 1946, pp. 266-268). However, in an important group of anemias (hypochromic microcytic) the reduction of mean corpuscular hemoglobin is more pronounced than is decrease in mean corpuscular volume, which is denoted by a decreased mean corpuscular hemoglobin concentration (Wintrobe, 1946, pp. 266-268). A discussion of anemia classification is presented by

Wintrobe (1946, pp. 286-292).

Hemoglobin

Hemoglobin determinations were made by the cyanmethemoglobin method (Crosby et al., 1954) using the Spectronic 20 colorimeter (Bausch and Lomb), and Bausch and Lomb cuvettes (Cat. No. 33-29-33). The Spectronic 20 colorimeter was performance-tested for wavelength scale setting and meter reading function according to the methods and procedures outlined by Bausch and Lomb Optical Company.

A slight modification was made in the prescribed technique in order to economize on the amount of blood taken from one animal. In the standard procedure, 0.02 ml blood is added to a colorimeter tube containing 5.0 ml of Drabkin's solution.* The procedure was modified to use only 0.01 ml blood. Dilutions were made with Yankee Lambda micropet, 0.01 ml, TC 20°C, ± 1.0 percent. The reading of percent transmission was made as usual, and the amount of hemoglobin for the respective transmission was then doubled. The modified method was previously checked for accuracy and reproducibility against the standard method and found highly satisfactory (Ewing, 1962).

The modification in the standard procedure for hemoglobin determination by the cyanmethemoglobin method was used throughout the present study.

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*Composed of: 1 g sodium bicarbonate, C.P.
52 mg potassium cyanide, C.P.
198 mg potassium ferricyanide, C.P.
Dissolved in distilled water to exactly one liter.

Hematocrit

Hematocrit determinations were made by the microhematocrit method of McGovern et al. (1955). Heparinized capillary tubes, 1.3 to 1.5 mm in diameter and 75 mm in length, were filled to approximately three-fourths the total length directly with blood from the tail. The unfilled end of the capillary tube was sealed by use of a low flame nicroburner. The tubes were centrifuged in an International micro-capillary centrifuge, Model MB*, for a period of four minutes at 11,500 r.p.m. Hematocrits were read directly from a micro-capillary card reader with use of a hand lens for greater magnification.

Two determinations of hematocrit value were taken on each animal, one at the beginning of sampling, and the second as the last step of sampling after blood had been taken for a number of different determinations. These "first and last" samples seldom differed more than one percent, and in most cases were the same. In case of difference between the two determinations, the results were averaged and the mean recorded as the hematocrit value for the mouse. Earlier work (Ewing, 1962) had shown that the percentage of solids in the blood collected from the tail did not change noticeably during the course of collection. According to McGovern et al. (1955), the results of the microhematocrit method, using either venous or capillary blood from the human, are comparable to those obtained with the standard Wintrobe method using human venous blood.

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*International Equipment Company, Boston, Massachusetts.

Erythrocyte count

Erythrocyte counts were made by the standard procedure outlined by Wintrobe (1946, pp. 251-252). Adams oval bead blood diluting pipettes were used for dilutions. Erythrocyte counts were made on the Spencer Bright-Line Improved Neubauer hemocytometer. Gowers' solution, using the Mallory and Wright modification as suggested by Ch'u and Forkner (1938), was selected as the blood diluent for erythrocyte count.

Both chambers of the hemocytometer were filled from a single dilution, with several drops of the pipette contents discarded between filling of the chambers. Corpuscles were allowed to settle before counts were made. Both wells of the hemocytometer were counted and the calculated average of the two counts recorded as the erythrocyte count for the sample. When counts of the two wells differed more than 250,000 corpuscles, the preparation was discarded, the hemocytometer cleansed, both chambers refilled from the same dilution pipette used previously, and the counts repeated. If a difference of over 15 corpuscles per square was noted, the count was repeated on another filling from the same dilution. This double method of checking for difference in count within a chamber and difference between chambers, while not taking into account a possible dilution error, would tend to reduce error from improper filling of the chamber, and from uneven distribution of erythrocytes within a chamber.

According to Wintrobe (1946, p. 253), counts of less variation than 18 corpuscles among the squares show satisfactory distribution within a chamber. He also states that counts agreeing within 200,000 corpuscles/ mm^3 blood can be made from the same sample using human blood, and that

two or more counts should be averaged for the final result. The standards used for erythrocyte count determinations in the present study closely approximate those set forth by Wintrobe (1946, p. 253).

Mean corpuscular volume

The original formula for "corpuscular volume" was introduced by Wintrobe (1929). Later publications (Haden, 1932; Wintrobe, 1932) referred to the measurement as "mean corpuscular volume". The formula allows calculation of mean corpuscular volume in absolute terms, expressed as cubic micra (μ^3). The formula used for calculation was:

$$MCV = \frac{\text{Volume (cc. per 1000 cc.)}}{\text{R.B.C. (in millions)}}$$

where volume refers to volume of packed corpuscles per 1000 cubic centimeters of whole blood (hematocrit x 10), and R.B.C. refers to the erythrocyte count in millions of corpuscles per cubic millimeter of whole blood.

Mean corpuscular hemoglobin

Wintrobe (1929) introduced the term "corpuscular hemoglobin" and the formula for its calculation. In later publications (Haden, 1932; Wintrobe, 1932) the measurement is referred to as "mean corpuscular hemoglobin". The formula expresses mean corpuscular hemoglobin in absolute terms of micromicrograms ($\gamma\gamma g$). The formula used in calculation of mean corpuscular hemoglobin was:

$$MCH = \frac{\text{Hemoglobin (gm. per 1000 cc.)}}{\text{R.B.C. (in millions)}}$$

in which hemoglobin refers to grams of hemoglobin per 1000 cubic centi-

meters of whole blood (hemoglobin determination x 10), and erythrocyte count is expressed in millions of corpuscles per cubic millimeter of whole blood.

Mean corpuscular hemoglobin concentration

"Proportion of hemoglobin in cell" was suggested by Wintrobe (1929) to introduce the formula for calculation of "mean corpuscular hemoglobin concentration" (Haden, 1932; Wintrobe, 1932). The formula allows expression of the ratio of the weight of hemoglobin in the average corpuscle to the volume in which it is contained. The following formula was used in calculations:

$$\text{MCHC} = \frac{\text{Hemoglobin (gm. per 100 cc.)}}{\text{Volume (cc. per 100 cc.)}}$$

in which volume refers to the erythrocyte volume per 100 cubic centimeters of whole blood (hematocrit), and hemoglobin refers to grams per 100 cubic centimeters of whole blood (hemoglobin determination).

The distinction between mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration should be made clear. Mean corpuscular hemoglobin is the weight of hemoglobin in the average corpuscle in the blood examined. Mean corpuscular hemoglobin concentration is the ratio of the weight of hemoglobin in the average erythrocyte to the volume of the average erythrocyte in the blood examined, and is expressed as a percent.

Statistical Methods

Measured values for hemoglobin, hematocrit, and erythrocyte count, and calculated values for mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were analyzed for significance of linear and curvilinear (quadratic or second degree polynomial) regression on diet time (age less two months), following Snedecor (1956, pp. 413-472). Differences in mean values for each diet group, i.e., means calculated from the particular linear or quadratic regression formula, for seven and 14 months on the diet were tested for significance at the five percent level after Duncan (1955) using The New Multiple Range Test.

RESULTS

In recording results, time on diet rather than age of mice will be used as a measure of duration of exposure to experimental diets. Since mice were not placed on experimental regimen until approximately two months of age, time on diet differs from age by two months. This may be expressed mathematically as $X' = X - 2$, where X represents age in months, X' indicates time on diet in months, and 2 represents the acclimation period of two months. In the formulae for linear ($Y = a + bX$) and quadratic ($Y = a + bX + cX^2$) regression, X' was inserted as the independent variable (time on diet) in place of X. The dependent variable Y, therefore, is equal to a when X' is zero and X is two months of age, i.e., at the beginning of experimental conditions.

Utilization of time on diet rather than age allows for age effect and diet effect to be recorded, compared, and discussed using the same time basis.

Hemoglobin

Results of blood hemoglobin content for individual mice on the five diets are recorded in Tables 1, 2, 3, 4, and 5. Graphic depiction of the mathematical averages for a given age and time on diet is shown in Figures 2 and 3. Results of linear and quadratic regression fittings are shown in Table 6. Statistical evaluations for significant ($P<.05$) differences between calculated regression means for each diet group, at seven and 14 months on diet, are recorded in Table 7.

The control group (Purina Mouse Breeder Chow) exhibited a gradual

Table 1. Hemoglobin content values (g/100 ml blood) of control diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months) 4	6	9	12	16	20	24
	Time on diet at date of examination (months) 2	4	7	10	14	18	22
1	16.4	15.7	14.7	14.4	14.4	13.0	--
2	14.4	15.4	16.0	15.0	14.7	--	--
3	15.7	16.4	15.7	14.4	14.4	14.4	13.0
4	16.7	15.2	--	--	--	--	--
5	15.7	14.0	14.0	14.4	14.2	14.0	13.0
6	17.0	16.4	15.4	14.4	14.2	14.0	15.0
7	15.7	16.0	15.2	14.7	12.5	--	--
8	16.4	16.2	15.4	15.0	14.4	14.0	--
9	16.2	15.7	15.7	15.0	14.4	14.4	12.8
10	17.7	16.4	16.0	14.6	15.0	15.0	14.0
Total	161.9	157.4	138.1	131.9	128.2	98.8	67.8
Average	16.2	15.7	15.3	14.7	14.2	14.1	13.6

Table 2. Hemoglobin content values (g/100 ml blood) of Tween 20-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	15.5	--	--	--	--	--	--
2	16.6	15.6	14.2	14.0	--	--	--
3	17.0	--	--	--	--	--	--
4	15.0	15.0	13.3	14.2	14.2	--	--
5	16.2	15.4	15.0	14.4	14.5	15.2	14.0
6	15.2	15.0	13.6	14.0	13.9	14.7	--
7	16.7	16.0	14.4	13.8	13.0	--	--
8	16.4	15.0	14.0	13.8	14.4	--	--
9	17.4	16.0	14.4	14.8	15.0	11.2	--
10	17.4	15.4	14.7	14.0	14.0	15.7	13.0
Total	163.4	123.4	113.6	113.0	99.0	56.8	27.0
Average	16.3	15.4	14.2	14.1	14.1	14.2	13.5

Table 3. Hemoglobin content values (g/100 ml blood) of Tween 20-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	18.0	16.2	15.7	12.8	--	--	--
2	15.6	15.2	15.0	13.3	14.2	11.0	14.0
3	16.0	16.0	15.0	14.4	14.2	10.2	--
4	16.0	16.0	14.4	14.0	13.0	12.2	9.0
5	15.6	16.2	14.0	13.0	13.3	12.8	12.8
6	18.0	16.4	15.0	13.6	14.2	12.5	11.4
7	17.0	15.7	14.0	14.0	13.8	--	--
8	18.2	15.7	14.2	14.0	--	--	--
9	17.0	16.0	15.7	15.2	14.7	12.2	14.4
10	17.4	15.7	14.7	14.2	14.2	13.3	13.0
Total	168.8	159.1	147.7	138.5	111.6	84.2	74.6
Average	16.9	15.9	14.8	13.9	14.0	12.0	12.4

Table 4. Hemoglobin content values (g/100 ml blood) of Tween 60-5% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	16.0	16.2	15.4	14.0	14.4	13.6	--
2	16.4	15.4	14.4	12.8	13.6	13.6	--
3	16.0	15.7	14.0	13.0	12.2	13.6	13.8
4	16.0	16.0	14.2	13.6	13.6	14.0	--
5	17.0	16.2	15.7	14.4	14.0	12.6	12.2
6	16.2	16.0	15.0	15.0	14.4	13.0	--
7	17.2	16.4	15.2	14.2	14.2	13.8	--
8	16.0	16.0	15.0	15.0	14.2	10.2	--
9	15.2	15.0	14.4	14.0	13.3	12.0	12.8
10	16.4	15.3	15.0	14.4	13.0	13.6	14.2
Total	162.4	158.2	148.3	140.4	136.9	130.0	53.0
Average	16.2	15.8	14.8	14.0	13.7	13.0	13.3

Table 5. Hemoglobin content values (g/100 ml blood) of Tween 60-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	16.4	15.0	13.6	12.8	12.8	12.2	--
2	17.0	13.6	13.0	11.4	12.2	12.0	11.8
3	16.0	14.2	13.6	11.4	11.8	11.8	--
4	16.4	14.0	13.0	10.2	12.8	12.0	11.4
5	18.0	14.4	14.0	12.2	13.0	13.0	--
6	18.6	15.4	13.3	11.8	12.8	13.0	12.8
7	16.0	14.4	12.9	12.2	12.8	11.6	11.0
8	16.0	14.0	13.3	10.6	11.8	11.8	--
9	15.4	13.2	13.0	11.0	12.4	12.8	12.8
10	15.5	15.0	13.0	12.5	13.3	13.0	12.2
Total	165.3	143.2	132.7	116.1	125.7	123.2	72.0
Average	16.5	14.3	13.3	11.6	12.6	12.3	12.0

Figure 2. Average hemoglobin values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 20-5 percent, and Tween 20-10 percent

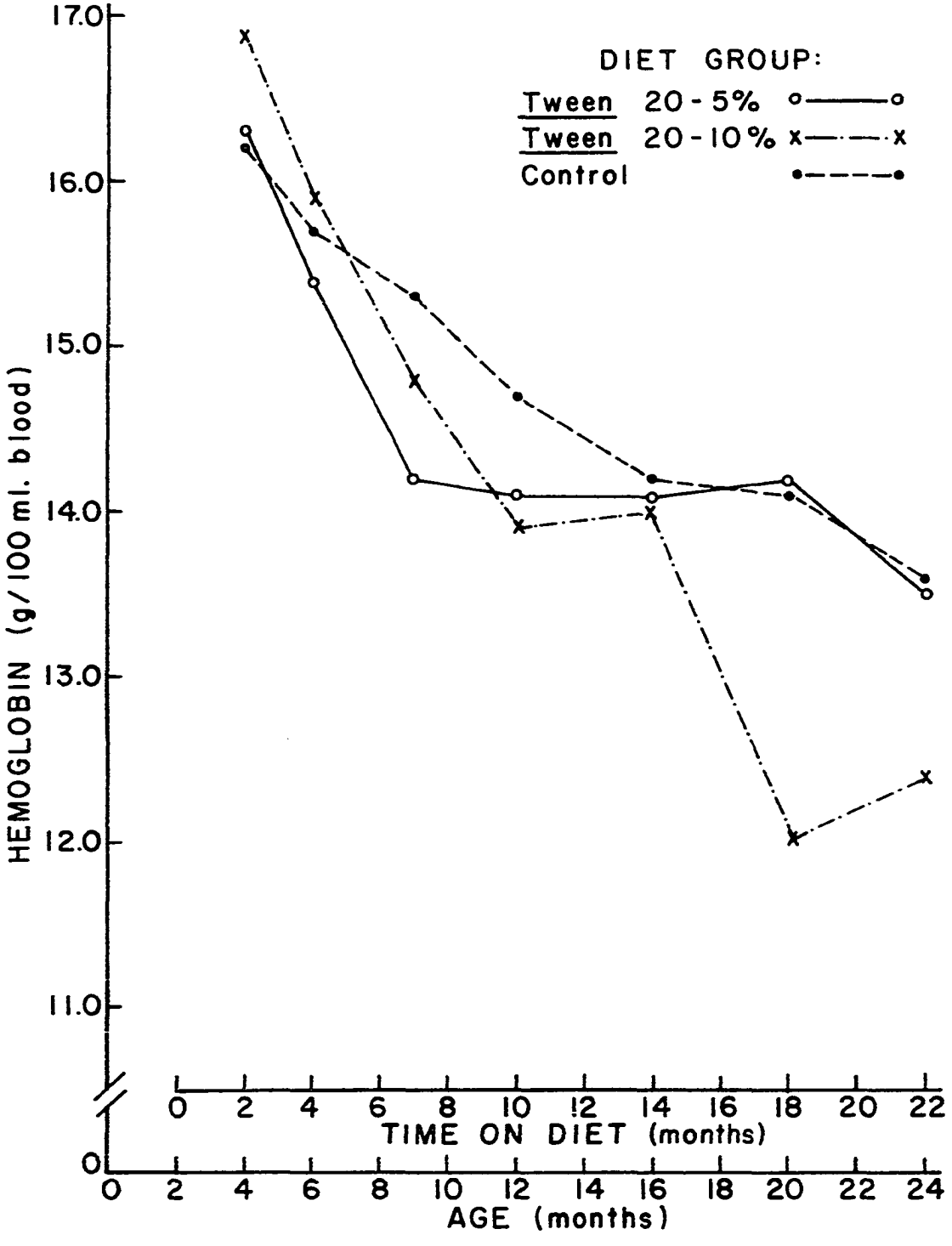
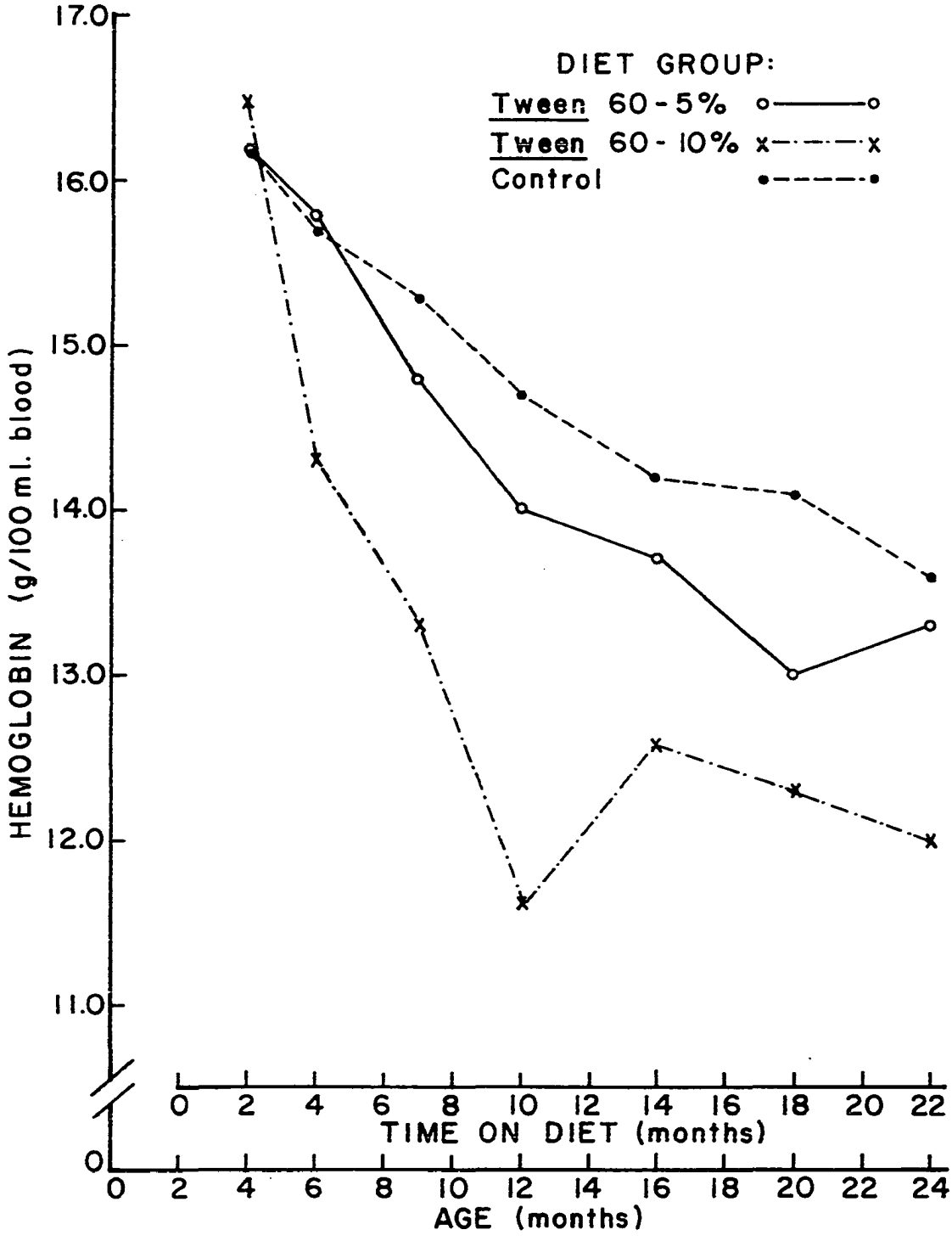

DIET GROUP:
Tween 20 - 5%
Tween 20 - 10%
Control
HEMOGLOBIN (g/100 ml. blood)
17.0
16.0
15.0
14.0
13.0
12.0
11.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Figure 3. Average hemoglobin values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 60-5 percent, and Tween 60-10 percent


DIET GROUP:
Tween 60 - 5%
Tween 60 - 10%
Control
HEMOGLOBIN (g/100 ml. blood)
17.0
16.0
15.0
14.0
13.0
12.0
11.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

decline in hemoglobin content throughout the course of the investigation (Table 1). Controls at two months on diet had an average value for 10 mice of 16.2 g/100 ml blood; at 22 months time on diet the five surviving animals had an average hemoglobin value of 13.6 g/100 ml blood.

Hemoglobin content determinations of mice on the Tween 20-5 percent diet are summarized in Table 2, which also includes the arithmetic means. The range of hemoglobin content was 16.3 g/100 ml blood for 10 mice at two months on diet to 13.5 g/100 ml blood for two mice at 22 months on diet.

Tween 20-10 percent diet group hemoglobin content results are summarized in Table 3, including arithmetic means. Values ranged from 16.9 g/100 ml blood for 10 mice at two months on diet to 12.0 g/100 ml blood for seven mice at 18 months on diet. At 22 months on diet, the six survivors had a slight increase to 12.4 g/100 ml blood.

Table 4 is a summary of data for hemoglobin content of mice on Tween 60-5 percent diet. Hemoglobin content decreased from the first examination period (16.2 g/100 ml blood for 10 mice) to the sixth examination period (13.0 g/100 ml blood for 10 mice) at 18 months on diet. However, at 22 months on diet, the four surviving mice showed a slight increase in hemoglobin content to 13.3 g/100 ml blood.

Results of Tween 60-10 percent diet group are recorded in Table 5, which includes the mathematical means. Hemoglobin content values ranged from 16.5 g/100 ml blood for 10 mice at two months on diet to 11.6 g/100 ml blood for 10 mice at 10 months on diet. Ten mice

exhibited a slight increase (to 12.6 g/100 ml blood) at 14 months on diet. The next two examination periods showed a slight decrease in hemoglobin content.

Linear and quadratic regression formulae, and significance levels, are included in Table 6. Controls were significant ($P<.01$) for linear

Table 6. Linear and quadratic regression formulae, and significance levels, for hemoglobin content (Y) on time on diet (X') of male $C_{57}BL/6$ Jax mice on different diets

Diet	Regression formula	Significance
Control	$Y = 16.26 - (0.1307)X'$	$P<.01$
Tween 20-5%	$Y = 16.87 - (0.3999)X' + (0.0218)(X')^2$	$P<.01$
Tween 20-10%	$Y = 17.61 - (0.4503)X' + (0.0095)(X')^2$	$P<.01$
Tween 60-5%	$Y = 17.06 - (0.3820)X' + (0.0092)(X')^2$	$P<.01$
Tween 60-10%	$Y = 17.28 - (0.7164)X' + (0.0231)(X')^2$	$P<.01$

regression of hemoglobin content on time on diet factor. Tween 20-5 percent, Tween 20-10 percent, Tween 60-5 percent, and Tween 60-10 percent diet groups exhibited quadratic regression of hemoglobin content on time on diet factor significant at less than the one percent level ($P<.01$). In Table 6, Y represents the dependent variable (hemoglobin content) and X' indicates the independent variable (time on diet factor).

Hemoglobin content regression means for seven and 14 months time on diet were calculated from the particular regression formula (Table 6) for each of the five diets. Significant differences between the calculated

regression hemoglobin means for each diet were tested at the five percent, or less, level of probability ($P<.05$) using The New Multiple Range Test (Duncan, 1955). Table 7 includes the calculated regression means for each group at seven and 14 months on diet, and the results of the

Table 7. Results of statistical analysis of calculated hemoglobin content regression means (g/100 ml blood) for different diet groups at seven and 14 months on diet

Diet	Tween 60-10%	Tween 20-5%	Tween 60-5%	Tween 20-10%	Control
Calculated regression mean at seven months on diet	13.40	14.70	14.84	14.92	15.34

Diet	Tween 60-10%	Tween 20-10%	Tween 60-5%	Tween 20-5%	Control
Calculated regression mean at 14 months on diet	11.78	13.17	13.51	13.79	14.43

statistical analysis. In Table 7, those regression means not underscored by a common line are statistically different ($P<.05$). For example, control hemoglobin content regression mean at seven months on diet is significantly different from each of the other four diet group hemoglobin regression means; but, Tween 20-5 percent diet group hemoglobin content regression mean at seven months on diet is not different from either Tween 20-10 percent or Tween 60-5 percent regression mean. However,

Tween 20-5 percent, Tween 20-10 percent, and Tween 60-5 percent diet group regression means are each different from Tween 60-10 percent diet group regression mean at the seven months on diet period.

Hematocrit

Tables 8, 9, 10, 11, and 12 contain the results of hematocrit determinations of individual mice on the five diets included in the present study. Mathematical means are shown graphically in Figures 4 and 5 according to age and time on diet. Results of linear and quadratic regression formula calculations are shown in Table 13. Statistical evaluations for significant ($P<.05$) differences between calculated regression means of each diet group at seven and 14 months time on diet are recorded in Table 14.

Hematocrit values of controls declined from 48.1 percent for 10 mice at two months on diet to 42.2 percent for seven mice at 18 months on diet (Table 8). At 22 months on diet, the arithmetic mean for five surviving mice increased slightly to 42.4 percent.

Tween 20-5 percent diet group mice exhibited a decrease in hematocrit value throughout the course of investigation (Table 9). Hematocrit values ranged from 48.2 percent for 10 mice at two months on diet to 40.0 percent for two survivors at 22 months on diet.

Mice on Tween 20-10 percent diet exhibited a decline of hematocrit value over the period of study. The range was from 50.0 percent for 10 mice at two months on diet to 37.8 percent for six mice at 22 months on diet (Table 10).

Table 8. Hematocrit values (ml packed erythrocytes/100 ml blood=%) of control diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	48.0	47.0	45.0	44.5	43.5	41.0	--
2	44.5	46.0	48.0	45.0	45.0	--	--
3	48.0	48.5	45.0	44.0	44.5	42.5	42.0
4	48.0	45.5	--	--	--	--	--
5	46.0	43.0	42.5	43.5	43.5	41.0	40.0
6	50.0	49.0	45.0	44.0	43.0	41.5	45.5
7	47.5	49.0	45.0	44.0	39.0	--	--
8	50.0	48.0	46.0	45.0	43.0	42.0	--
9	48.0	47.0	47.0	45.5	44.0	43.0	40.0
10	51.0	49.0	48.0	43.0	45.0	44.5	44.5
Total	481.0	472.0	411.5	398.5	390.5	295.5	212.0
Average	48.1	47.2	45.7	44.3	43.4	42.2	42.4

Table 9. Hematocrit values (ml packed erythrocytes/100 ml blood=%) of Tween 20-5% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	45.0	--	--	--	--	--	--
2	49.0	47.0	43.0	41.5	--	--	--
3	49.0	--	--	--	--	--	--
4	44.0	45.5	42.5	43.0	42.0	--	--
5	48.0	47.0	46.5	44.0	43.0	44.5	42.0
6	46.0	45.0	42.5	42.0	41.5	42.5	--
7	50.0	49.0	44.5	42.0	38.0	--	--
8	49.0	46.5	44.0	41.0	42.0	--	--
9	50.0	49.0	44.5	45.0	45.0	33.0	--
10	52.0	46.0	45.0	42.0	43.0	47.0	38.0
Total	482.0	375.0	352.5	340.5	294.5	167.0	80.0
Average	48.2	46.9	44.1	42.6	42.1	41.8	40.0

Table 10. Hematocrit values (ml packed erythrocytes/100 ml blood=%) of Tween 20-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	52.0	48.5	47.0	39.0	--	--	--
2	47.0	47.5	44.0	41.5	42.0	39.0	43.0
3	50.0	48.0	46.0	43.0	43.0	35.5	--
4	47.5	48.0	44.0	43.0	42.0	39.5	26.0
5	46.0	48.0	43.5	40.0	40.0	39.5	39.0
6	52.0	48.0	44.5	41.0	42.0	40.0	36.0
7	50.0	46.0	45.0	43.0	41.0	--	--
8	53.0	47.5	43.0	44.0	--	--	--
9	51.0	48.0	46.0	46.0	43.0	39.5	43.0
10	51.5	48.0	44.0	42.0	43.0	42.0	40.0
Total	500.0	477.5	447.0	422.5	336.0	275.0	227.0
Average	50.0	47.8	44.7	42.3	42.0	39.3	37.8

Table 11. Hematocrit values (ml packed erythrocytes/100 ml blood=%) of Tween 60-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	48.0	49.0	48.0	43.0	45.0	42.0	--
2	50.0	46.5	44.0	41.0	40.0	39.5	--
3	50.0	47.0	42.0	41.0	39.0	41.5	40.5
4	49.0	48.0	43.0	42.0	41.0	42.0	--
5	50.0	48.0	46.5	44.0	42.0	40.0	38.0
6	52.0	49.0	46.0	45.0	43.0	41.0	--
7	51.0	49.0	46.5	43.5	43.0	42.0	--
8	49.0	48.0	46.0	45.0	42.0	34.0	--
9	48.0	45.0	44.0	44.0	40.0	39.0	39.5
10	50.0	46.0	45.5	43.0	40.0	41.0	43.0
Total	497.0	475.5	451.5	431.5	415.0	402.0	161.0
Average	49.7	47.6	45.2	43.2	41.5	40.2	40.3

Table 12. Hematocrit values (ml packed erythrocytes/100 ml blood=%) of Tween 60-10% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	49.0	47.0	44.0	41.5	41.0	40.0	--
2	50.0	46.0	43.0	40.0	40.0	38.0	39.0
3	47.0	44.0	43.5	40.0	40.0	38.0	--
4	49.0	45.0	43.0	38.0	41.0	40.0	39.0
5	53.0	45.0	43.0	40.5	42.0	40.0	--
6	56.0	48.0	43.5	42.0	42.0	41.0	40.0
7	50.0	47.5	43.5	41.0	42.0	36.0	37.0
8	49.0	44.0	43.5	38.0	40.0	39.0	--
9	47.0	43.0	42.5	40.0	41.0	40.0	40.5
10	48.0	46.5	44.0	40.0	44.0	41.0	41.0
Total	498.0	455.5	433.5	401.0	413.0	393.0	236.5
Average	49.8	45.6	43.4	40.1	41.3	39.3	39.4

Figure 4. Average hematocrit values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 20-5 percent, and Tween 20-10 percent

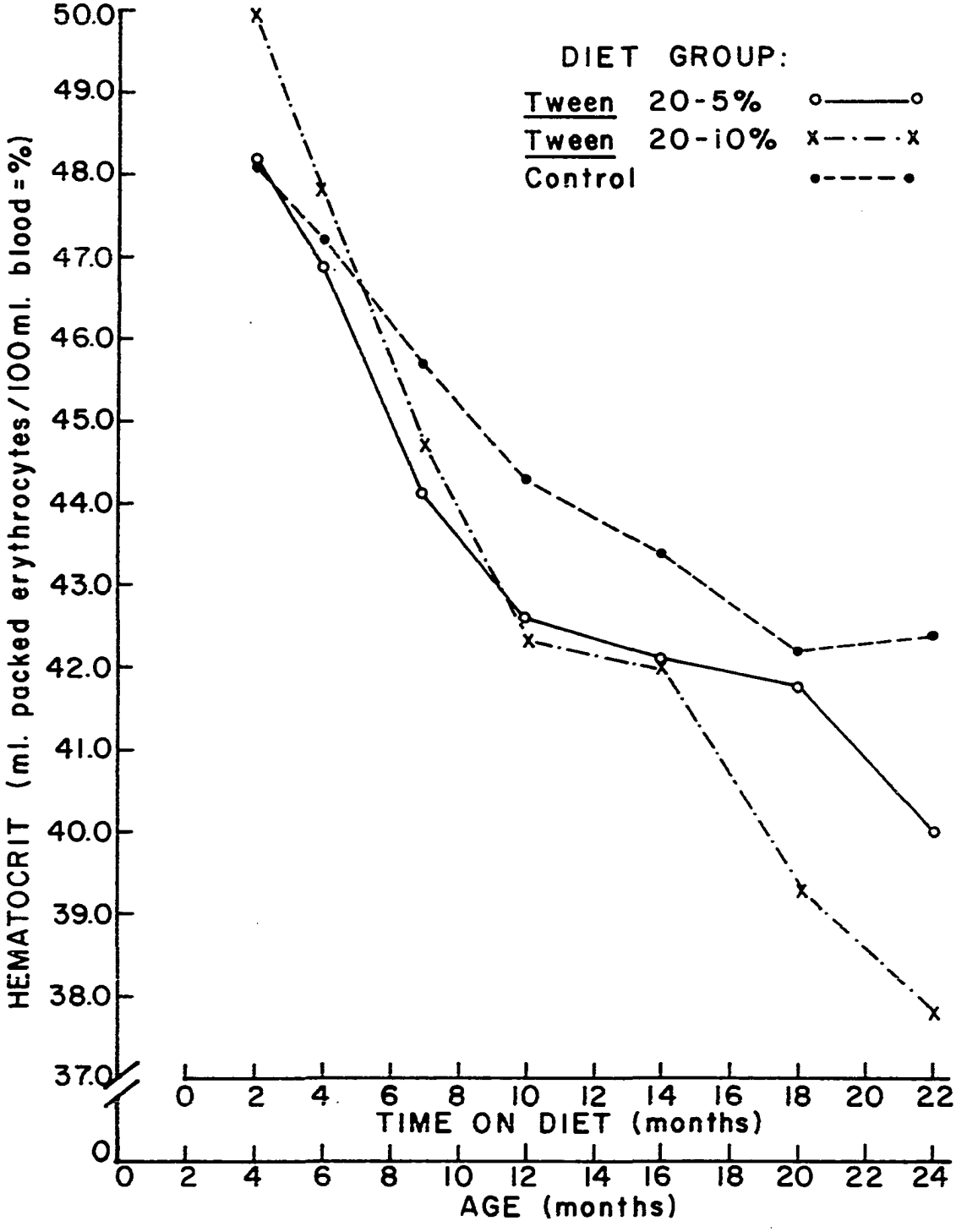
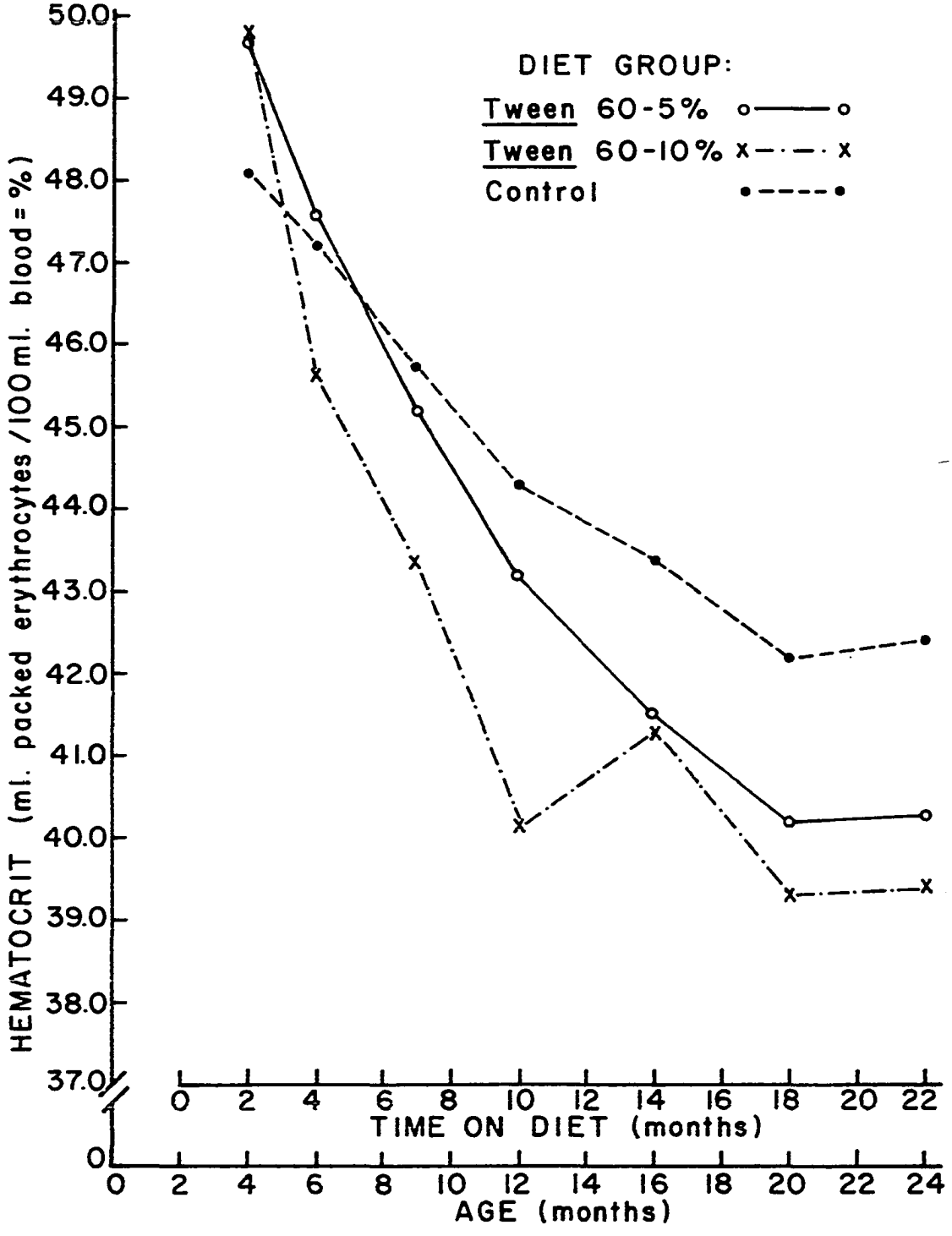

DIET GROUP:
Tween 20-5%
Tween 20-10%
Control
HEMATOCRIT (ml. packed erythrocytes/100ml. blood = %)
50.0
49.0
48.0
47.0
46.0
45.0
44.0
43.0
42.0
41.0
40.0
39.0
38.0
37.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Figure 5. Average hematocrit values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 60-5 percent, and Tween 60-10 percent


DIET GROUP:
Tween 60-5%
Tween 60-10%
Control
HEMATOCRIT (ml. packed erythrocytes /100ml. blood = %)
50.0
49.0
48.0
47.0
46.0
45.0
44.0
43.0
42.0
41.0
40.0
39.0
38.0
37.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Hematocrit values for mice on Tween 60-5 percent showed a decline from two months on diet until 18 months on diet with a range from 49.7 percent for 10 mice at two months to 40.2 percent for 10 mice at 18 months. At 22 months on diet, four surviving mice had an increase of 0.1 percent over the 18 month on diet results (Table 11).

Mice on Tween 60-10 percent diet ranged in arithmetic mean value from 49.8 percent for 10 mice at two months on diet to 39.3 percent for 10 mice at 18 months on diet. A rapid decline (4.2 percent in mean values) was noted between two and four months examination periods. In the 10 months to 22 months on diet interim, arithmetic mean hematocrit values showed a maximal difference of 2.0 percent (Table 12).

Table 13 includes quadratic regression formulae and significance levels. Control, Tween 20-5 percent, and Tween 20-10 percent diet groups exhibited quadratic regression of hematocrit on time on diet significant at less than the five percent level ($P<.05$). Tween 60-5 percent and Tween 60-10 percent diet group hematocrit values were significant at less than the one percent level ($P<.01$) for quadratic regression of hematocrit on time on diet. In Table 13, Y indicates the dependent variable (hematocrit), and X' represents the independent variable (time on diet).

Regression means for hematocrit values for each diet group at seven and 14 months on diet were calculated from the particular quadratic regression formula (Table 13). The New Multiple Range Test (Duncan, 1955) was used to test for significant ($P<.05$) differences between the calculated regression means of each of the five diet groups at seven and 14 months on diet. Table 14 includes the results of the calculated

Table 13. Linear and quadratic regression formulae, and significance levels, for hematocrit (Y) on time on diet (X') of male $C_{57}BL/6$ Jax mice on different diets

Diet	Regression formula	Significance
Control	$Y = 49.45 - (0.6482)X' + (0.0146)(X')^2$	$P<.05$
Tween 20-5%	$Y = 49.93 - (0.9426)X' + (0.0248)(X')^2$	$P<.05$
Tween 20-10%	$Y = 51.77 - (1.1006)X' + (0.0222)(X')^2$	$P<.05$
Tween 60-5%	$Y = 51.75 - (1.1323)X' + (0.0276)(X')^2$	$P<.01$
Tween 60-10%	$Y = 51.51 - (1.4301)X' + (0.0415)(X')^2$	$P<.01$

Table 14. Results of statistical analysis of calculated hematocrit regression means (%) for different diet groups at seven and 14 months on diet

Diet	Tween 60-10%	Tween 20-5%	Tween 60-5%	Tween 20-10%	Control
Calculated regression mean at seven months on diet	43.53	44.55	45.16	45.18	45.63

Diet	Tween 60-10%	Tween 20-10%	Tween 60-5%	Tween 20-5%	Control
Calculated regression mean at 14 months on diet	39.62	40.72	41.31	41.60	42.23

hematocrit regression means for each of the diet groups. Those regression means not underscored by a common line are statistically different.

Erythrocyte Count

Erythrocyte counts of individual mice on the five diets employed in the present investigation are recorded in Tables 15, 16, 17, 18, and 19. Figures 6 and 7 illustrate graphically the arithmetic means of erythrocyte count values versus age and time on diet for each of the five diets. Linear and quadratic regression formulae, and levels of significance, for each diet group are included in Table 20. Significant ($P<.05$) differences between calculated regression means for each diet group at seven and 14 months on diet are shown in Table 21.

Erythrocyte count mean values for control mice exhibited a decrease in value throughout the study (Table 15). Erythrocyte count values ranged from 9.97 million corpuscles/mm^3 blood for 10 mice at two months on diet to 9.08 million corpuscles/mm^3 blood for five survivors at 22 months on diet.

Mice on Tween 20-5 percent diet exhibited a range of from 9.88 million corpuscles/mm^3 blood for 10 mice at two months on diet to 8.70 million corpuscles/mm^3 blood for two mice at 22 months on diet (Table 16). A gradual decline in erythrocyte count average was shown through the first five examination periods, with a slight increase at 18 months on diet examination.

Tween 20-10 percent diet group mean values for erythrocyte count showed a decline in value during the course of investigation (Table 17).

Table 15. Erythrocyte counts (million corpuscles/mm^3 blood) of control diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	9.44	9.61	9.71	9.57	9.43	8.78	--
2	9.08	9.93	9.86	9.67	9.61	--	--
3	9.88	9.98	9.31	9.13	9.37	9.18	9.25
4	9.32	9.21	--	--	--	--	--
5	9.81	8.91	9.21	9.22	9.40	9.23	9.12
6	10.30	9.83	9.63	9.39	9.29	9.31	9.47
7	9.89	9.76	9.46	9.56	8.94	--	--
8	9.84	9.51	9.71	9.73	9.38	9.21	--
9	10.20	10.02	9.75	9.69	9.57	9.43	8.19
10	11.96	10.75	9.85	9.70	9.70	9.61	9.37
Total	99.72	97.51	86.49	85.66	84.69	64.75	45.40
Average	9.97	9.75	9.61	9.52	9.41	9.25	9.08

Table 16. Erythrocyte counts (million corpuscles/mm^3 blood) of Tween 20-5% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months) 4	6	9	12	16	20	24
	Time on diet at date of examination (months) 2	4	7	10	14	18	22
1	9.36	--	--	--	--	--	--
2	9.18	9.79	9.21	9.34	--	--	--
3	9.82	--	--	--	--	--	--
4	8.88	9.47	9.16	9.29	9.44	--	--
5	10.60	9.70	10.03	9.53	9.38	9.56	9.09
6	9.70	9.21	8.96	9.29	9.03	9.32	--
7	10.76	9.90	9.57	9.14	8.81	--	--
8	9.28	8.98	9.29	9.31	9.02	--	--
9	10.56	9.93	9.38	9.56	9.34	8.23	--
10	10.66	9.84	9.34	9.15	9.37	9.76	8.31
Total	988.0	76.82	74.94	74.61	64.39	36.87	17.40
Average	9.88	9.60	9.37	9.33	9.20	9.22	8.70

Table 17. Erythrocyte counts (million corpuscles/mm^3 blood) of Tween 20-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	10.61	10.02	9.83	9.17	--	--	--
2	8.36	9.60	9.43	9.35	9.34	9.01	9.38
3	10.02	9.52	9.56	9.59	9.26	7.97	--
4	10.41	9.68	9.49	9.38	8.93	8.56	7.29
5	8.93	9.58	8.92	8.49	8.79	8.69	8.69
6	11.92	10.28	9.39	9.21	9.17	9.04	8.37
7	9.50	9.40	9.61	9.72	8.85	--	--
8	10.63	10.20	9.39	9.45	--	--	--
9	10.06	9.82	9.58	9.44	9.51	9.29	9.32
10	9.98	9.78	9.41	9.28	9.39	9.42	9.14
Total	100.42	97.88	94.61	93.08	73.24	61.98	52.19
Average	10.04	9.79	9.46	9.31	9.16	8.85	8.70

Table 18. Erythrocyte counts (million corpuscles/mm^3 blood) of Tween 60-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	10.54	9.94	10.07	9.54	9.58	9.20	---
2	11.74	8.93	9.35	9.09	8.59	8.56	---
3	9.98	9.61	8.97	9.23	8.37	9.08	9.18
4	10.40	9.96	9.13	9.30	9.07	9.37	---
5	11.20	9.92	9.76	9.52	9.29	8.42	8.23
6	9.95	10.23	9.29	9.41	9.41	8.79	---
7	11.57	9.76	9.69	9.37	9.26	9.28	---
8	10.21	9.88	9.52	9.46	9.35	8.04	---
9	9.73	9.80	9.41	9.29	8.61	8.87	9.01
10	9.98	9.43	9.28	9.12	8.93	9.03	9.39
Total	105.30	97.46	94.47	93.33	90.46	88.64	35.81
Average	10.53	9.75	9.45	9.33	9.05	8.86	8.95

Table 19. Erythrocyte counts (million corpuscles/mm^3 blood) of Tween 60-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	10.22	9.28	9.39	9.15	8.91	8.60	--
2	10.21	9.82	9.27	8.59	8.58	8.12	8.21
3	9.76	9.18	9.31	8.81	8.87	8.79	--
4	10.19	9.12	9.43	7.93	9.03	8.32	8.16
5	11.02	10.03	9.48	8.68	9.29	9.12	--
6	12.01	9.97	9.37	8.75	9.09	9.02	9.23
7	10.03	9.46	9.67	8.97	8.78	8.31	8.08
8	10.21	9.53	9.16	8.89	8.42	8.09	--
9	9.93	9.14	9.38	8.63	9.23	8.83	8.39
10	9.87	9.89	9.53	9.03	9.51	9.21	8.67
Total	103.45	95.42	93.99	87.43	89.71	86.41	50.74
Average	10.35	9.54	9.40	8.74	8.97	8.64	8.46

Figure 6. Average erythrocyte count values of male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet: control, Tween 20-5 percent, and Tween 20-10 percent

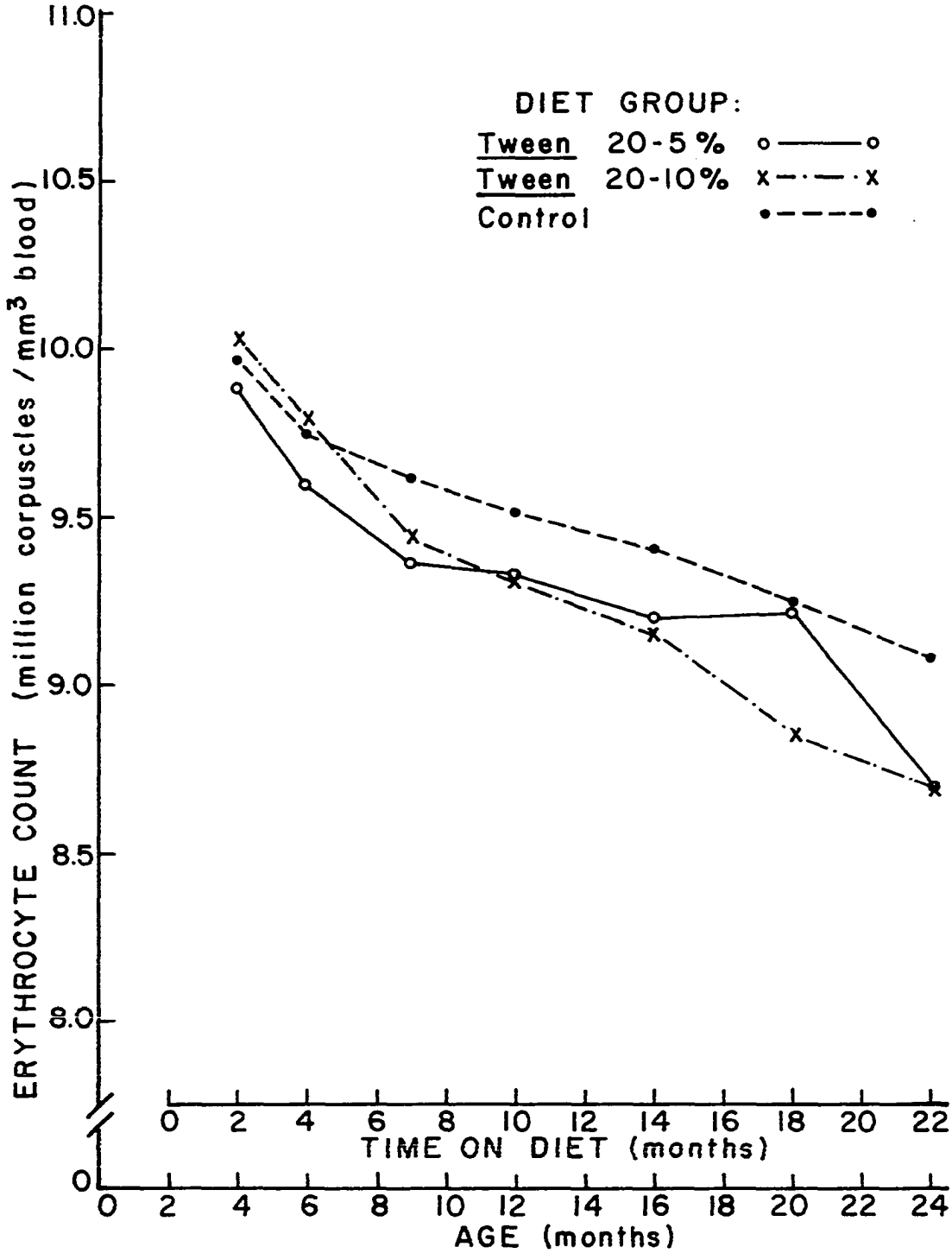

DIET GROUP:
Tween 20-5%
Tween 20-10%
Control
ERYTHROCYTE COUNT (million corpuscles / mm³ blood)
11.0
10.5
10.0
9.5
9.0
8.5
8.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Figure 7. Average erythrocyte count values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 60-5 percent, and Tween 60-10 percent

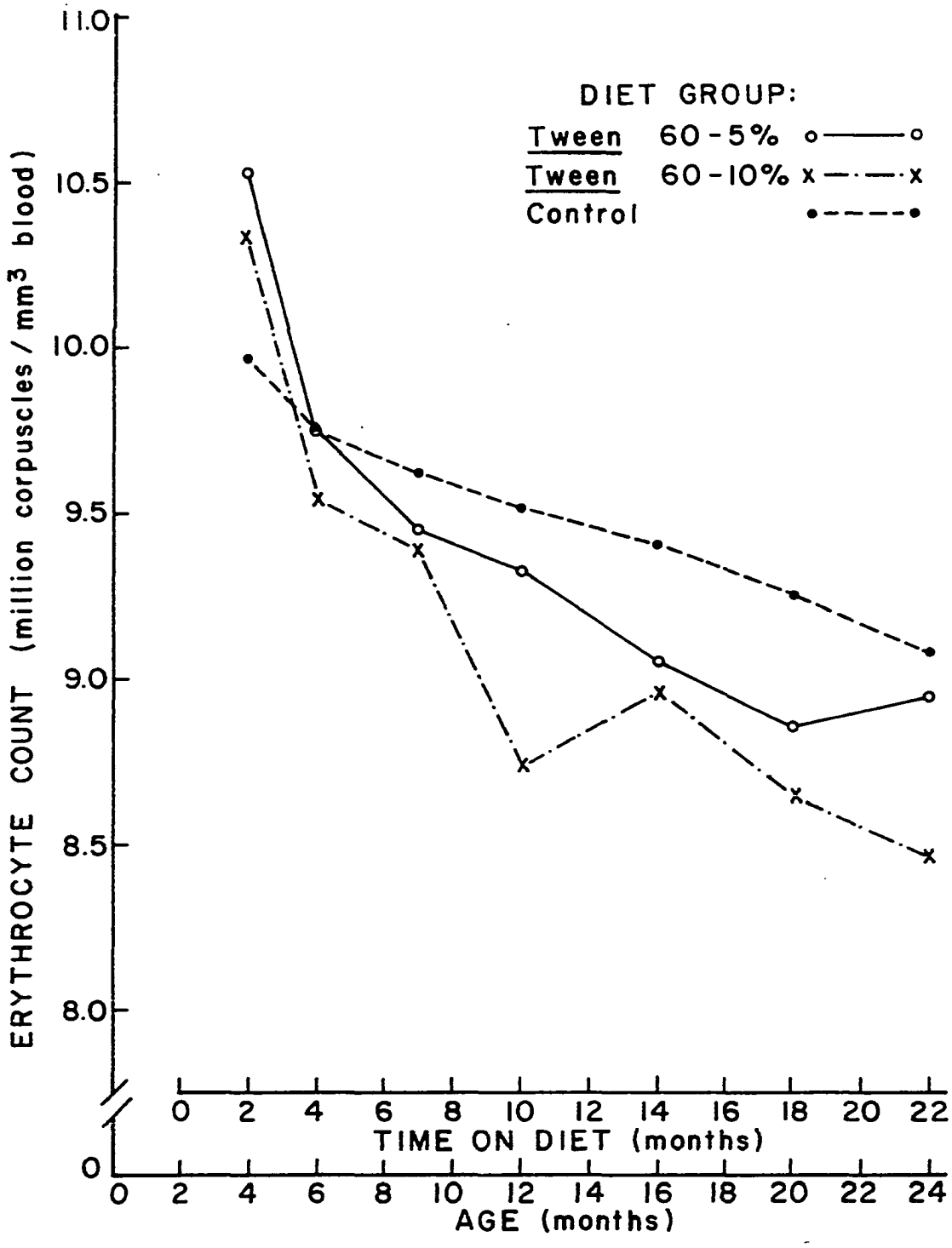

DIET GROUP:
Tween 60-5%
Tween 60-10%
Control
ERYTHROCYTE COUNT (million corpuscles / mm3 blood)
11.0
10.5
10.0
9.5
9.0
8.5
8.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Table 20. Linear and quadratic regression formulae, and significance levels, for erythrocyte count (Y) on time on diet (X') of male $C_{57}BL/6$ Jax mice on different diets

Diet	Regression formula	Significance
Control	$Y = 9.96 - (0.0407)X'$	$P<.01$
Tween 20-5%	$Y = 9.84 - (0.0467)X'$	$P<.01$
Tween 20-10%	$Y = 10.04 - (0.0654)X'$	$P<.01$
Tween 60-5%	$Y = 10.72 - (0.2091)X' + (0.0060)(X')^2$	$P<.01$
Tween 60-10%	$Y = 10.52 - (0.2056)X' + (0.0054)(X')^2$	$P<.01$

Table 21. Results of statistical analysis of calculated erythrocyte count regression means (million corpuscles/mm^3 blood) for different diet groups at seven and 14 months on diet

Diet	Tween 60-10%	Tween 20-5%	Tween 60-5%	Tween 20-10%	Control
Calculated regression mean at seven months on diet	9.35	9.52	9.55	9.59	9.67

Diet	Tween 60-10%	Tween 60-5%	Tween 20-10%	Tween 20-5%	Control
Calculated regression mean at 14 months on diet	8.70	8.96	9.13	9.19	9.39

Mean values ranged from 10.04 million corpuscles/mm^3 blood for 10 mice at two months on diet to 8.70 million corpuscles/mm^3 blood for six surviving mice at 22 months on diet.

Erythrocyte mean values of mice on Tween 60-6 percent diet ranged from 10.53 million corpuscles/mm^3 for 10 mice at two months on diet to 8.86 million corpuscles/mm^3 blood for 10 mice at 18 months on diet (Table 18). Four surviving mice, at 22 months on diet, exhibited an increase of 0.09 million corpuscles/mm^3 blood over the 18 months on diet average.

Erythrocyte count means for Tween 60-10 percent diet group ranged from 10.35 million corpuscles/mm^3 blood for 10 mice at two months on diet to 8.46 million corpuscles/mm^3 blood for six survivors at 22 months on diet (Table 19). A decrease in mean erythrocyte count was shown between two and 10 months time on diet, with an increase at the 14 months on diet examination, and then a decrease at both 18 and 22 months on diet examinations.

Significance levels of linear and quadratic regression formulae for erythrocyte count on time on diet for each of the five diets are included in Table 20. Control, Tween 20-5 percent, and Tween 20-10 percent diet groups exhibited a linear regression of erythrocyte count on time on diet significant at less than the one percent level ($P<.01$). Tween 60-5 percent and Tween 60-10 percent diet groups showed a quadratic regression of erythrocyte count on time on diet significant at less than the one percent level ($P<.01$). The dependent variable (erythrocyte count) is represented by Y, and the independent variable (time on diet) is indicated by X' in Table 20.

Erythrocyte count regression means were calculated for each diet from the particular regression formula (Table 20), and are recorded in Table 21. Significant ($P<.05$) differences between erythrocyte count regression means for each diet, at both seven and 14 months, are indicated in Table 21. Those means not underscored by a common line are statistically different.

Mean Corpuscular Volume

Results of mean corpuscular volume calculations for individual mice on each of five diets are recorded in Tables 22, 23, 24, 25, and 26. Arithmetic means of the results for each examination period are included. Figures 8 and 9 are graphic representations of mean corpuscular volume averages for each diet group versus age and time on diet. Linear and quadratic regression formulae, and significance levels, for mean corpuscular volume on time on diet are shown in Table 27. Statistical evaluations for significant ($P<.05$) differences between diet group calculated regression means, at seven and 14 months on diet, are included in Table 28.

Controls exhibited a decrease in mean corpuscular volume throughout the investigation from four months on diet until 22 months on diet examination in which an increase was observed (Table 22). A very slight increase (0.1 μ^3) was observed between the two month and four month examination periods. Mean corpuscular volume values ranged from 48.5 μ^3 for 10 mice at four months on diet to 45.6 μ^3 for seven mice at 18 months on diet.

Table 22. Mean corpuscular volume (μ^3) values of control diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	50.8	48.9	46.3	46.5	46.1	46.7	---
2	49.0	46.3	48.7	46.5	46.8	---	---
3	48.6	48.6	48.3	48.2	47.5	46.3	45.4
4	51.5	49.4	---	---	---	---	---
5	46.9	48.3	46.1	47.2	46.3	44.4	43.8
6	48.5	49.8	46.7	46.9	46.3	44.6	48.0
7	48.0	50.2	47.6	46.0	43.6	---	---
8	50.8	50.5	47.4	46.2	45.8	45.6	---
9	47.1	46.9	48.2	47.0	46.0	45.6	48.9
10	42.6	45.6	48.7	44.3	46.4	46.3	47.5
Total	483.8	484.5	428.0	418.8	414.8	319.5	233.6
Average	48.4	48.5	47.6	46.5	46.1	45.6	46.7

Table 23. Mean corpuscular volume (μ^3) values of Tween 20-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months) 4	6	9	12	16	20	24
	Time on diet at date of examination (months) 2	4	7	10	14	18	22
1	48.1	--	--	--	--	--	--
2	53.4	48.0	46.7	44.4	--	--	--
3	49.9	--	--	--	--	--	--
4	49.6	48.0	46.4	46.3	44.5	--	--
5	45.3	48.5	46.4	46.2	45.8	46.5	46.2
6	47.4	48.9	47.4	45.2	45.9	45.6	--
7	46.5	49.5	46.5	46.0	43.1	--	--
8	52.8	51.8	47.4	44.0	46.6	--	--
9	47.3	49.3	47.4	47.1	48.2	40.1	--
10	48.8	46.7	48.2	45.9	45.9	48.2	45.7
Total	489.1	390.7	376.4	365.1	320.0	180.4	91.9
Average	48.9	48.8	47.1	45.6	45.7	45.1	46.0

Table 24. Mean corpuscular volume (μ^3) values of Tween 20-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months) 4	6	9	12	16	20	24
	Time on diet at date of examination (months) 2	4	7	10	14	18	22
1	49.0	48.4	47.8	42.5	--	--	--
2	56.2	49.5	46.7	44.4	45.0	43.3	45.8
3	49.9	50.4	48.1	44.8	46.4	44.5	--
4	45.6	49.6	46.4	45.8	47.0	46.1	35.7
5	51.5	50.1	48.8	47.1	45.5	45.5	44.9
6	42.6	46.7	47.4	44.5	45.8	44.2	43.1
7	52.6	48.9	46.8	44.2	46.3	--	--
8	49.9	46.6	45.8	46.3	--	--	--
9	50.7	48.9	48.0	48.7	45.2	42.5	46.2
10	51.6	49.1	46.8	45.3	45.8	44.6	43.8
Total	500.6	488.2	472.6	453.6	367.0	310.7	259.5
Average	50.1	48.8	47.3	45.3	45.9	44.4	43.3

Table 25. Mean corpuscular volume (μ^3) values of Tween 60-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	45.5	49.3	47.7	45.1	47.0	45.7	--
2	42.9	52.1	47.1	45.1	46.6	46.1	--
3	50.1	48.8	46.8	44.4	46.6	45.7	44.2
4	47.1	48.2	47.1	45.2	45.2	44.8	--
5	44.6	48.4	47.6	46.2	45.2	47.5	46.2
6	52.3	47.9	49.5	47.8	45.7	46.6	--
7	44.1	50.2	48.0	46.4	46.4	45.3	--
8	48.0	48.6	48.3	47.6	44.9	42.3	--
9	49.3	45.9	46.8	47.4	46.5	44.0	43.8
10	50.1	48.8	49.0	47.2	43.7	45.4	45.8
Total	474.0	488.2	477.9	462.4	457.8	453.4	180.0
Average	47.4	48.8	47.8	46.2	45.7	45.3	45.0

Table 26. Mean corpuscular volume (μ^3) values of Tween 60-10% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	47.9	50.6	46.9	45.4	46.0	46.5	--
2	49.0	46.8	46.4	46.6	46.6	46.8	47.2
3	48.2	47.9	46.7	45.4	45.1	43.2	--
4	48.1	49.3	45.6	47.9	45.4	48.1	47.8
5	48.1	44.9	45.5	46.7	45.2	43.9	--
6	48.2	48.1	46.4	48.0	46.2	45.5	43.4
7	49.9	50.2	45.0	45.7	47.7	43.3	45.8
8	48.0	46.2	47.5	42.7	47.5	48.2	--
9	47.3	47.0	45.3	46.3	44.4	45.3	48.3
10	48.6	47.0	46.2	44.3	46.3	44.5	47.3
Total	483.3	478.0	461.5	359.0	460.4	455.3	279.8
Average	48.3	47.8	46.2	45.9	46.0	45.5	46.6

Figure 8. Average mean corpuscular volume values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 20-5 percent and Tween 20-10 percent

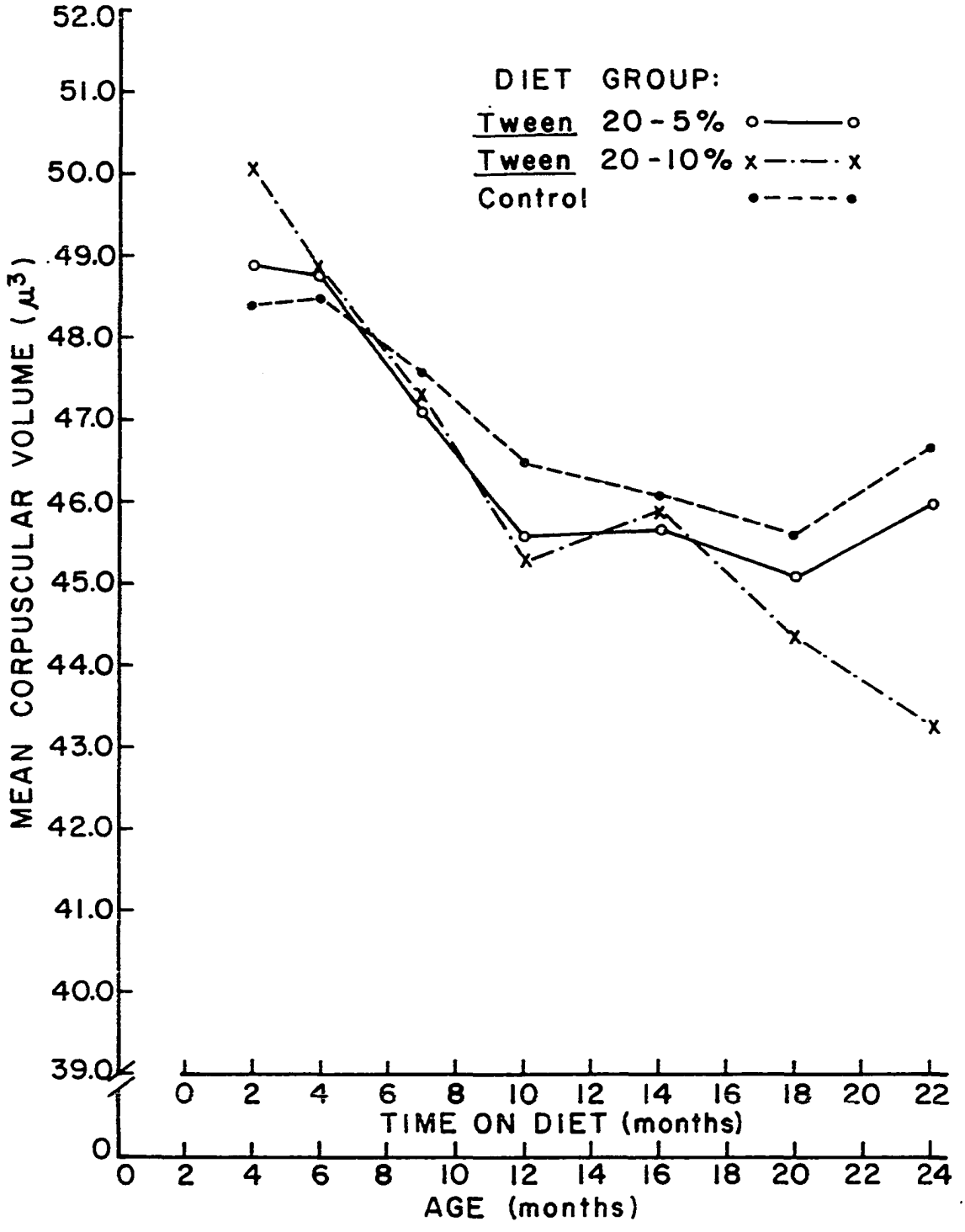

DIET GROUP:
Tween 20-5%
Tween 20-10%
Control
MEAN CORPUSCULAR VOLUME (μ³)
52.0
51.0
50.0
49.0
48.0
47.0
46.0
45.0
44.0
43.0
42.0
41.0
40.0
39.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Figure 9. Average mean corpuscular volume values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 60-5 percent, and Tween 60-10 percent

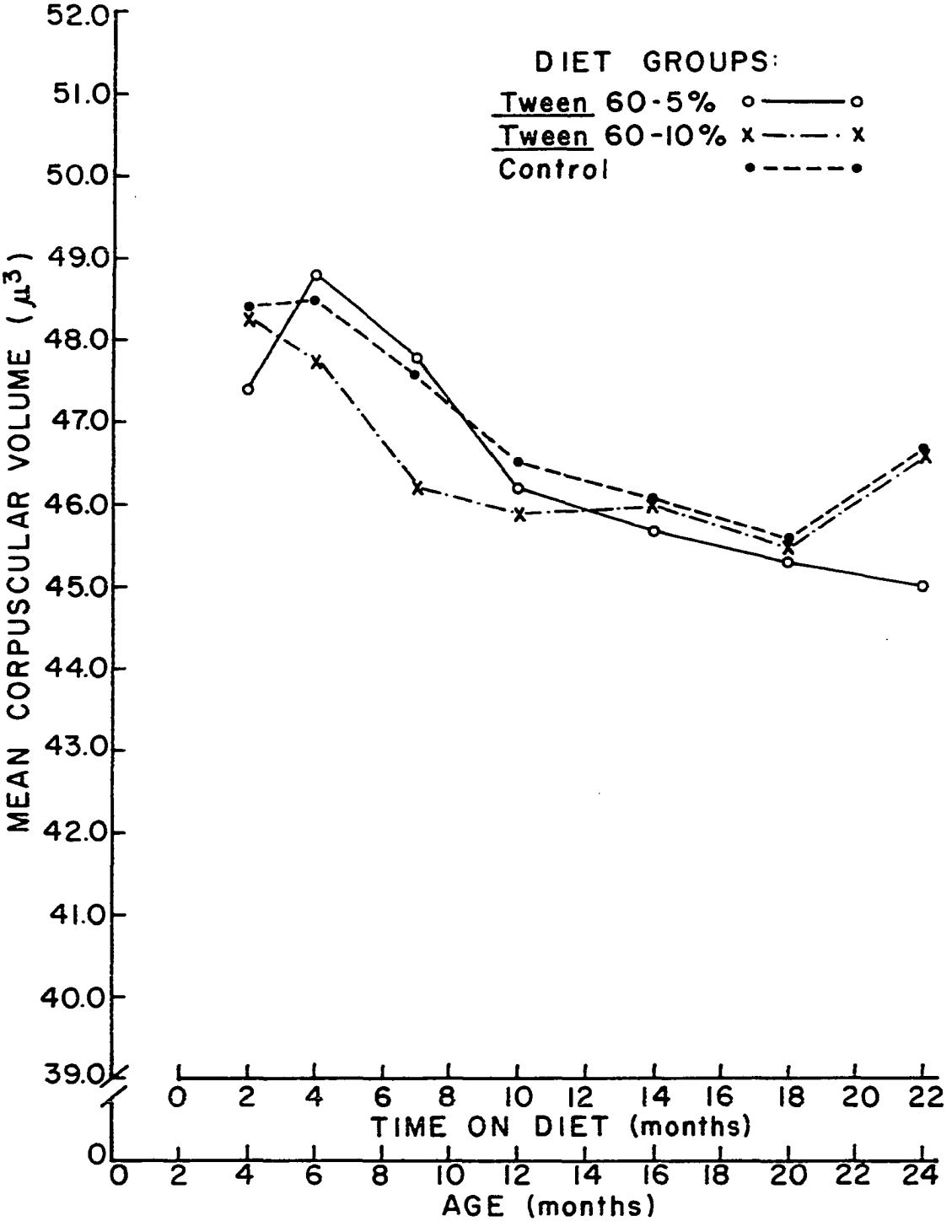

DIET GROUPS:
Tween 60-5%
Tween 60-10%
Control
MEAN CORPUSCULAR VOLUME (μ3)
52.0
51.0
50.0
49.0
48.0
47.0
46.0
45.0
44.0
43.0
42.0
41.0
40.0
39.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Table 27. Linear and quadratic regression formulae, and significance levels, for mean corpuscular volume (Y) on time on diet (X') of male $C_{57}BL/6$ Jax mice on different diets

Diet	Regression formula	Significance
Control	$Y = 49.54 - (0.4092)X' + (0.0120)(X')^2$	$P<.05$
Tween 20-5%	$Y = 50.37 - (0.6104)X' + (0.0183)(X')^2$	$P<.05$
Tween 20-10%	$Y = 49.93 - (0.3230)X'$	$P<.01$
Tween 60-5%	$Y = 48.57 - (0.1796)X'$	$P<.01$
Tween 60-10%	$Y = 49.31 - (0.5133)X' + (0.0177)(X')^2$	$P<.01$

Table 28. Results of statistical analysis of calculated mean corpuscular volume regression means (μ^3) for different diet groups at seven and 14 months on diet

Diet	Tween 60-10%	Tween 20-5%	Control	Tween 60-5%	Tween 20-10%
Calculated regression mean at seven months on diet	46.58	47.00	47.26	47.30	47.67

Diet	Tween 20-10%	Tween 20-5%	Tween 60-10%	Tween 60-5%	Control
Calculated regression mean at 14 months on diet	45.41	45.41	45.59	46.05	46.15

Mice on Tween 20-5 percent diet exhibited a range for mean corpuscular volume from 48.9 μ^3 for 10 mice at two months on diet to 45.1 μ^3 for four mice at 18 months on diet. A decrease in mean corpuscular volume was observed through the first four examination periods (Table 23).

Table 24 is a summary of data for Tween 20-10 percent diet group mean corpuscular volume determinations. Values ranged from 50.1 μ^3 for 10 mice at two months on diet to 43.3 μ^3 at 22 months on diet for the six surviving mice. A decline in mean corpuscular volume was observed through the first four examination periods with an increase at the 14 months on diet examination. A decrease in mean corpuscular volume was noted at 18 and 22 months on diet examinations.

Results of Tween 60-5 percent diet group mean corpuscular volume calculations are recorded in Table 25. The range of values for the investigation interim was from 48.8 μ^3 for 10 mice at four months on diet to 45.0 μ^3 for the four survivors at 22 months on diet. An increase was noted between the two and four months on diet examinations. From four months on diet through 22 months on diet a gradual decline in mean corpuscular volume was observed.

Tween 60-10 percent diet group average mean corpuscular volume ranged from 48.3 μ^3 for 10 animals at two months on diet to 45.5 μ^3 for 10 mice at 18 months on diet (Table 26). A decrease in mean value was found over the first four examination periods with an increase at the fifth examination. It should be noted that in the interim from seven months on diet to 22 months on diet the maximal difference in arithmetic means was only 1.1 μ^3.

Results of linear and quadratic regression formulae, including significance levels, are included in Table 27. Quadratic regression of mean corpuscular volume on time on diet was significant ($P<.05$) for the control diet group. Linear regression of mean corpuscular volume on time on diet was significant ($P<.01$) for Tween 20-10 percent and Tween 60-5 percent diet groups. Quadratic regression of mean corpuscular volume on time on diet was significant for Tween 20-5 percent diet group ($P<.05$) and Tween 60-10 percent diet group ($P<.01$) animals. In Table 27, Y represents the dependent variable (mean corpuscular volume) and X' indicates the independent variable (time on diet).

Mean corpuscular volume regression means for each diet, at seven and 14 months on diet, were calculated from the specific regression formula (Table 27). Statistical differences between regression means for each diet were tested at the five percent, or less, level at both seven and 14 months on diet. Table 28 includes the results of the statistical analysis. Those means not underscored by a common line are statistically different.

Mean Corpuscular Hemoglobin

Results of mean corpuscular hemoglobin for individual mice on each of five diets, including arithmetic means for each examination period, are recorded in Tables 29, 30, 31, 32, and 33. Figures 10 and 11 illustrate graphically the arithmetic means for each of the diet groups versus time on diet and age. Linear and quadratic regression formulae, with significance levels, are shown in Table 34. Statistical evaluations for

Table 29. Mean corpuscular hemoglobin (γγg) values of control diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	17.4	16.3	14.9	15.0	15.3	14.8	--
2	15.9	15.5	16.2	15.5	15.3	--	--
3	15.9	16.4	16.9	15.8	15.4	15.7	14.1
4	17.9	16.5	--	--	--	--	--
5	16.0	15.7	15.2	15.6	15.1	15.2	14.3
6	16.5	16.6	16.0	15.3	15.3	15.0	15.8
7	15.8	16.4	16.1	15.4	14.0	--	--
8	16.7	17.0	15.9	15.4	15.4	15.2	--
9	15.8	15.7	16.1	15.5	15.0	15.3	15.6
10	14.8	15.3	16.2	15.1	15.5	15.6	15.0
Total	162.7	161.4	143.5	138.6	136.3	106.8	74.8
Average	16.3	16.1	15.9	15.4	15.1	15.3	15.0

Table 30. Mean corpuscular hemoglobin (γγg) values of Tween 20-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	16.6	--	--	--	--	--	--
2	18.1	15.9	15.4	15.0	--	--	--
3	17.3	--	--	--	--	--	--
4	16.9	15.8	14.5	15.3	15.0	--	--
5	15.3	15.9	15.0	15.1	14.4	15.9	15.4
6	15.7	16.3	15.2	15.1	15.4	15.8	--
7	15.5	16.2	15.0	15.1	14.8	--	--
8	17.7	16.7	15.1	14.8	16.0	--	--
9	16.5	16.1	15.4	15.5	16.0	13.6	--
10	16.3	15.7	15.7	15.3	14.9	16.1	15.6
Total	165.9	128.6	121.3	121.2	106.5	61.4	31.0
Average	16.6	16.1	15.2	15.2	15.2	15.4	15.5

Table 31. Mean corpuscular hemoglobin (γγg) values of Tween 20-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	17.0	16.2	16.0	14.0	--	--	--
2	18.7	15.8	15.9	14.2	15.2	12.2	14.9
3	16.0	16.8	15.7	15.1	15.3	12.8	--
4	15.4	16.5	15.2	14.9	14.6	14.3	12.6
5	17.5	16.9	15.7	15.3	15.1	14.7	14.7
6	15.1	16.0	16.0	14.8	15.5	13.8	13.6
7	17.9	16.7	15.0	14.4	15.6	--	--
8	17.1	15.1	15.1	14.8	--	--	--
9	16.9	16.3	16.4	16.1	15.5	13.1	15.5
10	17.4	16.0	15.5	14.5	15.1	14.1	14.2
Total	169.0	162.3	156.5	148.1	121.9	95.0	85.5
Average	16.9	16.2	15.7	14.8	15.2	13.6	14.3

Table 32. Mean corpuscular hemoglobin (γγg) values of Tween 60-5% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	15.2	16.3	15.3	14.7	15.0	14.8	--
2	14.0	17.2	15.4	14.1	15.8	15.9	--
3	16.0	16.3	15.6	14.1	14.6	15.0	15.0
4	15.4	16.1	15.6	14.6	15.0	14.9	--
5	15.2	16.3	16.1	15.1	15.1	15.0	14.8
6	16.1	15.6	16.1	15.9	15.3	14.8	--
7	14.9	16.8	15.7	15.2	15.3	14.9	--
8	15.6	16.2	15.8	15.9	15.2	12.7	--
9	15.6	15.3	15.3	15.1	15.4	13.5	14.2
10	16.4	16.2	16.2	15.8	14.6	15.1	15.2
Total	154.4	162.3	157.1	150.5	151.3	146.6	59.2
Average	15.4	16.2	15.7	15.1	15.1	14.7	14.8

Table 33. Mean corpuscular hemoglobin (γγg) values of Tween 60-10% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	16.0	16.2	14.5	14.0	14.4	14.2	--
2	16.7	13.8	14.1	13.3	14.2	14.8	14.4
3	17.0	15.5	14.6	12.9	13.3	13.4	--
4	16.1	15.4	13.8	12.9	14.2	14.4	13.8
5	16.3	14.4	14.8	14.1	14.0	14.3	--
6	15.5	15.4	14.2	13.5	13.9	14.4	13.9
7	16.0	15.2	13.3	13.6	14.6	14.0	13.6
8	15.7	14.7	14.5	11.9	14.0	14.6	--
9	15.5	14.4	13.9	12.7	13.4	14.5	15.3
10	15.7	15.2	13.6	13.8	14.0	14.1	14.1
Total	160.5	150.2	141.3	132.7	140.0	142.7	85.1
Average	16.1	15.0	14.1	13.3	14.0	14.3	14.2

Figure 10. Average mean corpuscular hemoglobin values of male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet: control, Tween 20-5 percent, and Tween 20-10 percent

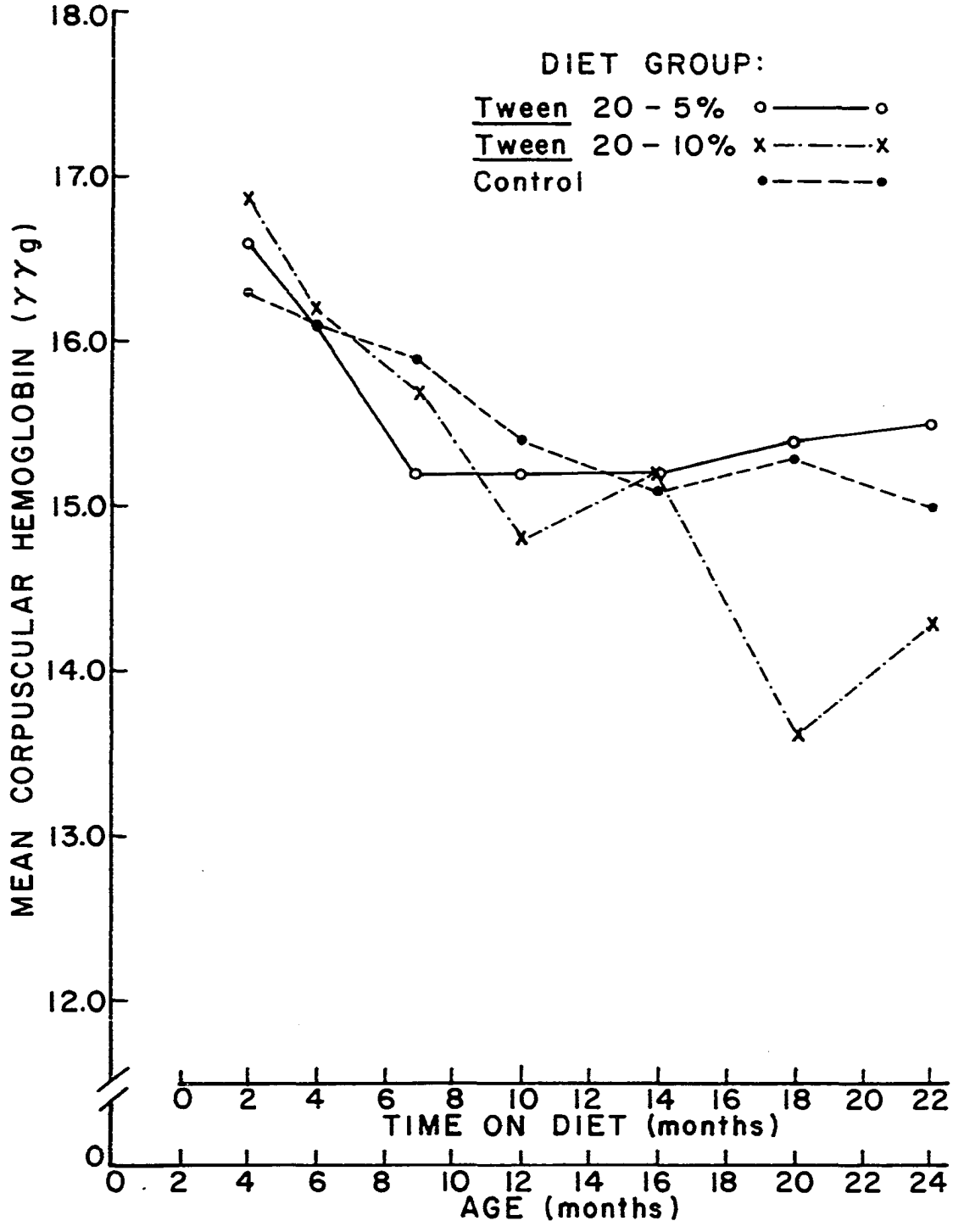

DIET GROUP:
Tween 20 - 5%
Tween 20 - 10%
Control
MEAN CORPUSCULAR HEMOGLOBIN (γγg)
18.0
17.0
16.0
15.0
14.0
13.0
12.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Figure 11. Average mean corpuscular hemoglobin values of male C57BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 60-5 percent, and Tween 60-10 percent

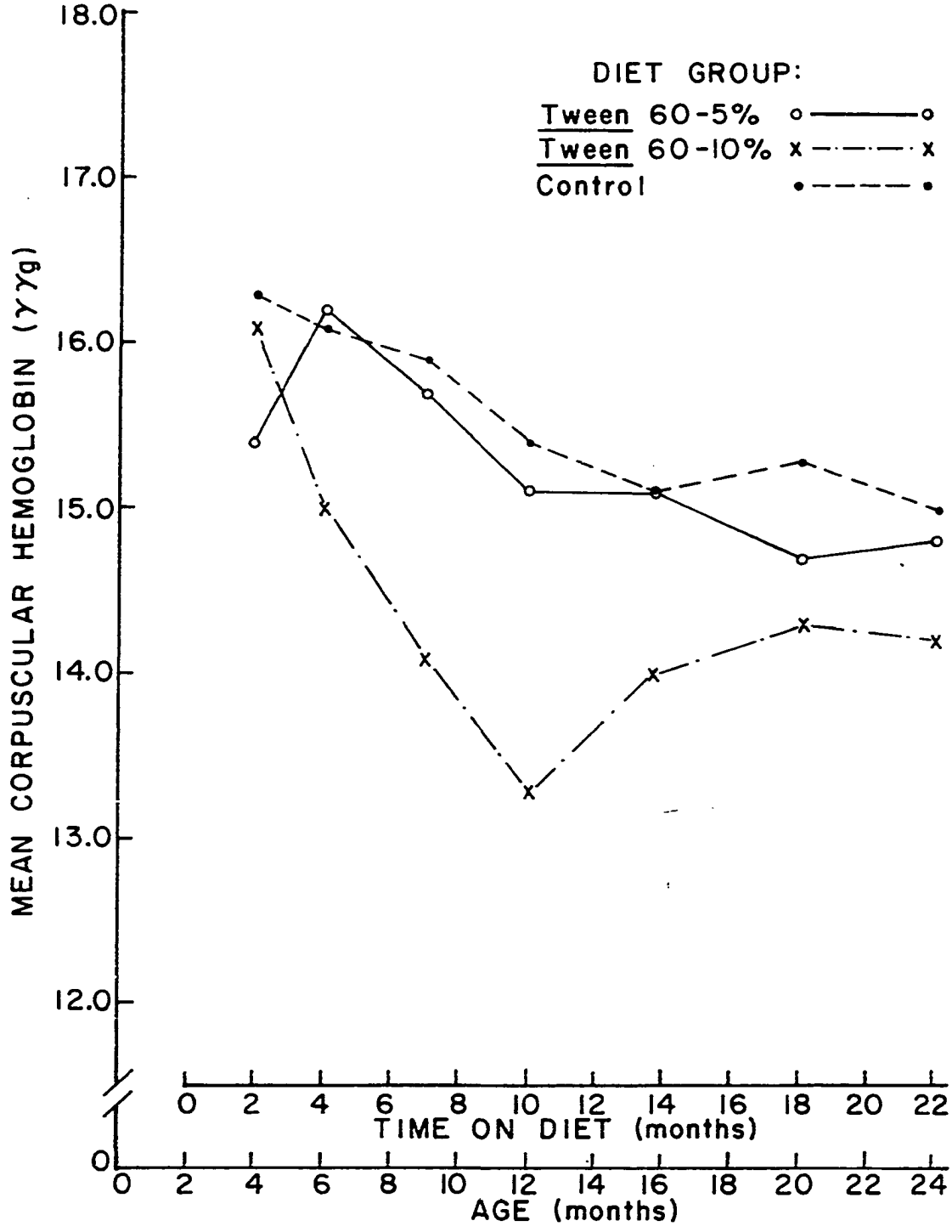

DIET GROUP:
Tween 60-5%
Tween 60-10%
Control
MEAN CORPUSCULAR HEMOGLOBIN (γγg)
18.0
17.0
16.0
15.0
14.0
13.0
12.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Table 34. Linear and quadratic regression formulae, and significance levels, for mean corpuscular hemoglobin (Y) on time on diet (X') of male $C_{57}BL/6$ Jax mice on different diets

Diet	Regression formula	Significance
Control	$Y = 16.35 - (0.0705)X'$	$P<.01$
Tween 20-5%	$Y = 17.08 - (0.3081)X' + (0.0114)(X')^2$	$P<.01$
Tween 20-10%	$Y = 17.40 - (0.2986)X' + (0.0067)(X')^2$	$P<.05$
Tween 60-5%	$Y = 15.99 - (0.0654)X'$	$P<.01$
Tween 60-10%	$Y = 16.61 - (0.4439)X' + (0.0162)(X')^2$	$P<.01$

significant ($P<.05$) differences between calculated regression means of diet groups, at seven and 14 months on diet, are recorded in Table 35.

Controls exhibited a range from 16.3 γγg for five survivors at 22 months on diet. A gradual decline in mean corpuscular hemoglobin was noted through the first five examination periods. From the 10 month on diet examination to the 22 months on diet examination period a maximal difference on only 0.4 γγg was observed (Table 29).

Mean corpuscular hemoglobin calculations of mice on Tween 20-5 percent diet are summarized in Table 30, which includes mathematical means. The range for mean corpuscular hemoglobin was from 16.6 γγg for 10 mice at two months on diet to 15.2 γγg for eight mice at both seven and 10 months on diet, and for seven mice at 14 months on diet. A slight increase was observed at the 18 and 22 months on diet examination periods.

Tween 20-10 percent diet group mice exhibited a decrease in mean corpuscular hemoglobin through the first four examination periods

Table 35. Results of statistical analysis of calculated mean corpuscular hemoglobin regression means (γγg) for different diet groups at seven and 14 months on diet

Diet	Tween 60-10%	Tween 20-5%	Tween 60-5%	Tween 20-10%	Control
Calculated regression mean at seven months on diet	14.30	15.48	15.53	15.63	15.86

Diet	Tween 60-10%	Tween 20-10%	Tween 20-5%	Tween 60-5%	Control
Calculated regression mean at 14 months on diet	13.58	14.53	15.01	15.07	15.36

(Table 31). Mean values ranged from 16.9 γγg for 10 mice at two months on diet to 13.6 γγg for seven mice at 18 months on diet.

Table 32 is a summary of mean corpuscular hemoglobin data for individual mice on Tween 60-5 percent diet, including the arithmetic means for each examination period. Mean corpuscular hemoglobin values ranged from 16.2 γγg for 10 mice at four months on diet to 14.7 γγg for 10 mice at 18 months on diet. An increase in mean corpuscular hemoglobin was noted between the two and four months on diet examination periods.

Tween 60-10 percent diet group results are recorded in Table 33. A decrease in value was noted through the first four examination periods.

Mean values ranged from 16.1 γγg for 10 mice at two months on diet to 13.3 γγg for 10 mice at 10 months on diet.

Results of linear and quadratic regression formulae, including significance levels, are recorded in Table 34. Control and Tween 60-5 percent diet groups exhibited linear regression of mean corpuscular hemoglobin on time on diet significant at less than the one percent level ($P<.01$). Tween 20-10 percent diet group exhibited significant ($P<.05$) quadratic regression of mean corpuscular hemoglobin on time on diet. Quadratic regression of mean corpuscular hemoglobin on time on diet was significant ($P<.01$) for both Tween 20-5 percent and Tween 60-10 percent diet groups.

Mean corpuscular hemoglobin regression means, for seven and 14 months on diet, were calculated from the particular regression formula (Table 34) and are recorded in Table 35. Regression means for each of the five diets, at seven and 14 months on diet, were tested for significant ($P<.05$) differences. Those means not underscored by a common line are statistically different.

Mean Corpuscular Hemoglobin Concentration

Mean corpuscular hemoglobin concentration values of individual mice on each of five diets are recorded in Tables 36, 37, 38, 39, and 40. Graphic depiction of arithmetic means of each diet group for each examination period versus age and time on diet is shown in Figures 12 and 13. Calculated regression means, at seven and 14 months on diet for each diet group, were tested for statistical difference ($P<.05$). Results of the

Table 36. Mean corpuscular hemoglobin concentration (%) values of control diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	34.2	33.4	32.7	32.4	33.1	31.7	--
2	32.4	33.5	33.3	33.3	32.7	--	--
3	32.7	33.8	34.9	32.7	32.4	33.9	30.9
4	34.8	33.4	--	--	--	--	--
5	34.1	32.6	32.9	33.1	32.6	34.1	32.5
6	34.0	33.5	34.2	32.7	33.0	33.7	33.0
7	33.1	32.7	33.6	33.4	32.0	--	--
8	32.8	33.8	33.5	33.3	33.5	33.3	--
9	33.8	33.4	33.4	33.0	32.7	33.5	32.0
10	34.7	33.5	33.3	34.0	33.3	33.7	31.5
Total	336.6	333.6	301.8	297.9	295.3	233.9	159.9
Average	33.7	33.4	33.5	33.1	32.8	33.4	32.0

Table 37. Mean corpuscular hemoglobin concentration (%) values of Tween 20-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	34.4	--	--	--	--	--	--
2	33.9	33.2	33.0	33.7	--	--	--
3	34.7	--	--	--	--	--	--
4	34.1	33.0	31.3	33.0	33.8	--	--
5	33.8	32.8	32.3	32.7	33.7	34.2	33.3
6	33.0	33.3	32.0	33.3	33.5	34.6	--
7	33.4	32.7	32.4	32.9	34.2	--	--
8	33.5	32.3	31.8	33.7	34.3	--	--
9	34.8	32.7	32.4	32.9	33.0	33.9	--
10	33.5	33.5	32.7	33.3	32.5	33.4	34.2
Total	339.1	263.5	257.9	265.5	235.0	136.1	67.5
Average	33.9	32.9	32.2	33.2	33.6	34.0	33.8

Table 38. Mean corpuscular hemoglobin concentration (%) values of Tween 20-10% diet male C_{57}BL/6 Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	34.6	33.4	33.4	32.8	--	--	--
2	33.2	32.0	34.1	32.0	33.8	28.2	32.6
3	32.0	33.3	32.6	33.5	33.0	28.7	--
4	33.7	33.3	32.7	32.6	31.0	30.9	34.6
5	33.9	33.8	32.2	32.5	33.3	32.4	32.8
6	34.6	34.2	33.7	33.2	33.8	31.3	31.7
7	34.0	34.1	31.1	32.6	33.7	--	--
8	34.3	33.1	33.0	31.8	--	--	--
9	33.3	33.3	34.1	33.0	34.2	30.9	33.2
10	33.8	32.7	33.4	33.8	33.0	31.7	32.5
Total	337.4	333.2	330.3	327.8	265.8	214.1	197.4
Average	33.7	33.3	33.0	32.8	33.2	30.6	32.9

Table 39. Mean corpuscular hemoglobin concentration (%) values of Tween 60-5% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	33.3	33.1	32.1	32.6	32.0	32.4	--
2	32.8	33.1	32.7	31.2	34.0	34.4	--
3	32.0	33.4	33.3	31.7	31.3	32.8	34.0
4	32.7	33.3	33.0	32.4	33.2	33.3	--
5	34.0	33.8	33.8	32.7	33.3	31.5	32.1
6	31.2	32.7	32.6	33.3	33.5	31.7	--
7	33.7	33.5	32.7	32.6	33.0	32.9	--
8	32.7	33.3	32.6	33.3	33.8	30.0	--
9	31.7	33.3	32.7	31.8	33.3	30.8	32.4
10	32.8	33.3	33.0	33.5	32.5	33.2	33.0
Total	326.9	332.8	328.5	325.1	329.9	323.0	131.5
Average	32.7	33.3	32.9	32.5	33.0	32.3	32.9

Table 40. Mean corpuscular hemoglobin concentration (%) values of Tween 60-10% diet male $C_{57}BL/6$ Jax mice at different ages and lengths of time on diet

Animal number	Age at date of examination (months)						
	4	6	9	12	16	20	24
	Time on diet at date of examination (months)						
	2	4	7	10	14	18	22
1	33.4	31.9	30.9	30.9	31.2	30.5	--
2	34.0	29.6	30.2	28.5	30.5	31.6	30.2
3	34.0	32.3	31.3	28.5	29.5	31.1	--
4	33.4	31.1	30.2	26.8	31.2	30.0	29.2
5	34.0	32.0	32.6	30.1	31.0	32.5	--
6	32.1	32.1	30.6	28.1	30.5	31.7	32.0
7	32.0	30.3	29.7	29.8	30.5	32.2	29.9
8	32.7	31.8	30.6	27.9	29.5	30.2	--
9	32.8	30.7	30.6	27.5	30.2	32.0	31.6
10	32.3	32.3	29.5	31.3	30.2	31.7	29.8
Total	330.7	314.1	306.2	289.4	304.3	313.5	182.7
Average	33.1	31.4	30.6	28.9	30.4	31.4	30.5

Figure 12. Average mean corpuscular hemoglobin concentration values of male C_{57}BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 20-5 percent, and Tween 20-10 percent

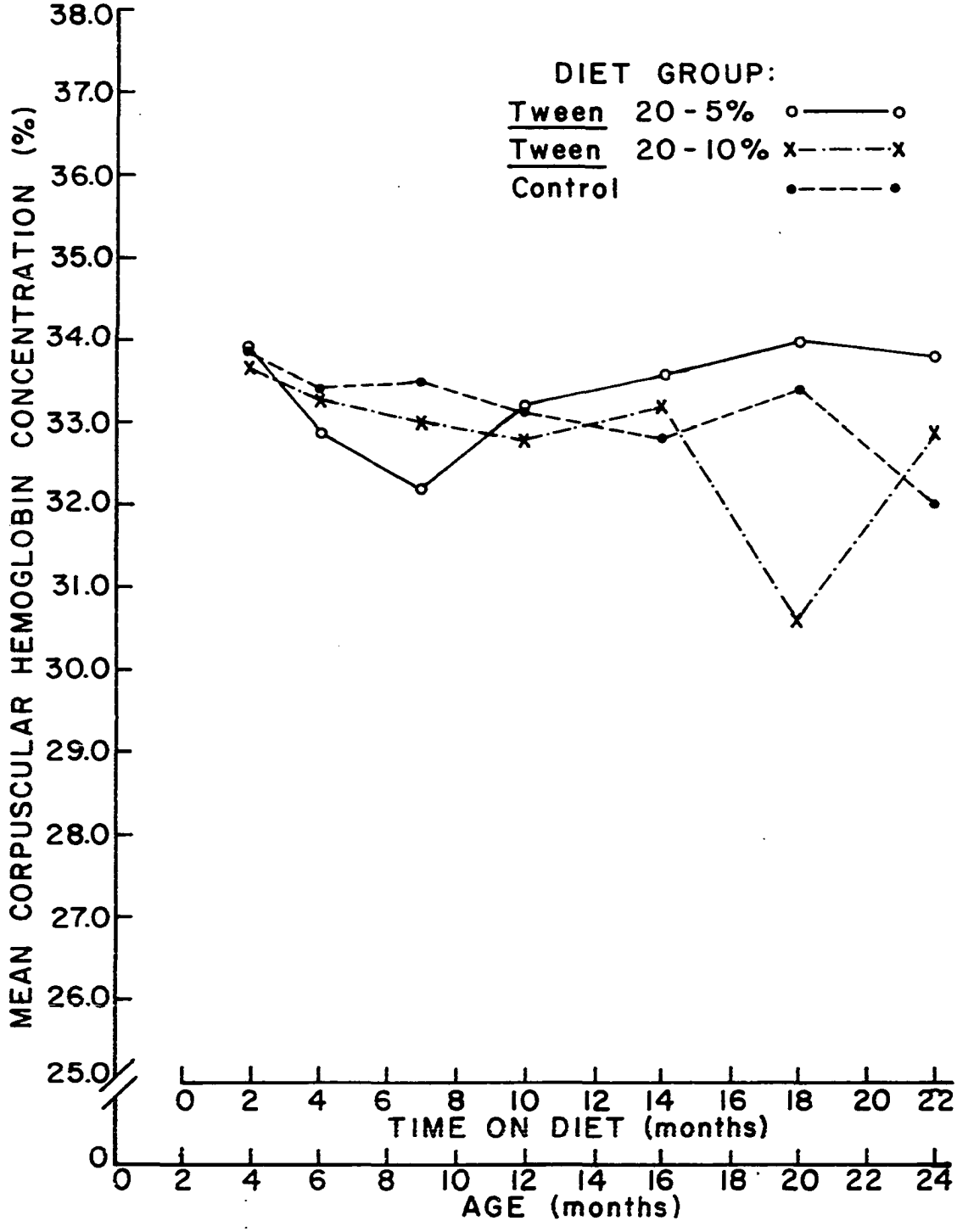
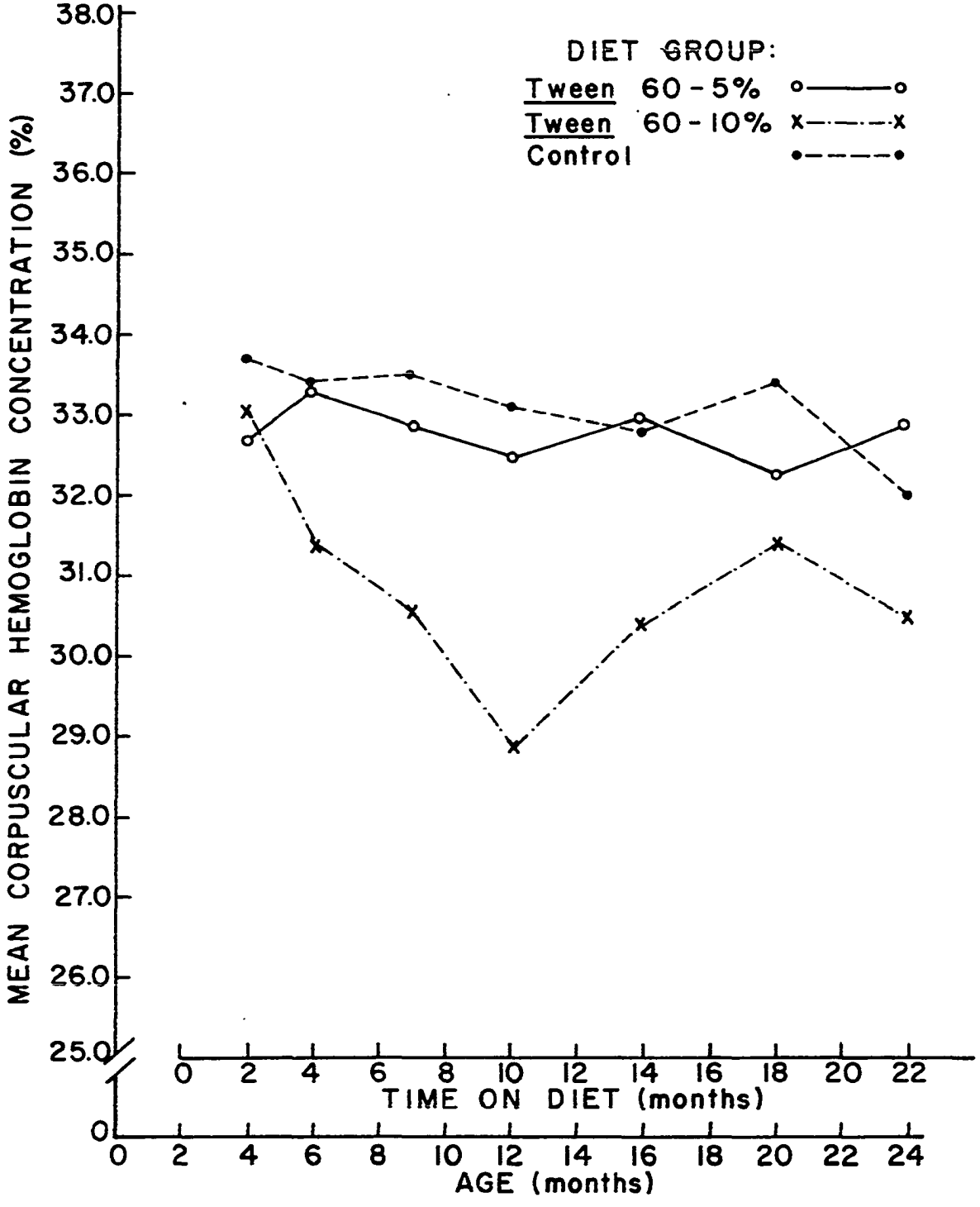

DIET GROUP:
Tween 20 - 5%
Tween 20 - 10%
Control
MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (%)
38.0
37.0
36.0
35.0
34.0
33.0
32.0
31.0
30.0
29.0
28.0
27.0
26.0
25.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

Figure 13. Average mean corpuscular hemoglobin concentration values of male C57BL/6 Jax mice at different ages and lengths of time on diet: control, Tween 60-5 percent, and Tween 60-10 percent


DIET GROUP:
Tween 60 - 5%
Tween 60 - 10%
Control
MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (%)
38.0
37.0
36.0
35.0
34.0
33.0
32.0
31.0
30.0
29.0
28.0
27.0
26.0
25.0
0
0 2 4 6 8 10 12 14 16 18 20 22
TIME ON DIET (months)
0 2 4 6 8 10 12 14 16 18 20 22 24
AGE (months)

statistical analysis are shown in Table 42.

The control diet group exhibited a range in mean corpuscular hemoglobin concentration from 33.7 percent for 10 mice at two months on diet to 32.0 percent for five surviving mice at 22 months on diet. Maximal difference in mean corpuscular hemoglobin concentration through the first six examination periods was only 0.9 percent (Table 36).

Mean corpuscular hemoglobin concentration calculations for Tween 20-5 percent diet group are summarized in Table 37, which includes arithmetic means. A decrease in mean corpuscular hemoglobin concentration was observed through the first three examination periods, then a slight increase was noted during later examinations. Values ranged from 34.0 percent for four mice at 18 months on diet to 32.2 percent for eight mice at seven months on diet.

Tween 20-10 percent diet group results for mean corpuscular hemoglobin concentration are summarized in Table 38. Mean values ranged from 33.7 percent for 10 mice at two months on diet to 30.6 percent for seven mice at 18 months on diet. A gradual decline in value was noted through the first four examination periods.

Table 39 is a summary of mean corpuscular hemoglobin concentration calculations for mice on Tween 60-5 percent diet. Maximal difference in arithmetic means for the seven examination periods was only 1.0 percent. Mean corpuscular hemoglobin concentration averages ranged from 33.3 percent for 10 animals at four months on diet to 32.3 percent for 10 mice at 18 months on diet.

Mice on Tween 60-10 percent diet exhibited a range in mean values of

from 33.1 percent for 10 mice at two months on diet to 28.9 percent for 10 mice at 10 months on diet. A decrease in mean corpuscular hemoglobin concentration was observed during the first 10 months time on diet.

Linear and quadratic regression formulae, and significance levels, are recorded in Table 41. Linear regression of mean corpuscular

Table 41. Linear and quadratic regression formulae, and significance levels, for mean corpuscular hemoglobin concentration (Y) on time on diet (X') of male $C_{57}BL/6$ Jax mice on different diets

Diet	Regression formula	Significance
Control	$Y = 33.74 - (0.0546)X'$	$P<.01$
Tween 20-5%	$Y = 33.85 - (0.1944)X' + (0.0104)(X')^2$	$P<.01$
Tween 20-10	$Y = 33.72 - (0.0853)X'$	$P<.01$
Tween 60-5%	$Y = 32.98 - (0.0204)X'$	N.S.*
Tween 60-10%	$Y = 33.63 - (0.5778)X' + (0.0220)(X')^2$	$P<.01$

*N.S. indicates not significant.

hemoglobin concentration on time on diet was significant ($P<.01$) for both control and Tween 20-10 percent diet groups. Tween 20-5 percent and Tween 60-10 percent diet groups exhibited significant ($P<.01$) quadratic regression of mean corpuscular hemoglobin concentration on time on diet. Neither linear nor quadratic regression of mean corpuscular hemoglobin concentration on time on diet was significant for mice on Tween 60-5 percent diet.

Mean corpuscular hemoglobin concentration regression means were

calculated from the particular regression formula (Table 41) for seven and 14 months time on diet for each of the five diets.* Results are recorded in Table 42. Calculated regression means, at both seven and 14 months on diet, were tested for significant ($P<.05$) differences for each

Table 42. Results of statistical analysis of calculated mean corpuscular hemoglobin concentration regression means (%) for different diet groups at seven and 14 months on diet

Diet	Tween 60-10%	Tween 60-5%	Tween 20-5%	Tween 20-10%	Control
Calculated regression mean at seven months on diet	30.66	32.84	33.00	33.13	33.35

Diet	Tween 60-10%	Tween 20-10%	Tween 60-5%	Control	Tween 20-5%
Calculated regression mean at 14 months on diet	29.85	32.53	32.69	32.97	33.17

of the diet groups. In Table 42, those regression means not underscored by a common line are statistically different.

*The linear regression formula for mice on Tween 60-5 percent diet, though not statistically significant, was used for calculation of regression means for statistical analysis of differences between diet groups at both seven and 14 months time on diet.

DISCUSSION

Control Diet Group

Establishment of a hematological pattern for the $C_{57}BL/6$ Jax mouse was one of the primary purposes of the present research.

One of the more interesting aspects of the study was development of an experimental design which would permit use of a limited number of animals and still maintain biological and statistical validity. Although animal care, cost of maintenance, and facilities were not limiting factors in the present study, these factors many times are a deterrent. The experimental design, including the method of statistical analysis, is noteworthy since it does, in effect, preclude some possible limiting factors.

Only 50 animals and 10 cage units were used in the investigation. If different animals had been used for each age group, the animal number would have been 350, at least 70 cage units would have been necessary, the volume of food and Tween used would have quadrupled, and the maintenance time would have tripled. It is apparent that use of the same animals throughout the experimental period reduced animal, equipment, and feeding costs to a minimum.

The use of linear and quadratic regression formula fittings to the raw data, and calculation of the regression mean for a given time on diet, allowed inclusion of the total number of examinations for a particular diet group over the 22 month period, rather than just the determinations from 10 (or fewer) mice for the specific diet time. The error of each

hematological determination for a diet group throughout the experiment was thus included while, at the same time, increasing the statistical degrees of freedom. While this type of experimental design is applicable only to certain investigative procedures, it is important since it establishes that experimental research can be accomplished with limited facilities and funds.

Results of successive determinations on a single group of 10 mice at seven different ages indicate a decline in hemoglobin content, hematocrit, erythrocyte count, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration with increased age. Statistical evaluation of linear and quadratic regression of each value on time on control diet (age less two months) indicates a mathematical regularity of pattern (Tables 6, 13, 20, 27, 34, and 41).

Linear regression of hemoglobin content, erythrocyte count, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration on time on diet were significant at less than the one percent level. Hematocrit and mean corpuscular volume exhibited quadratic regression on time on diet significant at less than the five percent level. However, through the first four examination periods (two, four, seven, and 10 months on diet) it has been shown that hematocrit and mean corpuscular volume exhibit linear regression on age (Ewing, 1962). Apparently, subsequent to this span of time, hematocrit and mean corpuscular volume values do not decrease at the prior rate, thereby introducing the second degree polynomial factor (quadratic regression). It should be noted that both hematocrit and mean corpuscular volume increased at the 22 months on

diet examination. Since mean corpuscular volume is directly proportional to hematocrit, and inversely proportional to erythrocyte count, the increase in mean corpuscular volume would be expected.

The decrease in hematological values throughout the investigation interim of 22 months does not indicate an anemia. Maximal differences between the highest mean values and lowest mean values for each determination are not of a magnitude suggestive of anemia. However, the decrease in hematological values with increased age does indicate need for careful selection of age limits in hematological studies. It is probable that age could have an effect on experimental research involving the erythron, and, therefore, on any investigation that involves dependence upon erythron functions.

Results of the present study indicate that hemoglobin content, hematocrit, erythrocyte count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration values decrease with increased age in a manner that may be expressed mathematically. Although earlier investigations (DeKock, 1931; Francis and Strong, 1938; and Strong and Francis, 1940) suggest a decrease in certain hematological values with increased age, the present investigation is the first attempt (as disclosed through a literature survey by the author) to formulate a mathematical expression for this phenomenon. It must be emphasized that no attempt has been made to show any differences, statistical or otherwise, between values of control animals at different ages. An investigation of possible differences between values at different ages seems warranted from the results of the present study.

Tween 20-5 Percent Diet Group

Hematological values of mice on Tween 20-5 percent diet exhibited a general decrease throughout the course of investigation. Linear regression of erythrocyte count on time on diet was significant at less than the one percent level. Hemoglobin content, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration exhibited a statistically significant quadratic regression of value on time on diet (Tables 6, 13, 20, 27, 34, and 41).

Hemoglobin content, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration differed from controls in exhibiting quadratic regression of value on time on diet. Hemoglobin content of mice on Tween 20-5 percent diet decreased to 14.2 g/100 ml blood at seven months on diet, while control animals did not reach this level until 14 months on diet. However, the experimental diet mice, from seven to 18 months on diet, remained at nearly the same value, while controls continued a slight decrease for the period. Both groups at 22 months on diet exhibited about the same decrease. Mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration approximate this same pattern for control and Tween 20-5 percent diet groups. The modification in regression type, from linear to curvilinear, is indicatory of a response to the experimental diet. Quadratic regression may characterize development of a tolerance to the immediate effect of Tween 20. Mean corpuscular volume, hematocrit, and erythrocyte count regression types were of the same type as the control diet group.

Significant ($P<.05$) differences between Tween 20-5 percent and

control diet groups were shown for hemoglobin and hematocrit regression means at both seven and 14 months on diet. Erythrocyte count regression means were statistically different at 14 months on diet but not at seven months on diet. No statistically significant differences were found between control and Tween 20-5 percent diet groups for mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Calculated regression mean values, at seven and 14 months, were greater for controls for all determinations except mean corpuscular hemoglobin concentration at 14 months on diet. After only two months on diet, all mean values except erythrocyte count were higher for Tween 20-5 percent diet mice than for controls. However, at four months on diet, only mean corpuscular volume was greater for Tween 20-5 percent diet mice. It thus appears that Tween 20 fed at the five percent level exerted an immediate stimulatory influence upon the erythron of the C_{57}BL/6 Jax mouse, but that this condition was rapidly reversed and effected a rapid decrease in hematological values until the 10 months on diet examination.

Differences between certain hematological values of control and Tween 20-5 percent diet animals are not of a magnitude indicative of an anemia in the experimental diet group. However, these differences, coupled with a change in regression type, are indicatory of response to the Tween 20-5 percent diet. It appears that Tween 20 at the five percent diet level influences a change in the hematological picture of the C_{57}BL/6 Jax mouse, though apparently not of pathological nature. There is suggestion that the "influence" exerted by Tween 20 is only temporary, and animals develop

a tolerance to the experimental diet. A systematic histological study of hemopoietic tissues, and of possible development of extramedullary hemopoietic tissue, appears warranted for affirmation of the postulation.

Tween 20-10 Percent Diet Group

Mice fed Tween 20 at a 10 percent level for 22 months exhibited a general decrease in hematological values during the investigation. Linear regression of erythrocyte count, mean corpuscular volume, and mean corpuscular hemoglobin concentration on time on diet were statistically significant ($P<.01$). Hemoglobin content, hematocrit, and mean corpuscular hemoglobin values showed a statistically significant quadratic regression on time on diet (Tables 6, 13, 20, 27, 34, and 41).

Quadratic regression of hemoglobin content and mean corpuscular hemoglobin differed from linear regression shown by the control diet group. Mean corpuscular volume regression was linear as compared to the quadratic regression exhibited by the control animals. Hematocrit, erythrocyte count, and mean corpuscular hemoglobin concentration were of the same type as those of controls.

Hemoglobin content of mice on Tween 20-10 percent diet decreased rapidly through the first 10 months on diet to 13.9 g/100 ml blood while control animals did not decline nearly so rapidly (14.1 g/100 ml blood at 18 months on diet). Hematocrit, mean corpuscular volume, erythrocyte count, and mean corpuscular hemoglobin exhibited this same rapid decrease. At 14 months on diet, only erythrocyte count and hematocrit continued to decrease. At 18 months on diet, all determinations exhibited a decrease.

Hemoglobin content, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration increased at the 22 months on diet examination.

Significant ($P<.05$) differences between Tween 20-10 percent and control diet groups for hemoglobin content, mean corpuscular volume, and mean corpuscular hemoglobin regression values were found at both seven and 14 months on diet. Hematocrit and erythrocyte count regression values were statistically different at 14 months on diet but not at seven months. No differences were found between controls and Tween 20-10 percent diet groups for mean corpuscular hemoglobin concentration. In all cases of significant differences, Tween 20-10 percent diet group animal values were lower than control values.

Significant differences between regression values for hemoglobin content, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were shown for Tween 20-5 percent and Tween 20-10 percent diet groups at 14 months on diet. Mice on the 10 percent diet level were always lower than mice on the five percent level. Tween 20-5 percent fed mice exhibited quadratic regression of mean corpuscular hemoglobin concentration while Tween 20-10 percent diet mice showed linear regression for the same determination. No other statistical differences were noted between the two Tween 20 diet levels.

It appears that Tween 20 fed at the 10 percent level throughout the "adult" life-span of $C_{57}BL/6$ Jax mice does manifest a change in hematological pattern from the "normal". Decrease of hematological values are not indicatory of an anemia. However, the statistical differences shown between certain hematological values of control and Tween 20-10 percent

diet mice are indicative of an effect on the erythron by the experimental diet. The alteration in hematological pattern, as evidenced by differing regression types, does indicate some functional response to Tween 20. It appears that there is development of a tolerance to Tween 20 between 10 and 14 months on diet, but general decreases at 18 and 22 months indicate this tolerance is only temporary.

Results indicate little difference in hematological pattern between Tween 20-5 percent and Tween 20-10 percent diet groups through the first 10 months of feeding. Both groups follow the same general deviation from the control diet mice. After the 10 month examination, Tween 20-5 percent diet group animals exhibited a leveling of values comparable to controls. Tween 20-10 percent diet group values increased at the 14 months examination, then decreased sharply at the 18 months examination. Therefore, it appears that animals fed at the five percent level may develop a permanent tolerance to Tween 20 effect, while animals fed at the 10 percent level probably develop only a temporary tolerance.

Tween 60-5 Percent Diet Group

Hemoglobin content, hematocrit, and erythrocyte count values of mice on Tween 60-5 percent diet demonstrated a general decrease during the 22 months on diet investigation. Mean corpuscular volume and mean corpuscular hemoglobin increased between the two and four month on diet examination periods, and then exhibited a decrease throughout the investigation. Mean corpuscular hemoglobin concentration values were erratic over the course of investigation.

Quadratic regression of hemoglobin content and erythrocyte count on time on diet differed from linear regression of control animals. Mean corpuscular volume regression on time on diet was linear as compared to quadratic regression shown by control mice. Hematocrit and mean corpuscular hemoglobin regression types were linear as were controls. Mean corpuscular hemoglobin concentration was not significant for either linear or quadratic regression on time on diet. This was due to a negligible slope (Table 41, b=0.0204) of the line. Mean corpuscular hemoglobin concentration regression values were, however, calculated from the formula for linear regression in Table 41.

Significant ($P<.05$) differences between Tween 60-5 percent and control diet groups, at seven months on diet, were shown for hemoglobin content, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration regression values. At 14 months on diet, significant differences between calculated regression means of control and Tween 60-5 percent diet groups were found to exist between hemoglobin content, hematocrit, erythrocyte count, and mean corpuscular hemoglobin. In each instance, the values of Tween 60-5 percent diet mice were lower than control values.

Values for hematological determinations of Tween 60-5 percent diet mice at 10 months on diet were comparable in value to controls at 14 months on diet. At 14 months on diet, Tween 60-5 percent hematological values were highly comparable to the 22 months examination period values of control animals. There is no suggestion of development of a tolerance to Tween 60 at the five percent level until the 22 months on diet examina-

tion which included only four surviving animals. Six mice expired during the interim between 18 and 22 months on diet, which may suggest that some mice did not develop a tolerance and only those which did were able to survive. It is possible, however, that the mice simply died from old age. The rapid decline in hematological values through the 10 month on diet examination is indicatory of a physiological effect of Tween 60 on the erythron.

The deviations in regression pattern for certain hematological values, the statistically significant differences between control and Tween 60-5 percent diet mice, and the more rapid decline of hematological values of experimental diet animals are strongly suggestive of a detrimental effect by Tween 60 at the five percent level. The effect is not manifest as an apparent anemia. There may be a tolerance developed after chronic ingestion of Tween 60 at the five percent level. Results of the present study indicate the need for a systematic histological investigation to substantiate or abrogate the postulation.

Tween 60-10 Percent Diet Group

Hemoglobin content, hematocrit, erythrocyte count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration values decreased through the first four examination periods (two, four, seven, and 10 months on diet). At 14 months on diet, all hematological values increased. Hemoglobin content and erythrocyte count values decreased at both the 18 and 22 months on diet examinations. Hematocrit and mean corpuscular volume decreased at 18 months but

increased at 22 months on diet. Mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration continued to increase at 18 months but decreased at 22 months on diet.

A precipitous decline in hemoglobin content, hematocrit, erythrocyte count, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration occurred through the 10 month on diet examination. The values increased at 14 months time on diet and then exhibited a slight general decrease over the remainder of the investigation. This pattern for all hematological determinations was responsible for the significance of quadratic regression on time on diet. Control and Tween 60-10 percent diet groups exhibited quadratic regression of hematocrit and mean corpuscular volume on time on diet. It should be noted, however, that the quadratic regressions for hematocrit of control and Tween 60-10 percent diet groups are drastically different in value. The quadratic regression of Tween 60-10 percent diet hematocrit values exhibited a much more rapid decrease than did the control quadratic regression. This means that while the regression type was the same, there was a considerable difference in actual structure of the curves.

Significant ($P<.05$) differences between control and Tween 60-10 percent diet group mice were found to exist for hemoglobin content, hematocrit, erythrocyte count, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration at both seven and 14 months on diet. No differences were found between controls and Tween 60-10 percent fed mice for mean corpuscular volume at either seven or 14 months on diet.

No apparent anemia could be ascertained in mice on Tween 60-10 per-

cent diet. However, at 10 months on diet, a very slight hypochromasia (decreased mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration) was apparent. The deviation in regression types, the significant differences between hematological values of controls and experimental diet mice, and the precipitous decline of hematological values of experimental diet mice through 10 months on diet are indicatory of a deleterious effect on the erythron. The subsequent increase of 14 month examination values, and then general decrease in later examinations, suggests development of a tolerance to the Tween 60 effect. A systematic histological investigation of hemopoietic tissues, and of possible sites of extramedullary hemopoiesis, appears warranted for affirmation or abrogation of the postulation.

Significant ($P<.05$) differences existed between Tween 60-5 percent and Tween 60-10 percent diet groups for all determinations except mean corpuscular volume at both seven and 14 months on diet. Animals on the 10 percent level always exhibited lower values than animals on the five percent level at the same examination period. Mean corpuscular volume and mean corpuscular hemoglobin regression types differed for the two Tween 60 diets. No other statistical differences were noted.

Results indicate that Tween 60, at both the five and 10 percent diet levels, does evoke an alteration in hematological pattern in C_{57}BL/6 Jax mice. Both diet groups follow the same general hematological pattern for hemoglobin content, hematocrit, and erythrocyte count through the first 10 months of feeding, although the 10 percent level effect is greatly pronounced in rate and amount of decrease. Animals on the five percent

diet continue a general decrease throughout the investigation until the 22 months examination. Tween 60-10 percent diet mice exhibit an increase at 14 months on diet, and then a decrease in values at both 18 and 22 months examination periods. Tween 60-10 percent diet mice develop a slight hypochromasia and rapidly approach an anemic condition at the 10 months on diet examination. Apparently, the effect of Tween 60, at the 10 percent level, elicits a physiological response of the erythron, or development of extramedullary hemopoietic tissue to meet the needs of the animal. However, the response appears to be only temporary.

There were no apparent deleterious effects of either Tween 20 or Tween 60 upon life-span or general growth and health. It should be noted, however, that animals on Tween 60-10 percent and Tween 20-10 percent diets did exhibit much softer stools than other diet groups. Animals on Tween 60-10 percent diet maintained a soft, glistening coat throughout the investigation while other diet group mice did not. The increase in hematological values exhibited by several groups at the 22 months examination may have been due to body dehydration from old age rather than to any experimental effect.

SUMMARY AND CONCLUSIONS

1. A study of the effect of chronic ingestion of certain nonionic surface active agents ("Tweens") on the hematology of the C_{57}BL/6 Jax mouse was initiated as one phase of a long-term investigation currently in progress in the Department of Zoology and Entomology at Iowa State University.

2. An experimental design, including statistical analysis, was adopted in an attempt to determine the applicability and practicability of a specific type longitudinal study in hematological research. Although this general type of design is common to many research areas, no comparable design could be found in the literature involving the hematology of small animals.

3. Establishment of a "normal" hematological pattern for C_{57}BL/6 Jax mice was an integral part of the present study.

4. Ten male C_{57}BL/6 Jax mice were placed on each of five diets at the approximate age of two months.

5. Control mice were fed Purina Mouse Breeder Chow, ad libitum. Two nonionic emulsifiers, Tween 20 and Tween 60, were fed to different groups of mice at five and 10 percent diet levels. Experimental diets were fed on a food additive-Purina Mouse Breeder Chow total weight-percentage basis.

6. Blood samples were collected from the tail of each mouse at the approximate ages of four, six, nine, 12, 16, 20, and 24 months. Time on diet differed from age by minus two months.

7. Hematological determinations of hemoglobin content, hematocrit,

erythrocyte count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were completed for each blood sample.

8. Linear and quadratic regression formulae were developed, and tested for statistical significance, for each hematological determination of each diet group. Experimental diet group regression formulae were compared with control regression types as a means of determining hematological pattern deviation.

9. The New Multiple Range Test (Duncan, 1955) was utilized to test for statistical differences between hematological determination regression means of each diet group at both seven and 14 months on diet.

10. Hematological determination values of control C_{57}BL/6 Jax mice decreased as age increased in a manner which could be expressed mathematically. Controls exhibited linear regression of hemoglobin content, erythrocyte count, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Hematocrit and mean corpuscular volume showed quadratic regression on time on diet. The magnitude of decrease for any determination was not characteristic of an anemia. It is apparent, however, that age must be considered in any experimentation involving the erythron or dependence on erythron function(s).

11. Tween 20-5 percent diet mice showed quadratic regression of hemoglobin content, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration on time on diet. Erythrocyte count regressed linearly on time on diet. Hemoglobin content, hematocrit, and erythrocyte count decreased at a more pronounced

rate and magnitude than did control animal values. Several statistically significant differences were shown, at seven and 14 months on diet, between controls and Tween 20-5 percent diet mice. Hematological values were consistently lower than control values at equal lengths of time on diet. Tween 20, fed at the five percent level, apparently induced a change in the hematological picture of the C_{57}BL/6 Jax mouse, although it was not pathological (i.e., an anemia was not discernible). Mice on this diet appeared to develop a tolerance to the Tween 20 effect at approximately 10 months on diet.

12. Linear regression of erythrocyte count, mean corpuscular volume, and mean corpuscular hemoglobin concentration was significant for Tween 20-10 percent diet mice. Quadratic regression of hemoglobin content, hematocrit, and mean corpuscular hemoglobin on time on diet was significant for the diet group. In general, values decreased at a more rapid rate and greater amount than did controls. Significantly lower values were consistently shown, at both seven and 14 months on diet, when compared with control values. Tween 20, ingested at the 10 percent level, apparently exerted a profound effect on the hematological pattern of the C_{57}BL/6 Jax mouse. It appeared that a tolerance to this effect was developed prior to any perceptible development of an anemia.

13. Tween 20, fed at either the five or 10 percent level over a 22 month period, apparently induces a deviation from "normal" hematological pattern in the C_{57}BL/6 Jax mouse. It appears that the magnitude of change in pattern is related to diet concentration, the 10 percent diet level exerting greater and more rapid change than the five percent diet level.

14. Hemoglobin content, hematocrit, and erythrocyte count exhibited significant quadratic regression on time on diet for mice fed Tween 60-5 percent. Linear regression of mean corpuscular volume and of mean corpuscular hemoglobin on time on diet was significant for the diet group mice. Mean corpuscular hemoglobin concentration did not show any significant regression on time on diet. Hematological values of Tween 60-5 percent diet mice were consistently lower than controls, and significant differences were shown for several determinations at both seven and 14 months on diet. No anemia was apparent in mice on this diet. Tween 60, fed at the five percent level, effected a more rapid decrease in hemoglobin content, hematocrit, and erythrocyte count than shown in control mice, especially during the first 10 months of feeding. It appeared that a tolerance to the Tween 60-5 percent diet effect was developed during the last 12 months of the investigation.

15. Tween 60-10 percent diet group mice exhibited significant quadratic regression of all hematological determinations on time on diet. Hematocrit regression, though showing the same type as controls, displayed a more pronounced rate and magnitude of decrease. In general, all values decreased at a much greater rate and amount than did control values. Statistically lower values (compared with controls) were shown for all hematological determinations, except mean corpuscular volume, for Tween 60-10 percent diet mice at both seven and 14 months on diet. Animals, at 10 months on diet, were in a condition approximating early stages of anemia. Slight hypochromasia was noted. However, there was an apparent response of the erythron, or possibly, development of extramedullary

hematopoiesis, which resulted in a resurgence of values at the 14 months examination. After this increase, a general decrease was found at the 18 and 22 months examination periods. Tween 60, fed at the 10 percent level, elicited an alteration in hematological pattern in the C_{57}BL/6 Jax mouse.

16. Tween 60, fed at either the five or 10 percent level, changes the hematological pattern in C_{57}BL/6 Jax mice. The magnitude and rate of decrease appear to be directly related to the concentration of Tween 60 present in the diet, the greater the quantity the greater and more rapid the alteration.

17. The present investigation demonstrated the practicability of a longitudinal experimental design in collection and analysis of hematological data using a rather small number of experimental animals. This type design would be applicable to other types of investigation in which experimental funds or facilities were limited.

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