EFFECTS OF OVERFEEDING NURSING OSBORNE-MENDEL AND S5B/P1 MALE RATS ON GASTROCNEMIUS MUSCLE WEIGHT AND DNA, PROTEIN, AND TRIGLYCERIDE CONTENT

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ABSTRACT

EFFECTS OF OVERFEEDING NURSING OSBORNE-MENDEL AND S5B/P1 MALE RATS ON GASTROCNEMIUS MUSCLE WEIGHT AND DNA, PROTEIN, AND TRIGLYCERIDE CONTENT

By

Gloria J. Harris

Male Osborne-Mendel and S5B/P1 rats were nursed by dams fed either a high fat (44% w/w) or low fat (3% w/w) diet beginning at parturition. At birth, the pups were grouped into litter sizes of either three or six and raised as such until weaning at 24 days of age. One-half of the pups from each litter size were supplemented with a milk mixture during the suckling period, whereas the others were handled but not supplemented. At weaning, one-half of the rats from each treatment were sacrificed and the remaining animals were fed the same diet as their respective dams up until 105 days of age.

Disregarding the effects of diet or treatments, Osborne-Mendel rats had mean body weights of 79±11.9 and 525±11.9 grams compared to 51±11.9 and 289±11.9 grams for the S5B/P1 rats at 24 and 105 days of age, respectively. These weights between strains were significantly different

at both ages ($P<0.01$). Within the Osborne-Mendel strain at 105 days of age, those rats fed the high fat diet weighed 588 ± 11.9 grams compared to 460 ± 11.9 grams for those fed the low fat diet. These values were significantly different ($P<0.01$).

Gastrocnemius muscle weights were not significantly different between comparative groups of S5B/Pl and Osborne-Mendel rats at weaning. Mean weights for all groups were 0.21 ± 0.06 and 0.31 ± 0.06 grams for S5B/Pl and Osborne-Mendel rats, respectively, at 24 days of age. At 105 days of age, the Osborne-Mendel rats had a significantly ($P<0.01$) greater gastrocnemius muscle weight than comparative groups of S5B/Pl rats. The mean tissue weight for all groups was 1.67 ± 0.06 and 2.58 ± 0.06 grams for S5B/Pl and Osborne-Mendel rats, respectively.

The content of gastrocnemius muscle DNA significantly differed ($P<0.01$) between Osborne-Mendel and S5B/Pl rats at both ages. The mean total DNA content for all groups in gastrocnemius muscle tissue in the Osborne-Mendel strain was 210 ± 25.7 and 635 ± 25.7 ug DNA at 24 and 105 days of age, respectively, In contrast, at 24 and 105 days of age, all groups of S5B/Pl rats had a mean total of 138 ± 25.7 and 410 ± 25.7 ug DNA, respectively, in gastrocnemius muscle tissue.

The protein content in gastrocnemius muscle did not significantly differ between Osborne-Mendel and S5B/Pl rats at 24 days of age. The S5B/Pl rats had an average of

42±20.5 mg protein for all groups, while the Osborne-Mendel rats had an average of 57±20.5 mg protein for all groups at weaning. At 105 days, a significant ($P<0.01$) difference existed between strains. Osborne-Mendel rats had a mean total tissue protein content of 58.3±20.5 mg for all groups, whereas the S5B/P1 rats had only 370±20.5 mg protein when all values were grouped. In general, muscle protein increased in proportion to muscle weight.

For comparative groups, Osborne-Mendel rats had a significantly ($P<0.01$) greater muscle triglyceride content than S5B/P1 rats at both ages. At 24 and 105 days of age, the mean values for all S5B/P1 groups were 0.34±0.37 gm/100 gm tissue triglycerides and 0.96±0.37 gm/100gm tissue triglycerides, respectively. The mean values for all groups in the Osborne-Mendel strain were 0.83±0.37 gm/100 gm and 3.5± 0.37 gm/100 gm tissue triglycerides at 24 and 105 days of age, respectively.

Within the Osborne-Mendel strain, all groups of rats fed the high fat diet had a significantly ($P<0.01$) greater muscle triglycerides content than those fed the low fat diet at 105 days of age. Osborne-Mendel rats fed the low fat diet had a mean value of 2.75±0.37 gm/100 gm tissue triglycerides, while those fed the high fat diet had 4.25±0.37 gm/100 gm tissue triglycerides.

Supplementation and litter size had no effect on muscle weight, DNA, protein, and triglyceride content.

However, there were significant interactions of these variables with age, strain, and diet.

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A THESIS

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In memory of my father

Mr. John Anderson Harris

and

to my mother

Mrs. Cloteal M. Harris

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INTRODUCTION

Obesity is one of the major nutritional diseases affecting the human population in affluent societies today. This disease is the result of excessive caloric intake over caloric expenditure or requirement. The net result is an increase of fat deposition within the body.

The etiology of obesity is yet to be elucidated. However, it is known that genetic influences, hormonal abnormalities, and emotional disturbances facilitate the occurrence of obesity (Gordon, 1970).

Nutritional obesity produced by feeding a high fat diet on an ad libitum basis to experimental strains of rats is well documented (Mickelsen, et al., 1955; Schemmel et al., 1969, 1970 a & b, 1972, 1973). These researchers noted that an increase in body weight due to an increase in body fat was the most obvious change resulting from feeding rats the high fat diet. This technique for producing obesity was felt to provide a good comparative model for the type of obesity that has become a serious clinical problem with human subjects.

Does an increased muscle mass also accompany an increased body weight? Body protein was found to increase

only after the 100th day of life in rats fed a high fat diet at weaning (Schemmel et al., 1969). This increase was attributed to an increase in protein in body compartments not specifically representative of muscle. Schemmel et al., (1973) subsequently showed that pups nursed by dams fed a high fat diet did accrue more body protein by weaning than those nursed by dams fed a grain diet. However, whether or not this persisted until adulthood was not clearly elucidated due to great individual variability in total body protein among older rats.

The primary purpose of this study was to assess whether or not gastrocnemius muscle weight, protein, DNA and triglycerides were increased in Osborne-Mendel and S5B/P1 rats subjected to various treatments, prior to weaning, known to produce obesity in some strains of rats. The Osborne-Mendel strain readily becomes obese when fed a high fat diet, while the S5B/P1 strain does not (Schemmel et al., 1970). The Osborne-Mendel and S5B/P1 strains served as obesity-susceptible and obesity-resistant models in this study.

REVIEW OF LITERATURE

Structure of Skeletal Muscle

Of the three types of muscle cells in the mammalian, avian, amphibian, and reptilian body, skeletal muscle cells predominate, accounting for 40 to 45% of the total body weight (Vander, 1975). This tissue is attached to the skeleton and is responsible for body movements.

An individual skeletal muscle cell is termed a fiber. Each fiber is surrounded by a membranous sheath, the sarcolemma. Numerous cylindrical subunits in parallel orientation called myofibrils compose each fiber. Interstitial spaces between myofibrils are filled with sarcoplasm. This fluid contains the mitochondria and sarcoplasmic reticulum (Zierler, 1974).

Myofibrils are highly organized structures with a distinctive repeating pattern. One unit of this pattern is called a sarcomere. Each sarcomere is composed of two types of protein units (myofilaments): thick and thin (Yost, 1972). The thin filaments contain the protein, actin, and are called I bands. Thin filaments attach to the ends of each sacromere. The thick filaments contain the protein, myosin, and are called A bands. These

filaments are located centrally within the sarcomere. The proteinaceous myofilaments are responsible for the contractility of each sarcomere (Yost, 1972).

Each sarcomere is bound on either end by a structure known as a Z-line. This structure is the anchoring point for thin filaments of adjacent sarcomeres (Guyton, 1971). Invaginations of the sarcolemma at the Z-line or junction between the A and I bands form the transverse tubular system (T-system). This system maintains intimate contact among sarcomeres in the myofibrils and carries interstitial fluid directly to every sarcomere (Zierler, 1974).

Development of Muscle Fibers

Skeletal muscle development begins during embryogenesis. Mesenchyme from the germinal mesoderm layer of the embryo differentiates into myogenic precursor cells (Fischman, 1972). Obligatory cell divisions of the myogenic precursor cells eventually results in presumptive myoblasts (Holtzer and Bischoff, 1970). Presumptive myoblasts are replicating cells which do not fuse or synthesize contractile proteins. The next stage of development, resulting from the proliferation of presumptive myoblasts, is characterized by the presence of myoblasts. These are mononucleated, non-replicating cells capable of fusion (Holtzer and Bischoff, 1970).

Fusion is a process of merger between myoblasts to form multinucleated, post-mitotic myotubes. It may occur

between mononucleated myoblasts, mononucleated myoblasts and multinucleated myotubes, and nascent multinucleated myotubes (Holtzer, 1970).

Fusion begins when primed, homotypic cells "recognize" other homotypic cells of the same origin and undergo surface interactions. Okazaki and Holtzer (1965) illustrated this by labeling cells from other tissues and incubating them in cultures in which myotubes were forming. The labeled, nonmyogenic cells were not incorporated into the forming myotubes. Thus, only true muscle cells are destined to undergo myogenesis.

Myotubular formation is followed by a period of development in which cell-specific protein synthesis occurs (Herrmann et al., 1970). The bulk of the actin and myosin myofilaments appear during this period (Fischman, 1970; Fischman, 1972).

With continued contractile protein synthesis, the emergence of definite sarcomeres occurs. Concurrently, the sarcoplasmic reticulum and transverse tubular systems develop. Myogenesis is completed with the final organization of the myofibrils and neural innervation (Holtzer, 1970).

Biochemical Aspects of Myogenesis

During myogenesis, muscle cells undergo two distinct developmental periods. The first is characterized by the mitotic activity of myogenic cells prior to fusion. It is

during this time that DNA synthesis predominates. Subsequent to fusion, myofibrillar protein synthesis occurs to the greatest extent.

Much evidence has accumulated within the last two decades to substantiate these statements. Stockdale and Holtzer (1961) studied DNA synthesis by tracing the incorporation of tritiated thymidine into myotubes by means of radioautography. These researchers found that the nuclei of the myotubes did not synthesize DNA. Morphologically, it was shown that myotubes were formed by the fusion of myoblasts, and that mitotic activity ceased after fusion.

These findings were also confirmed when a histophotometric method was used (Cox and Simpson, 1970). Reptilian muscle cells, representing an oncoming fusion population, did not incorporate tritiated thymidine. This was not observed in mononucleated myoblasts. These investigators concluded that nuclei within the myotubes had withdrawn from the mitotic cycle and that nuclear replication had occurred prior to fusion. A study of the morphological aspect of cellular differentiation and the onset of cell-specific protein synthesis showed that the appearance of the contractile protein apparatus synchronously followed fusion of the mononuclear cells (Coleman and Coleman, 1968).

Studies in which metabolic cycles have been inhibited have also proved useful in elucidating the chronological patterns involved in myogenesis. Bischoff and Holtzer (1970) used a pyrimidine analogue

(5-bromodeoxyuridine) to block synthesis of cell components while allowing cellular proliferation to continue. It was shown that cells containing nuclei with bromodeoxyuridine-DNA failed to fuse. The synthesis of the contractile protein apparatus was also inhibited.

Shainberg et al., (1969) showed that varying concentrations of Ca^{++} affected fusion. At the higher concentrations (1400uM $CaCl_2$), normal fusion and formation of multinucleated fibers were seen microscopically. Increasing the concentration of $CaCl_2$ in some cultures allowed fusion to occur.

A recent investigation by Celotti et al., (1973) substantiates previous studies most thoroughly. These researchers studied DNA, RNA and protein synthesis in chick embryo and rat skeletal muscle cells. DNA and RNA synthesis was very active during cell division, but at the onset of myoblast fusion, a severe reduction was observed in their synthesis. After fusion, protein synthesis increased to its highest level.

The mechanisms controlling myogenesis are unknown, but considerable data indicate that enzymatic systems are involved. O'Neill and Strohman (1969) found a marked decrease in DNA-polymerase activity at the onset of fusion. Separation of the myotubes from myoblasts showed that DNA-polymerase activity in the fused cells was lost, while activity was still exhibited in the single cells. In chick embryo musculature cells, a 100-fold decrease in

DNA-polymerase activity was observed from the tenth day of embryonic life to hatching (Stockdale, 1970). This decrease paralleled the loss of mononucleated skeletal muscle precursor cells during development in vivo.

Yaffe and Fuchs (1967) exposed 7 day old cultures of rat skeletal muscle to radioactive uridine. A decrease in the incorporation of labeled uridine into RNA following fusion of myoblasts into multinucleated muscle fibers was observed. The investigators attributed this decrease in incorporation to a reduction in the rate of RNA synthesis following cell fusion. This study substantiated the theory that RNA synthesis decreased immediately before the contractile protein apparatus came into being.

A study of the mechanisms of the synthesis of the contractile protein apparatus during embryonic differentiation demonstrated that actin synthesis was more predominant initially than myosin synthesis (Heywood and Rich, 1968). Tropomyosin synthesis increased at a later stage. Microphotographs of the protein components of myotubes have since shown that the actin filaments do appear prior to the myosin filaments (Herrman et al., 1970).

Growth

Body, organ, and tissue growth have been assessed by increases in weight for decades. The obvious fault with such a procedure is that it gives little information on the mechanisms of cellular growth.

The first study of cellular growth by chemical means was conducted by Enesco and Leblond (1962). These researchers determined cell number by a procedure based on the constancy of DNA per nucleus (6.2 ug) in diploid cells. The number of nuclei was obtained by dividing total DNA by the amount of DNA per nucleus. As one nucleus per cell is present in most structures, it was felt that the number of nuclei represented cell number.

Three stages of growth are known to occur at the cellular level (Enesco and Leblond, 1962; Winick and Noble, 1965; Winick and Noble, 1967). Hyperplastic growth is seen during the first stage. Weight, protein and DNA content proportionally increase with cell division predominantly occurring. An increase in DNA slower than the increase in protein and weight is seen during the second stage. Hyperplasia with concomitant hypertrophy describes this stage. Hypertrophic growth is exhibited during the third stage. Net protein and weight increase at the same rate, but DNA does not increase.

Enesco (1961) attributed growth of muscles to a hypertrophy of pre-existing muscle fibers with an increase in nuclear material within these fibers. A subsequent study provided similar results (Enesco and Puddy, 1964). Histometric and DNA determinations of gastrocnemius muscle in male Sherman rats showed that muscle growth was due to an increase in size of the fiber rather than number of fibers.

An increase in the number of nuclei with age was also observed.

The source of this increase in nuclear material was investigated by Moss and Leblond (1964). These researchers showed that "satellite" cells underwent mitosis, after which they donated daughter cells to be incorporated into the muscle fiber nuclei to provide additional DNA.

Winick and Noble (1965) observed growth patterns of body organs of the rat from 10 days after conception until adulthood. Each organ grew according to the three phases of cellular growth, despite different time patterns. The beginning of the last two phases, hyperplasia accompanied with hypertrophy and hypertrophy, were dependent on a slowing down and finally cessation of DNA synthesis. Total organ analysis and labeled thymidine incorporation techniques indicated that DNA synthesis ceased prior to net protein synthesis.

Other evidence indicates that the amount of DNA and the number of nuclei in skeletal muscle of the developing rat increases 10 fold for males from birth to 14 weeks of age. In female rats, growth is more accelerated during this period than males. The female rat has larger muscle cells during early postnatal life, but the male eventually has larger muscle cells (Cheek et al., 1971). An investigation of growth processes in quadriceps of male Sprague-Dawley rats up to 150 days of age demonstrated that DNA plateaued at about 90 days post-parturition. Afterwards

sarcoplasmic and myofilbrillar protein content of rat muscle quadriceps increased (Gordon et al., 1966).

Cheek et al., (1971) studied skeletal muscle growth in adolescent boys and reported that DNA increments could become as great as 20 fold during postnatal growth. For girls, this was approximately 14 fold. A 2 to 3 fold increase in muscle cell size during growth occurred in adolescent boys and girls.

Because a need for a comparative model from which extrapolations might possibly be made for applications to human muscle development existed, Cheek et al., (1975) extensively studied muscle development in subprimates (Macaca mulatta). These researchers reported that DNA in gastrocnemius muscle increased 8-fold from midgestation to term. The concentration of DNA in gastrocnemius muscle increased with maturity. The protein:DNA ratio increased 3-fold from midgestation to birth. At about 120 days of age, protein concentration reached a stable value.

Factors Affecting Muscle Growth

Effects of Exercise

Work (exercise) of skeletal muscle fibers results in muscle hypertrophy. Usually the number of myofibrils per fiber increases, resulting in an increase in the diameter of the fiber (Goldspink, 1964). This increase in the contractile portion of the fiber is how skeletal muscle adapts itself to an increase in the intensity of exercise

(Goldspink, 1964). An electron microscopic study of differentiating muscle fibers showed that indeed, fiber hypertrophy resulted as the number of myofibrils increased (Goldspink, 1970).

Compensatory hypertrophy was induced in the soleus and plantaris muscles of rats by sectioning the tendons of the gastrocnemius muscle (Goldberg, 1968). The plantaris increased in weight by 20% and the soleus by 40% in weight within a week. Histological examinations of the muscles indicated that the increased weight correlated with an increase in the diameter of the muscle fibers. Compensatory hypertrophy induced in hypophysectomized and alloxan-diabetic rats suggested that neither growth hormone nor insulin were necessary for work-induced hypertrophy to occur (Goldberg, 1968).

Examinations of rats subjected to running exercises showed an increase in the concentration of sarcoplasmic proteins in muscle fibers (Gordon et al., 1967). When these rats performed weight lifting exercises--reaching for food pellets with weights strapped onto their backs--an increase in the concentration of myofibrillar proteins was observed.

Hamosh et al., (1967) reported that tenotomy of the gastrocnemius and plantaris muscles caused compensatory hypertrophy of the soleus in rats within a week. In addition, DNA and RNA content of the soleus muscle increased.

Effects of Hormones

Hormones are known to affect muscle development. Usually they work in concert to regulate developmental changes. Although some hormones produce catabolic effects, most of the hormonal influence on musculature results from the anabolic effects of androgens, growth hormone, and insulin.

Androgens.--Androgens are assumed to have an indirect, if not direct, effect on muscle development, because the musculature in males is almost always larger than that in females (Goldspink, 1972). Breuer and Florini (1965) reported that androgens influenced the rate and extent of skeletal muscle growth in rats. Testosterone administration reversed the decrease in the synthetic activity of skeletal muscle ribosomes observed in castrated, immature male rats. These investigators attributed this effect to an increase in chromosomal mRNA production.

Buresova et al., (1969) found that testosterone increased the incorporation of ^{14}C-leucine into proteins of rat skeletal muscle. However, high concentrations of testosterone produced an almost complete block of protein synthesis.

Growth Hormone.--Growth hormone acts on every tissue in the body and possesses glycotrophic, pancreotrophic, glycostatic, diabetogenic, ketogenic, and lipotrophic properties (Donovan, 1970). A most interesting study by

Cheek et al., (1965) showed that hypophysectomized Sprague-Dawley rats and hereditary panhypopituitary Snell Smith mice exhibited reduced skeletal muscle growth when compared to control animals. The hypophysectomized rats showed a 50% reduction in muscle cell number by the third week of the experimental period. At the end of the nine week experimental period, Snell Smith mice had less DNA than that found in one-week old controls. Postnatal pituitary insufficiency was thought to contribute to the inhibition of DNA replication. Cheek et al., (1965) felt that growth hormone was probably concerned with cell multiplication.

Growth hormone administration to human patients with hypopituitarism increased cellular multiplication. A definite growth spurt, characterized by a rapid gain in muscle cell number was observed (Cheek et al., 1966).

The yield and activity of ribosomes from thigh muscle in hypophysectomized rats was shown to increase after growth hormone administration (Florini and Breuer, 1966). These investigators felt that growth hormone increased protein synthesis, although they noted that its action could possibly have been due to a decrease in protein degradation.

Conversely, Beach and Kostyo (1968) found an increase in DNA, rather than protein, in skeletal muscles of hypophysectomized rats following growth hormone treatment. Their study indicated that the increase in DNA

occurring with normal growth was dependent upon pituitary growth hormone.

Cheek and Graystone (1969) and Cheek and Hill (1970) looked at the effects of growth hormone, insulin, and epinephrine on growth in muscle, brain and liver of hypophysectomized rats. Results indicated that insulin was predominantly responsible for growth in cell size while growth hormone affected cell number to a greater extent. Epinephrine negated the anabolic effects seen with growth hormone.

The endocrine system appears to play a role in regulating myogenesis by affecting the activity of some enzymes in embryonic muscle development (Love et al., 1969). Chick leg musculature cells were studied from normal, hypophysectomized, and pituitary transplant embryos. In hypophysectomized embryos, there was a continued increase in DNA due to myoblast proliferation. No myotubular formation was observed. Glucose-6-phosphate dehydrogenase remained active throughout the experimental period. These phenomena were opposite of those seen in the normal embryo and, to a degree, the pituitary transplant embryo. That is, myotubes formed and glucose-6-phosphate dehydrogenase activity decreased to immeasurable amounts during embryonic life.

Turner (1972) reported that growth hormone stimulated protein synthesis in diaphragm muscle from non-hypophysectomized rabbits by increasing the uptake rate of

certain amino acids by the muscle cells. It was concluded that the stimulation of cellular protein synthesis from existing amino acid pools was the role of growth hormone in non-hypophysectomized animals.

The effect of growth hormone on the synthesis of nuclear and cytoplasmic DNA was explored by Goldspink and Goldberg (1975). The incorporation of labeled thymidine into nuclear DNA of liver, kidney, and muscle was depressed in hypophysectomized, male Charles River rats. Hypophysectomy had no effect on thymidine incorporation into mitochondrial DNA. The results indicated that growth hormone affected the synthesis of nuclear DNA more so than cytoplasmic DNA.

Many of the anabolic effects of growth hormone appear to be mediated through a factor called "sulfation factor" or "somatomedin" (Tanner, 1972). "Somato" connotes a relationship to the soma, its target tissue. "Medin" indicates that it is an intermediary substance. This agent is found in the serum of normal animals, provided they have an adequate supply of growth hormone.

Insulin.--The action of insulin on protein metabolism is similar to the effects of growth hormone. Insulin injection in normal rats causes growth of muscle mass, which is attributed to an increase in the muscle protein:DNA ratio (Cheek et al., 1971). This effect is also seen in tissues of hypophysectomized rats.

Recent evidence indicates that insulin acts through an intermediate translation factor which enhances the ability of ribosomes to bind mRNA and hence to form polysomes. The net result is to activate the ribosomes so that they can synthesize protein (Wool et al., 1972). Although individual effects of insulin and growth hormone can be demonstrated, experimental evidence strongly supports a synergistic influence of these hormones on muscle development.

Effects of Nutrition

Undernutrition.--The effect of the level of nutrition on muscle growth has long been recognized. Obviously, malnutrition has been the subject of most research. The effect of undernutrition before and/or after weaning delays growth and development to an extent which varies with its severity. Rats deprived of adequate nourishment during suckling gained weight more rapidly after rehabilitation, but did not catch up to normal rats (McCance and Widdowson, 1962).

Similar results were obtained in piglets subjected to severe undernutrition at weaning. Rehabilitation produced a rapid increase in weight and growth in size with catch-up growth being almost complete by one year of age (McCance and Widdowson, 1962).

Winick and Noble (1966) reduced cell number in gastrocnemius muscle of rats by feeding them a diet

restricted to 50% of the total caloric intake of controls prior to weaning. This reduction was seen even after refeeding. When the insult occurred after weaning, complete recovery was possible.

Protein-calorie malnutrition produced a reduced muscle mass in infants studied by Cheek et al., (1970). The subjects ranged in age from four months to 2 1/2 years of age. The loss of cellular size accounted for the greatest loss of muscle mass. Protein/DNA and RNA/DNA ratios were significantly reduced even after rehabilitation. The results suggested that either a prolonged period of recovery was necessary or a permanent alteration in the ability to synthesize protein had occurred.

Hill et al., (1970) examined the effect of protein-calorie and isolated caloric restriction on the cellular growth of muscle and liver in weanling rats. The experimental period lasted from days 21 through 48 days of life. The calorie restricted diet caused a slowing in DNA production, but it was adequate for protein synthesis in both liver and muscle. A reduction of carcass fat was also observed.

The protein-calorie restricted diet produced more dramatic effects. Protein and DNA synthesis were minimal. Ribosomal RNA was very low compared to controls. The calorie:protein ratio suggested that considerable protein was used to provide calories.

Howarth and Baldwin (1971) studied the effects of nutrition on protein and nucleic acid metabolism in gastrocnemius muscle of male Sprague Dawley rats at three weeks of age. Restriction of food availability (50-60% of ad libitum intake of controls) reduced DNA, RNA, and protein content in the muscle. Following rehabilitation, DNA and RNA accumulation exceeded that during normal growth. Protein synthesis after refeeding was similar to that during normal growth.

Hill et al., (1971) reported findings on the chemical composition of certain organs and the carcasses of fetal rhesus monkeys subjected to intrauterine growth retardation (IUGR). These experimental animals were removed from the uterus at the 100th day of gestation and the interplacental blood vessels supplying the placenta were ligated. The fetuses were then returned to the uterus. These animals were taken by cesarian section during the 156-160th days of gestation. Analyses showed that the muscle composition was similar to controls except that a lower percent fat (0.76%) was found in the IUGR group. Controls had 1.0% fat in the muscle. Total amounts of DNA, RNA, and protein were significantly lower than in controls.

Howarth (1972) fed male weanling Wistar strain rats, in groups of 15 each, protein-deficient diets containing 6, 12, and 18% casein. Controls were fed a diet with 24% casein. A fifth group, designated the initial group, was killed at the beginning of the experimental period to

determine DNA, RNA, and protein content of gastrocnemius muscle prior to feeding the deficient diets. Groups were killed after a 14 day feeding period.

Protein and DNA content increased at rates less than normal in rats fed the 12 and 18% diets when compared to those fed the 24% diet. The 6% diet inhibited DNA synthesis but did permit some protein synthesis. Howarth (1972) concluded that protein synthesis had priority over DNA synthesis when muscle growth was retarded by protein deprivation.

Overnutrition.--Studies on the effects of overnutrition during suckling (Widdowson and McCance, 1960; Lat, Widdowson, and McCance, 1961) showed that rats made to grow fast by an abundance of food during the suckling period attained greater increments in body weight. These animals were more active and inquisitive than rats raised 18 per litter also (Lat et al., 1960).

A study of the effects of overnutrition on growth was conducted by Winick and Noble (1967). By reducing litter size from twelve to six or three rats per litter, these researchers showed that organ and tissue cellularity could be increased by nutritional overfeeding. Those animals grown three or six per litter showed no difference in tissue analyses however. The increase in growth was attributed to an increase in cell division.

Overnutrition has also been produced by increasing the caloric intake of Osborne-Mendel pups during suckling.

This was accomplished by feeding dams a high fat diet (60% w/w) during lactation which doubled the milk fat content (Schemmel et al., 1973). Pups of these dams were found to have greater body weights, body fat, water and protein at weaning (21 days) when compared to pups fed a grain ration. The effects of this early feeding were no longer in evidence for body weight and body protein of these rats at 168 days of age.

Czajka-Narins and Hirsch (1974) used a supplementation technique during the pre-weaning period to produce adipose tissue hypercellularity in rat pups. Marked changes were seen in carcass composition. The percent fat increased significantly ($P<0.01$) in supplemented pups over controls quite early in the experimental period. At 20 days of age, supplemented animals had 21% carcass fat compared to 14% for controls. At 105 days of age, the percent protein in the carcass of supplemented rats was significantly greater ($P<0.05$) than in controls. Supplemented animals had 19% protein compared to 13% for controls.

METHODOLOGY

Experimental Animals, Rations, and Conditions

Eighty Osborne-Mendel and 80 S5B/P1 male rats were used in this study. Table 1 gives details of their assignments to their respective treatments.

At birth the pups were grouped into litter sizes of either three or six and raised as such until weaning at 24 days of age. Up until weaning they suckled from dams who were fed either a low fat or high fat diet beginning at parturition.

The low fat (LF) ration (3% fat, w/w) contained 3.8 digestible kilocalories per gram of ration eaten. The high fat (HF) ration (44% fat, w/w) contained 5.6 digestible kilocalories per gram of ration eaten. The composition of the rations are presented in Table 2. The animals were fed the rations and water on an ad libitum basis.

From birth until weaning, one-half of the pups from both litter sizes were supplemented with a milk mixture. The animals were force-fed between 8-9 a.m. and 4-5 p.m. daily. An intubation technique in which the animals were fed with a gastric syringe was used for the artificial feeding of the pups (Miller and Dymsza, 1963). To reduce

the variability caused by handling of the force-fed pups, the non-supplemented animals were also handled.

The composition of the milk mixture simulated that of dams fed either a low-fat or high-fat diet. The method found in The Official Methods of Analysis of the Association of Official Analytical Chemists was used for the determination of fat in the dams' milk (Horwitz, 1970). Analysis showed the fat content to be variable, but the amount of fat used to make up the simulated milks represented averages.

The percentages of fat in the milk mixtures with which the Osborne-Mendel pups were supplemented when nursed by dams fed the low fat and high fat diets were 18% and 24%, respectively. The percentages of fat in the milk mixtures with which the S5B/P1 pups were supplemented when nursed by dams fed the low-fat or high-fat diets were 15% and 20%, respectively. The four milk mixtures were made by adding the above percentages as ml of corn oil to 15 grams of non-fat dry milk solids, and making it to a final volume of 100 ml with distilled water. Milk mixtures were vigorously shaken before force-feeding to assure mixture of ingredients.

At weaning, animals given the same treatment were divided into groups of 12. One-half (6) of the rats from each treatment were killed at this time by light anesthetization with anhydrous ethyl ether and decapitation. Following this, the rats were exsanguinated.

The remaining animals, counterparts of those sacrificed, were fed the same ration as had been fed their respective dams. These rats were raised until 105 days of age. At this time, they were killed according to the procedure given above. In some instances, one rat was lost to the study. Therefore, all groups were reduced to 5 rats in order to equalize numbers. This was done randomly. Thus, thirty-two groups of five rats each were analyzed.

The rats sacrificed at 105 days of age were housed in individual wire screen cages (18 X 18 X 25 cm). A controlled temperature of 23° C $\pm$ 1° C was maintained in the laboratory throughout the study. Conditions were regulated such that each 12 hour period of light was followed by 12 hours of darkness.

Care was taken to never disrupt the natural activity patterns of the animals. Disturbances were kept at a minimum during cage cleaning, feeding, and handling.

Procedure for Removal of Gastrocnemius Muscle

Following excision of the left lower leg, a longitudinal slit was made along the posterior aspect of the limb's entire length. The skin was then pulled back for exposure of the muscles. The scissors were carefully inserted into the groove between the tendons of the plantaris and gastrocnemius muscles. A transverse cut of the tendons at the origin and insertion was made to free the gastrocnemius muscle.

The tissue was immediately quick-frozen on dry ice. After weighing, each tissue was individually wrapped in aluminum foil, placed into a self-sealing plastic bag, and stored at -18° C until analyses occurred.

Procedure for DNA Analysis

Samples

On the day of analysis, the frozen tissues were thawed at room temperature and placed into individual test tubes filled with cold deionized water; the volume of which would give a final 1:10 dilution of muscle tissue to water. All tubes were kept on ice in an ice chest.

For homogenization, each test tube was placed in a small ice bath and the contents homogenized at an intensity speed of 8 for one minute on a Polytron homogenizer.[1] After each tissue was homogenized, the generator was rinsed with deionized water, ethanol, and finally acetone. Tissue debris was removed before homogenizing another sample.

For analysis of DNA a 1 ml aliquot of the homogenate was pipetted into appropriate clean, labeled 15 ml corex centrifuge tubes containing 5 ml of cold 20% trichloro-acetic acid. The centrifuge tubes were vortexed for 15-20 seconds and then spun for 20 minutes at 5000 rpm in a

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[1]Brinkmann Willems Polytron, Brinkmann Instruments, Inc., Cantiague Road, Westbury, New York.

refrigerated centrifuge[2] held at -5° C. The supernatant was discarded and 6 ml of 5% trichloroacetic acid were added to the pellet. The centrifuge tubes were placed in a 90° C water bath for 30 minutes, after which they were cooled on ice for 10 minutes. The tubes were then re-spun for 20 minutes at 5000 rpm in the refrigerated centrifuge.

For the assay, DNA was determined by the procedure of Burton (1956). Duplicate 2 ml aliquots of the supernatant were placed into clean, labeled test tubes. To this volume, 4 ml of diphenylamine-acetaldehyde solution were added. The tubes were vortexed for 15 seconds and incubated at room temperature for 16-20 hours.

Standards

For the assay, the standard solution was diluted with 5% trichloroacetic acid into concentrations of 10, 20, 30, 70, and 100 ug in the following manner:

ug DNA	0	10	20	30	70	100
ml Standard	0.0	0.2	0.4	0.6	1.4	2.0
ml 5% TCA	2.0	1.8	1.6	1.4	0.6	0.0

[2]Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge Ivan SORVALL Inc., Newtown, Connecticut.

A blank containing 2 ml of 5% trichloroacetic acid was also run in duplicate. The blanks and standards were treated with the diphenylamine-acetaldehyde solution as were the unknowns and incubated for 16-20 hours at room temperature. This series of tubes was run for each batch of samples.

The blanks, standards, and unknowns were read on a DB spectrophotometer[3] at a wavelength of 595 nm. After placing appropriate sized blank samples into the reference and unknown standard liquid cells,[4] the instrument was zeroed to 100% transmission. Standards and unknowns were then read against the blank.

The instrument was then set at a wavelength of 700 nm and the blanks, standards, and unknowns were read again. The readings at 700 nm were subtracted from the readings at 595 nm to obtain the final readings. Readings at 700 nm were usually negligible, if any reading was obtained at all.

The % transmission values were converted to values of absorbance by using a standardized chart. The extinction coefficient of the standards was used to obtain the concentration of DNA in the unknowns. This was accomplished by dividing the absorbance reading by the extinction

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[3]Beckmann Spectrophotometer Model DB, Beckmann Instruments, Inc., Fullerton, California.

[4]Beckmann Liquid Cells, Beckmann Instruments, Inc., Fullerton, California.

coefficient. The following calculation was used to determine total tissue DNA:

$$\left[\frac{\text{ug DNA/tube}}{\text{aliquot size}} \times \frac{\text{ml of 5\% TCA}}{\text{sample size}}\right] \times \left[\text{total volume of homogenate}\right]$$

Mean total tissue DNA was obtained by taking an average of the duplicate values for each sample. If the concentration of DNA in duplicates of a sample differed by more than 5%, analysis of the sample was repeated. Appendix A includes details of reagent preparations and a procedural summary.

Procedure for Protein Analysis

Samples

Tissue, prepared as described for DNA, was analyzed for protein using Lowry's procedure (1951). Homogenate aliquots of 0.25 ml size were placed into appropriate clean, labeled 15 ml corex centrifuge tubes containing 5 ml of 10% trichloroacetic acid. The tubes were vortexed for 15 seconds and spun in a refrigerated centrifuge at -5° C for 20 minutes at 5000 rpm. The supernatant was decanted and 10 ml of 1N sodium hydroxide were added to the pellet in each tube. The pellet was dissolved by continuous vortexing.

Duplicate 0.5 ml samples were placed in a series of clean, labeled test tubes. The samples were made to a volume of 1 ml by adding 0.5 ml of deionized water. The tubes were mixed for 10 seconds by vortexing.

To each tube, 5 ml of a 2% sodium carbonate-0.02% sodium tartrate plus 0.5% cupric sulfate solution were added. The tubes were simultaneously being vortexed to insure proper complexing of the copper with protein.

After waiting 10 minutes, 0.5 ml of 1N Folin-Ciocalteau reagent was added to each tube while concurrently, mixing occurred. The tubes were then incubated at room temperature for 30 minutes to insure maximum color development.

Standards

For the assay, 1 ml of bovine albumin standard (BAS) was diluted with 9 ml of 1N sodium hydroxide in a 10 ml volumetric flask. The contents were thoroughly mixed and further dilutions for working standards were prepared in the following manner:

ml of BAS	BAS (ug)	ml deionized H_2O
0.0	0	1.0
0.1	50	0.9
0.2	100	0.8
0.3	150	0.7
0.4	200	0.6
0.5	250	0.5
0.6	300	0.4

A blank consisting of 1 ml of deionized water was also run in duplicate. The blanks and standards were treated with 5 ml of the 2% sodium carbonate-0.02% sodium tartrate plus 0.5% cupric sulfate solution in the same manner as the unknowns. After adding the Folin-Ciocalteau reagent, the tubes were incubated for 30 minutes. The blanks and standards were run each time unknowns were run.

Blanks, standards, and unknowns were read on a spectrophotometer (noted on page 27) at a wavelength of 740 nm. The instrument was adjusted to 100% transmission with appropriate sized samples of the blank in the reference and unknown liquid cells. Standards and unknowns were read against the blank. Absorbance values were obtained by conversion from % transmission values through the use of a standardized chart. The concentration of protein in the unknowns was obtained by dividing the absorbance values of the unknowns by the extinction coefficient of the standards. The following calculations were used to determine total tissue protein:

$$\left[\frac{\text{ug protein/tube}}{\text{aliquot size}} \times \frac{\text{ml of 1N NaOH}}{\text{sample size}}\right] \times \left[\text{total volume of homogenate}\right]$$

Mean total tissue protein was obtained by taking an average of the duplicate values. If values for protein concentration in duplicates of a sample differed by more than 5%, analysis of the sample was repeated. Details of

reagent preparations and a summary of procedures are given in Appendix B.

Procedure for the Fluorometric Determination of Triglycerides

Samples

The procedure for homogenization has been previously described. Within certain groups at 24 days of age, homogenate was pooled due to insufficient sample volume. Homogenate aliquots (0.5 ml) were pipetted with a micropipette[5] into a series of 15 ml corex centrifuge tubes each containing 9.5 ml of 99% isopropanol. The tubes were covered with parafilm and vortexed for 30 seconds. Approximately 2 gm zeolite mixture were added to each isopropanol extract. The tubes were covered again and vortexed for another 30 seconds.

The samples were then allowed to stand for 30 minutes, during which time they were inverted once very ten minutes and gently resuspended. The tubes were centrifuged at 2000 rpm for 15 minutes in a refrigerated centrifuge at 0° C. The supernatant from each centrifuge tube was carefully decanted into clean, labeled screw-capped culture tubes with Teflon-lined caps. They were stored in a refrigerator at 4° for approximately 48 hours before analysis.

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[5]Centaur Micropipette with polypropylene delivery tip. Cole-Palmer, Chicago, Illinois.

Standards

Triolein in a concentration of 1000 mg/100 ml served as the stock standard. The reagent was diluted with 99% isopropanol in volumetric glassware as shown in the following protocol:

mg Triolein/100 ml	50	100	150	200	250	300
ml of Standard	5	10	15	20	25	30
ml of 99% isopropanol	95	90	85	80	75	70

The standards were diluted and treated in the same manner as the unknowns except that 9.0 ml of 99% isopropanol and 0.5 ml of water were placed in the centrifuge tubes. To this, 0.5 ml of the appropriate stock standard was then added.

After extraction, the standards were stored under refrigeration in clean, labeled screw-capped culture tubes with Teflon-lined caps for 48 hours prior to analysis. A blank consisting of 9.5 ml of 99% isopropanol and 0.5 ml of deionized water was also extracted.

The semi-automated procedure used for the determination of triglycerides was based on the work of Kessler and Lederer (1965). This system of automated analysis is commercially available as the Technicon AutoAnalyzer.[6]

[6]Technicon Instruments Corporation, Tarrytown, New York.

The system used was composed of a fluorometer, recorder, heating bath, proportioning pump, sampler, and triglyceride platter.

The fluorometer was allowed 15-20 minutes to warm up before using. During this time, the heating bath was switched "on" and adjusted to 50° C. The recorder was "on" also, but the chart drive remained in the "off" position.

After checking to insure that tubing was in working condition, the triglyceride platter was fitted onto the proportioning pump. Reagents were pumped through the system in the following manner:

A. Solutions of deionized water and methanol:HCL were pumped consecutively for 30 minutes to clean all tubing.

B. Approximately 500 ml of 80% isopropanol and potassium hydroxide were put into individual 600 ml Erlenmeyer flasks and pumped for 8 minutes.

C. After the 8 minutes, 500 ml of the periodate and acetylacetone were put into individual Erlenmeyer flasks and pumped.

With all the reagents pumping through the system, the chart drive was switched "on" and a 5% baseline was set. The high standard (300 mg/100 ml) was then pumped through. If the high standard did not read 95% on the chart, it was adjusted to that value with the Full Scale Reference. The samples were read after switching the sampler to automatic.

For the assay, duplicates of the blank, standards, and unknowns were run. Approximately 2 ml of each extract were placed in individual 4 ml conical sample cups.

Sampling speed was 30 determinations/hour, giving a net rate of 15 samples/hour.

Approximately 0.1 ml of lipid extract was sampled into an air-segmented 80% isopropanol solution. The potassium hydroxide was added on-stream and saponification of the triglycerides to glycerol occurred on-stream in a 50° C heating bath. After saponification, the glycerol was oxidized to formaldehyde after adding the periodate reagent. The working acetylacetone reagent was added for condensation to give the fluorescent product, 3,5-diacetyl-1,4-dihydrolutidine.

Values were obtained by counting units from zero to the peak height and subtracting the baseline value to get the actual height. The peak height of the water blank was subtracted from peak heights of the standards and unknowns to get final heights. By dividing the final heights of the unknowns by the extinction coefficient of the standards, the concentration of triglycerides in the unknowns was determined. The following calculations were used to determine total mg triglyceride/100 gm tissue:

$$\frac{\text{units of final height}}{\text{extinction coefficient}} = \text{mg triglyceride/100 gm homogenate}$$

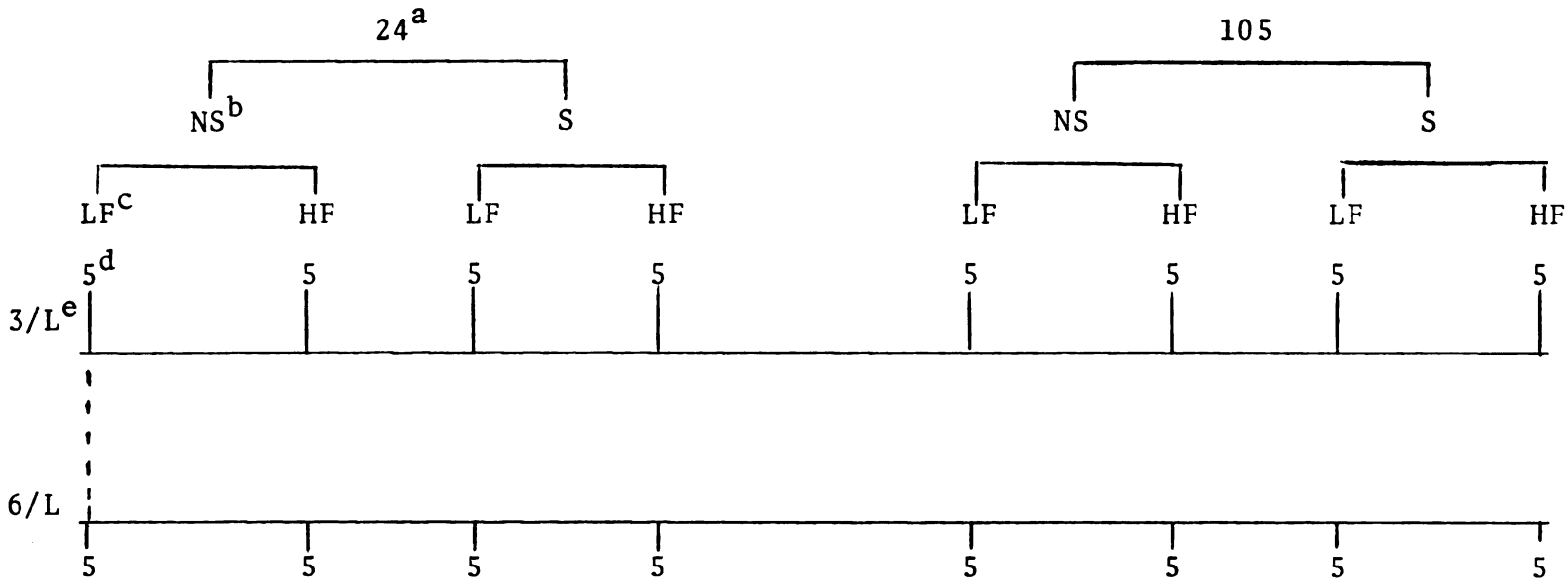
$$[\text{mg triglyceride/100 gm homogenate}] \times [\text{total volume homogenate}]$$

Details of the reagent preparations and a summary of the procedure used are given in Appendix C.

STATISTICAL ANALYSES

Means and standard errors of body weights, gastrocnemius muscle weights, and DNA, protein, and triglycerides were determined for the 32 groups. The significance of these five variables were determined by Yates' Method for Factorial Analysis (Cochran and Cox, 1957). This analysis of variance test measured mean differences of the main effects and interactions of the five independent variables (age, strain, diet, litter size, and supplementation). An explanation of the test is given in Appendix D.

TABLE 1.--Experimental Design.



[a]Refers to age (in days) at sacrifice.

[b]NS = non-supplemented; S = supplemented. See methodology for explanation.

[c]LF = low-fat diet; HF = high-fat diet.

[d]Number refers to animals per group. This design was repeated for the S5B/Pl and Osborne-Mendel strains.

[e]Pups were raised in litters of 3 or 6 rats.

TABLE 2.--Composition of Low Fat (LF) and High Fat (HF) Diets.

Ingredients	Diets (gm)	
	LF	HF
Protein (Casein)[a]	22.00	22.00
Salt Mix[b]	4.00	4.00
Non-Nutritive Bulk[c]	2.00	2.00
Vitamin Mix[d]	1.00	1.00
DL-Methionine[e]	0.10	0.10
Aureomycin[f]	0.01	0.01
Liver Mix[g]	1.50	1.50
Carbohydrate (Cerelose)[h]	66.39	7.00
Fat		
(Crisco)	-	29.51
(Corn Oil)	3.00	-
Total Weight	100.00	67.12

[a]Casein purchased from General Biochemical, Chagrin Falls, Ohio.

[b]Rogers and Harper Salt Mix purchased from General Biochemicals, Chagrin Falls, Ohio.

[c]Cellulose type purchased from General Biochemicals, Chagrin Falls, Ohio.

[d]A.O.A.C. Vitamin Mix purchased from General Biochemical, Chagrin Falls, Ohio. Supplied the following (gm/kgm diet): p-aminobenzoic acid, 0.10; Vitamin B_{12}, (0.1% in mannitol), 0.03; biotin, 0.004; calcium pantothenate, 0.04; choline, free base, 2.0; folic acid, 0.002; 1-inositol, 0.10; menadione, 0.005; niacin, 0.04; pyridoxine HCL, 0.04; riboflavin, 0.008; thiamine HCL, 0.005; dextrose, anhydrous, q.s.; (units/kgm): Vitamin A, 20,000.00; Vitamin D_2, 2,000.00; Vitamin E acetate, 100.00.

[e]Purchased from General Biochemicals, Chagrin Falls, Ohio.

[f]Provided by American Cyanamid Co., Princeton, New Jersey.

[g]Purchased from General Biochemicals, Chagrin Falls, Ohio.

[h]Purchased from Michigan State University General Stores, East Lansing, Michigan.

TABLE 2.--Continued.

ENERGY VALUE OF DIETS

Digestible Energy[a]

	Diets	
	LF	HF
Carbohydrate (kilocalories)	266.00	28.00
Protein (kilocalories)	88.00	88.00
Fat		
(Crisco) (kilocalories)	-	265.00
(Corn Oil) (kilocalories)	27.00	-
Total Digestible Energy (kilocalories)	381.00	381.00
Digestible Energy (kilocalories/gm)	3.81	5.67

[a]Values used for determining kilocalories were 4, 4, and 9 for 1 gm of protein, carbohydrate, and fat, respectively.

TABLE 3.--Body Weights (gms) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 24 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	49[d,e]	55	51	53	74	83	72	85
6/L	47	50	47	49	66	77	72	90

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low-fat diet; HF = high-fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±11.9.

[e]All groups of the same treatment were significantly different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

TABLE 4.--Body Weights (gms) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 105 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	280[d,e]	286	298	298	433	589[f]	456	562
6/L	279	282	259	329	470	571	481	634

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low-fat diet; HF = high-fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±11.9.

[e]All groups of identical treatment were significant different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

[f]Osborne-Mendel high-fat fed rats were significantly different ($P<0.01$) from those fed low-fat diets.

TABLE 5.--Gastrocnemius Muscle Weights (gms) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 24 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	0.21[d]	0.21	0.22	0.22	0.30	0.32	0.30	0.30
6/L	0.22	0.20	0.20	0.21	0.27	0.28	0.30	0.37

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low-fat diet; HF = high-fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ± 0.06.

[e]No significant difference between groups treated identically for S5B/P1 and Osborne-Mendel rats.

TABLE 6.--Gastrocnemius Muscle Weights (gms) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 105 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	1.75[d,e]	1.47	1.74	1.66	2.52	2.61	2.50	2.53
6/L	1.73	1.63	1.59	1.81	2.60	2.58	2.71	2.59

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low-fat diet; HF = high-fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±0.06.

[e]All groups of identical treatment were significantly different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

TABLE 7.--DNA Content of Gastrocnemius Muscle (ug) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 24 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	140[d,e]	104	128	178	240	210	196	213
6/L	141	116	165	133	199	183	204	237

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low-fat diet; HF = high=fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±25.7.

[e]All groups of identical treatment were significantly different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

TABLE 8.--DNA Content of Gastrocnemius Muscle (ug) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 105 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	408[d,e]	436	442	399	696	546	581	653
6/L	308	346	364	509	677	561	640	726

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low-fat diet; HF = high-fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±25.7.

[e]All groups of identical treatment were significantly different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

TABLE 9.--Protein Content of Gastrocnemius Muscle (mg) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 24 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	43[d,e]	44	47	41	65	58	57	58
6/L	42	40	43	38	52	55	57	55

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low fat diet; HG = high fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±20.5.

[e]No significant difference between groups treated identically for S5B/P1 and Osborne-Mendel rats.

TABLE 10.--Protein Content of Gastrocnemius Muscle (mg) of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 105 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	362[d,e]	368	439	348	507	636	592	523
6/L	362	377	362	347	540	675	662	529

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low fat diet; HF = high fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±20.5.

[e]All groups of identical treatment were significantly different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

TABLE 11.--Triglycerides Content of Gastrocnemius Muscle (gm/100 gm) in S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 24 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	0.32[d,e]	0.13	0.35	0.40	0.79	1.01	0.88	0.94
6/L	0.23	0.39	0.42	0.46	0.59	0.89	0.65	0.58

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low fat diet; HF = high fat diet.

[c]Pups raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±0.37.

[e]All groups of identical treatment were significantly different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

TABLE 12.--Triglycerides Content of Gastrocnemius Muscle (gm/100 gm) in S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 105 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS[a]		S		NS		S	
	LF[b]	HF	LF	HF	LF	HF	LF	HF
3/L[c]	1.35[d,e]	1.06	1.13	0.75	2.33	5.25[f]	2.64	3.30
6/L	0.54	1.10	0.86	0.96	2.83	3.83	3.25	4.73

[a]NS = non-supplemented; S = supplemented. See methodology for explanation.

[b]LF = low fat diet; HF = high fat diet.

[c]Pups were raised in litters of 3 or 6 rats.

[d]Standard error of each group of five rats is ±0.37.

[e]All groups of identical treatment were significantly different ($P<0.01$) for S5B/P1 and Osborne-Mendel rats.

[f]Osborne-Mendel high-fat fed rats were significantly different ($P<0.01$) from those fed low-fat diets.

TABLE 13.--Gastrocnemius Muscle Protein:DNA Ratio of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 24 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS		S		NS		S	
	LF	HF	LF	HF	LF	HF	LF	HF
3/L	0.30:1	0.42:1	0.36:1	0.23:1	0.27:1	0.27:1	0.29:1	0.27:1
6/L	0.29:1	0.34:1	0.26:1	0.28:1	0.26:1	0.30:1	0.27:1	0.23:1

TABLE 14.--Gastrocnemius Protein:DNA Ratio of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 105 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS		S		NS		S	
	LF	HF	LF	HF	LF	HF	LF	HF
3/L	0.89:1	0.84:1	0.99:1	0.87:1	0.72:1	1.16:1	1.0:1	0.80:1
6/L	1.17:1	1.08:1	0.99:1	0.68:1	0.79:1	1.20:1	1.0:1	0.72:1

TABLE 15.--Percent Protein of Gastrocnemius Muscle of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 24 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS		S		NS		S	
	LF	HF	LF	HF	LF	HF	LF	HF
3/L	20	20	21	18	21	18	19	19
6/L	19	20	21	18	19	19	19	14

TABLE 16.--Percent Protein of Gastrocnemius Muscle of S5B/P1 and Osborne-Mendel Rats Exposed to Various Treatments at 105 Days of Age.

	S5B/P1				Osborne-Mendel			
	NS		S		NS		S	
	LF	HF	LF	HF	LF	HF	LF	HF
3/L	20	25	25	20	20	24	23	20
6/L	20	23	22	19	20	26	24	20

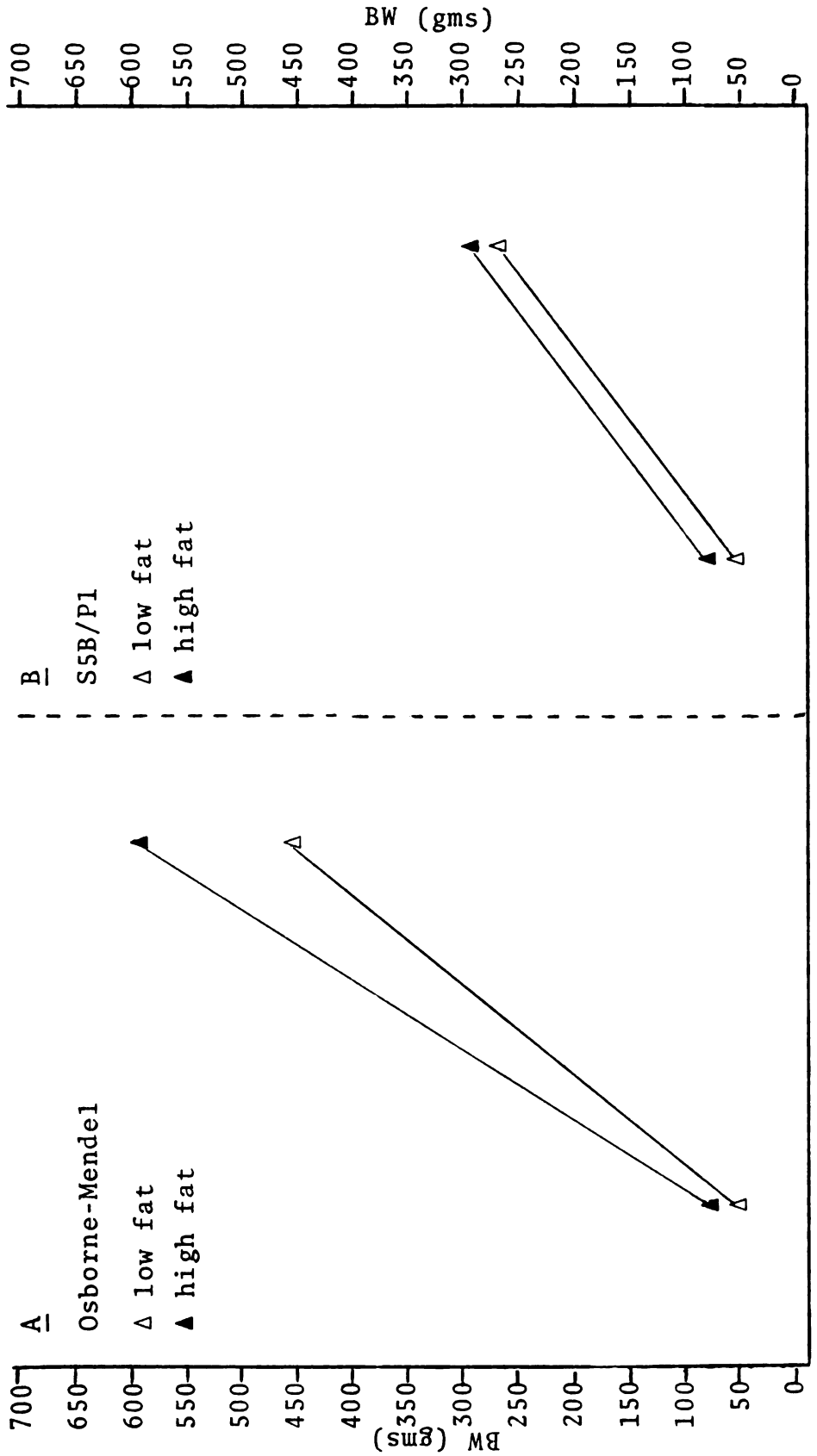


FIGURE 1.--Effect of a Low Fat or High Fat Diet on Body Weights of Osborne-Mendel and S5B/P1 Rats.

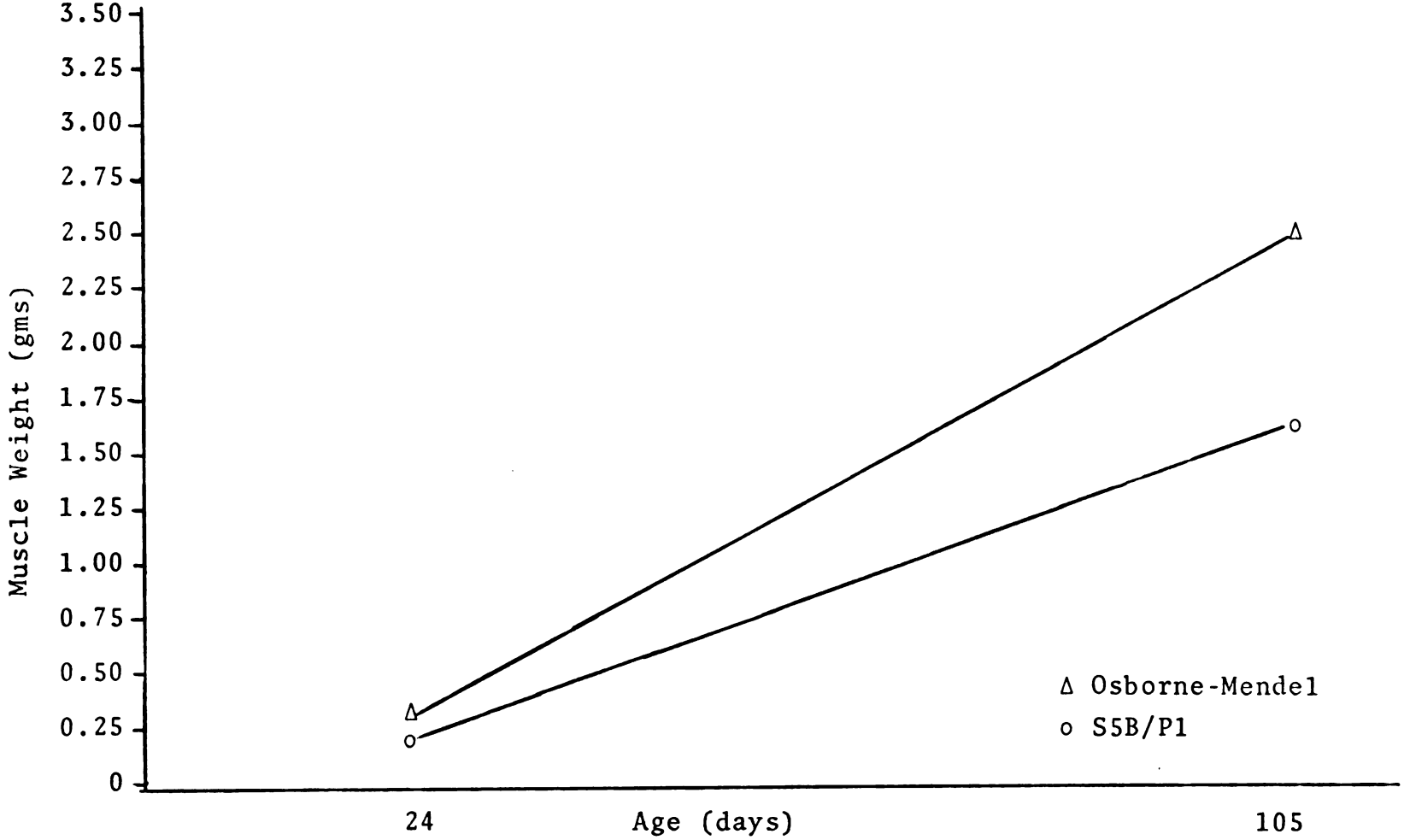


FIGURE 2.--Effect of Age on Gastrocnemius Muscle Weights in Osborne-Mendel and S5B/P1 Rats.

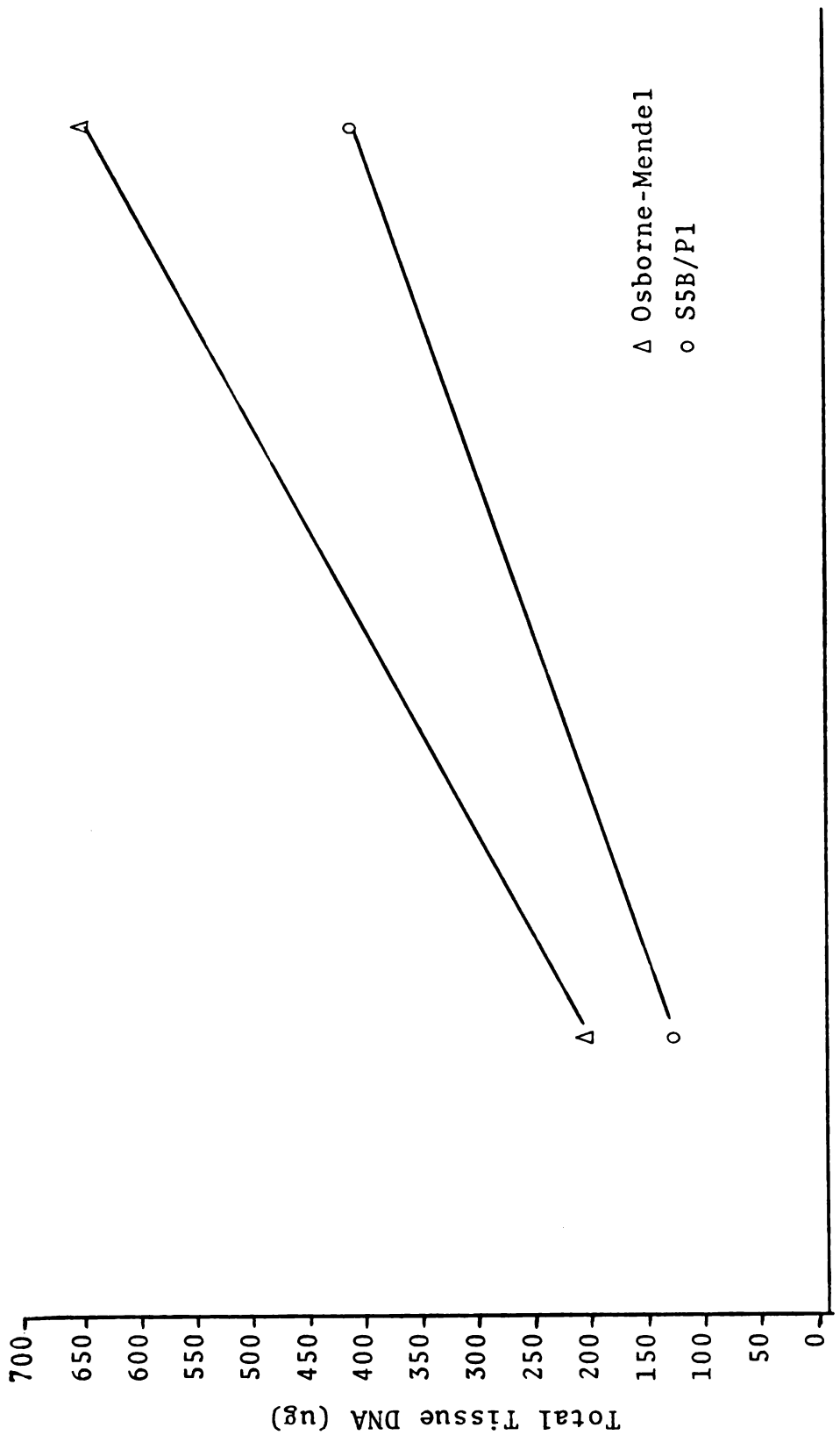


FIGURE 3.--Effect of Age on Gastrocnemius Muscle DNA in Osborne-Mendel and S5B/P1 Rats With Age.

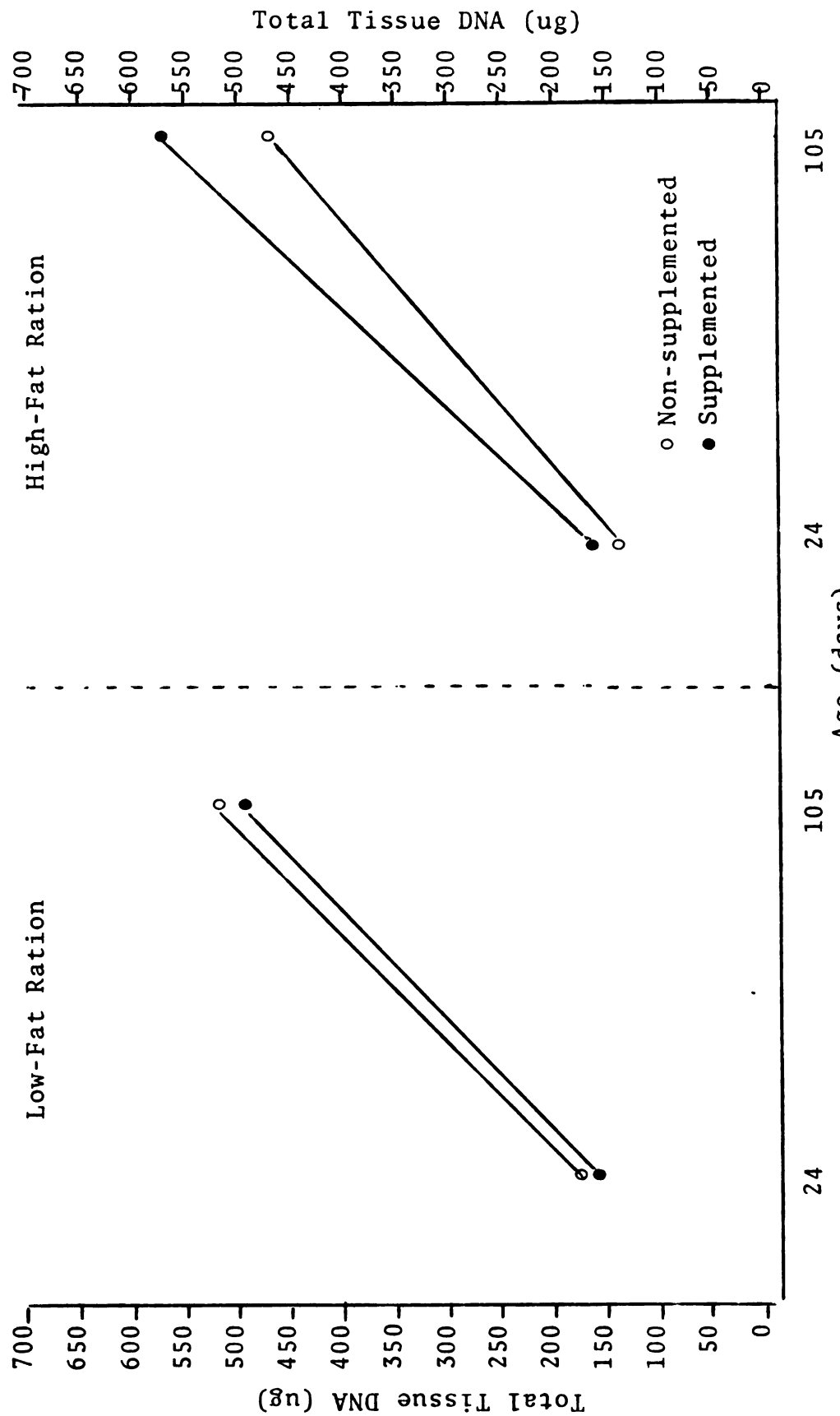


FIGURE 4.--Effect of Supplementation on Gastrocnemius Muscle DNA Content in Osborne-Mendel and S5B/P1 Rats Fed a Low-Fat or High-Fat Diet.

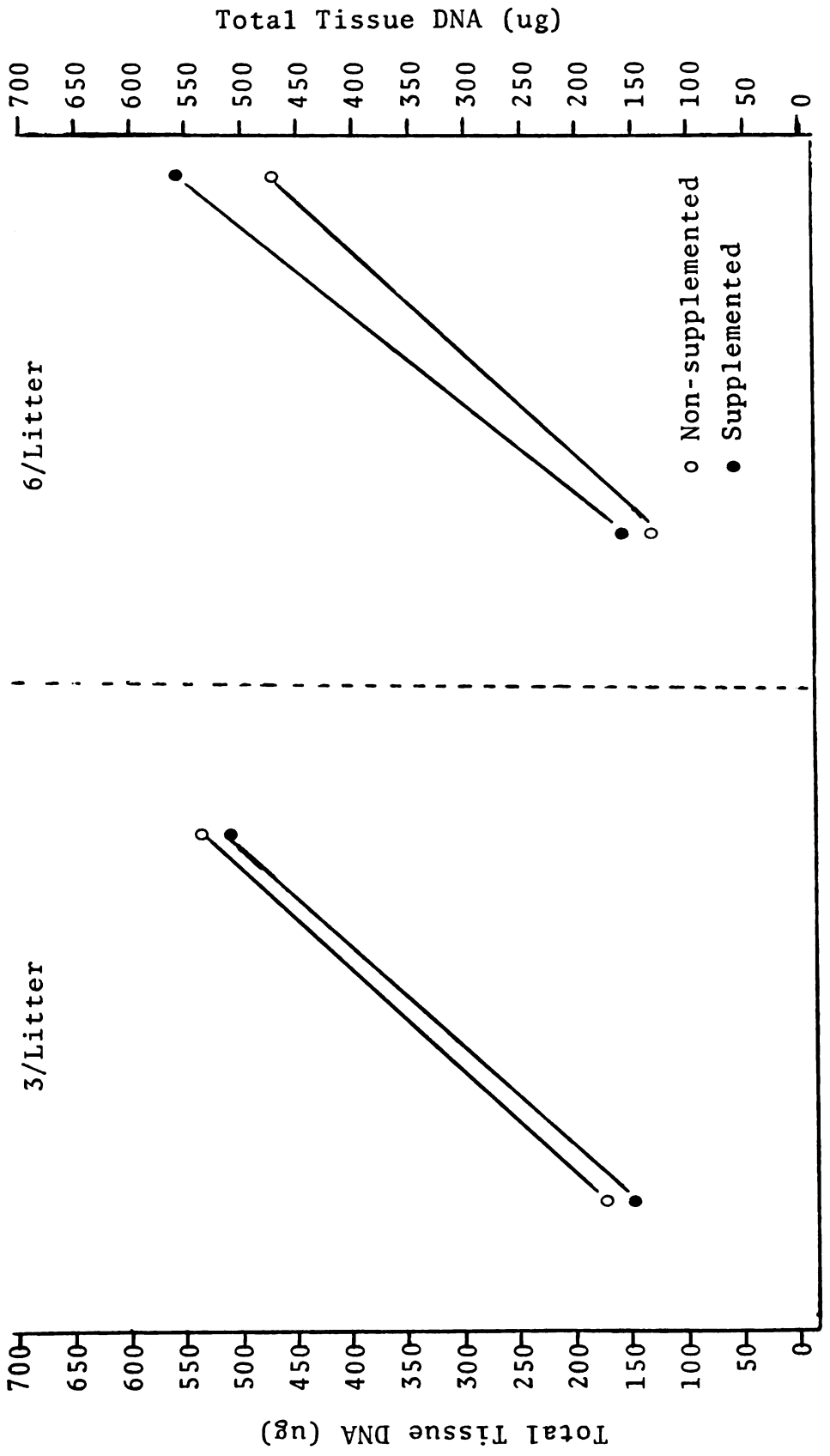


FIGURE 5.--Effect of Supplementation on Gastrocnemius Muscle DNA Content in Osborne-Mendel and S5B/P1 Rats Raised 3/Litter or 6/Litter.

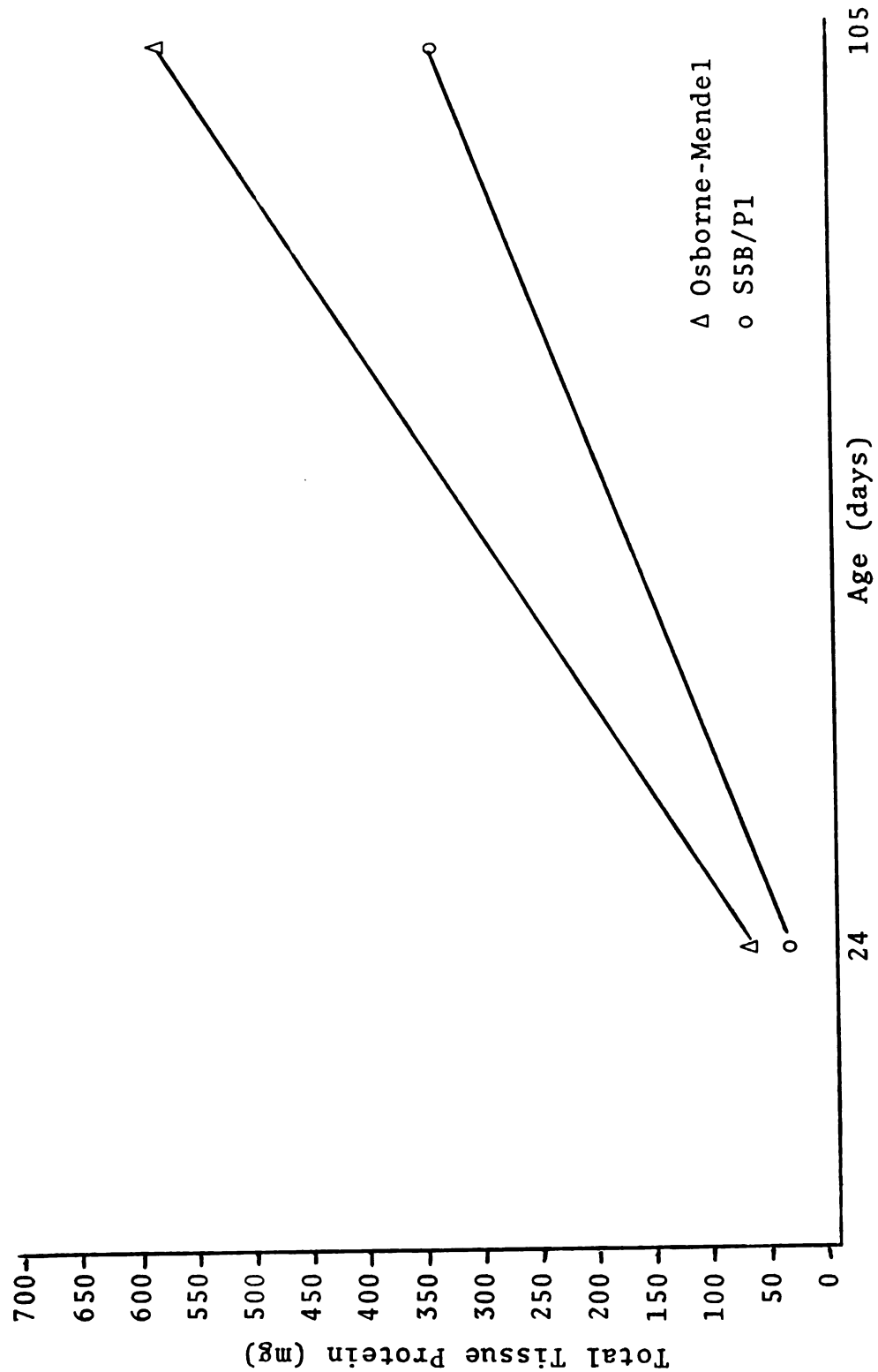


FIGURE 6.--Effect of Age on Gastrocnemius Muscle Protein in Osborne-Mendel and S5B/P1 Rats.

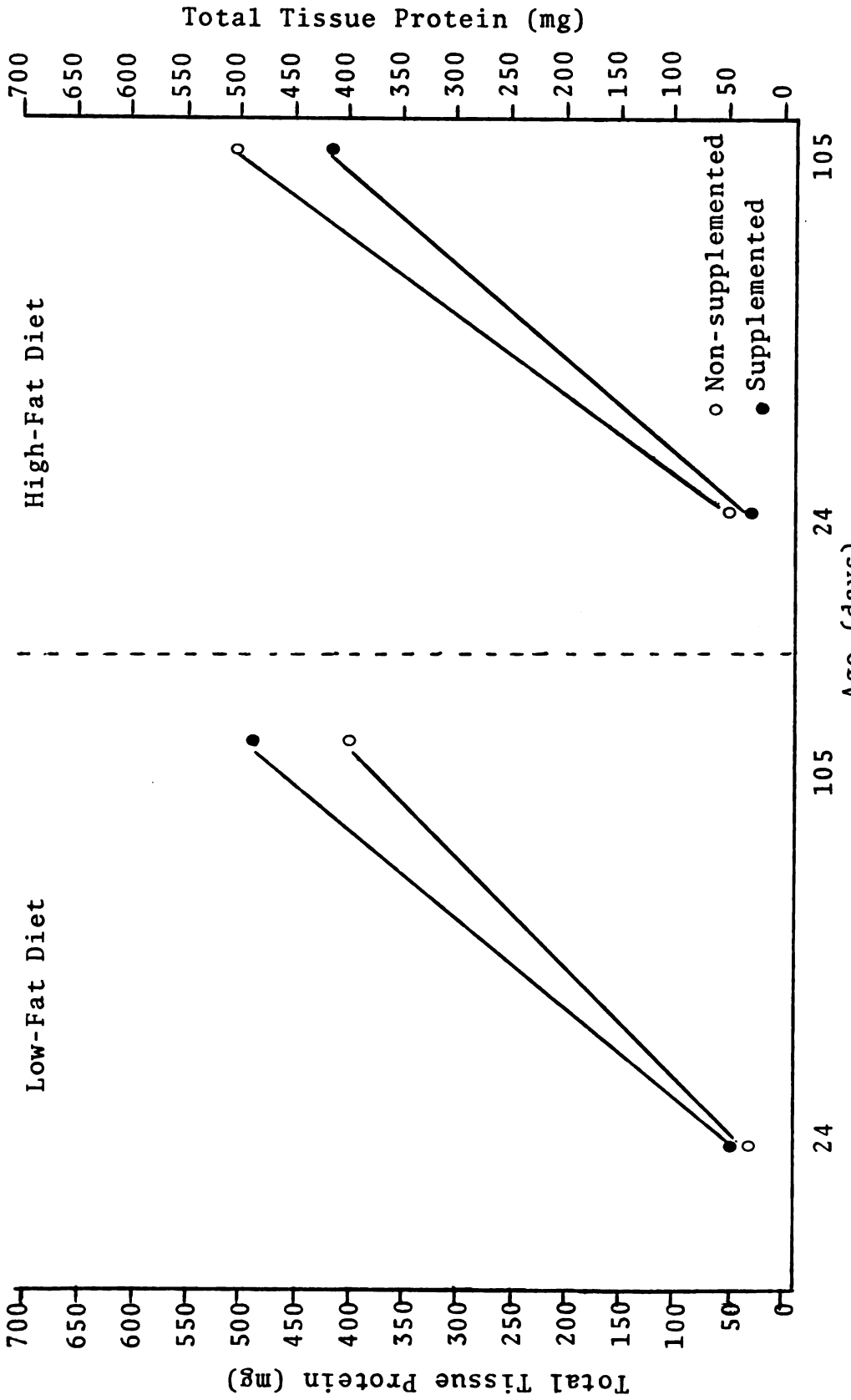


FIGURE 7.--Effect of Supplementation on Gastrocnemius Muscle Protein Content in Osborne-Mendel and S5B/P1 Rats Fed a Low-Fat or High-Fat Diet.

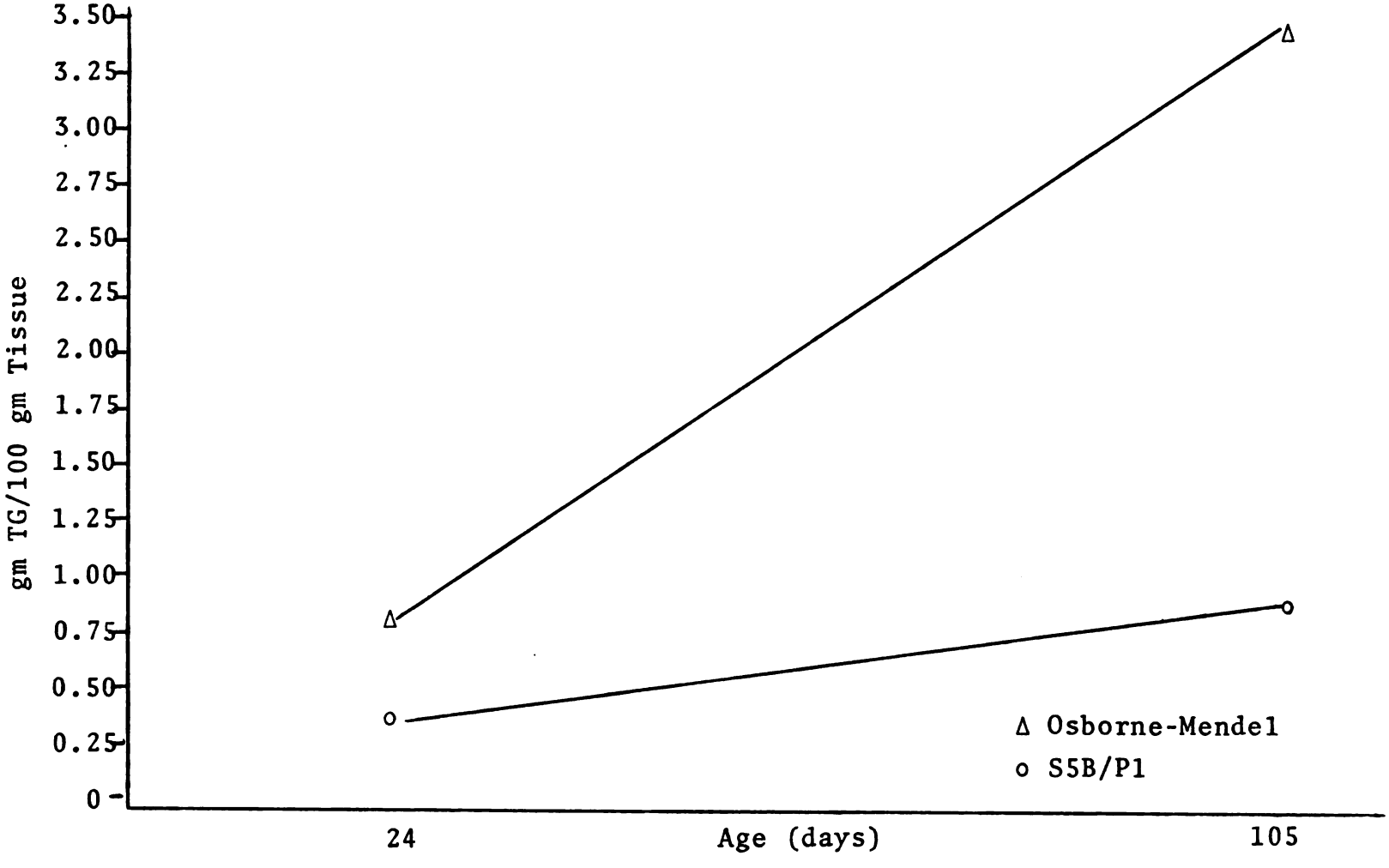


FIGURE 8.--Effect of Age on Gastrocnemius Muscle Triglycerides in Osborne-Mendel and S5B/P1 Rats.

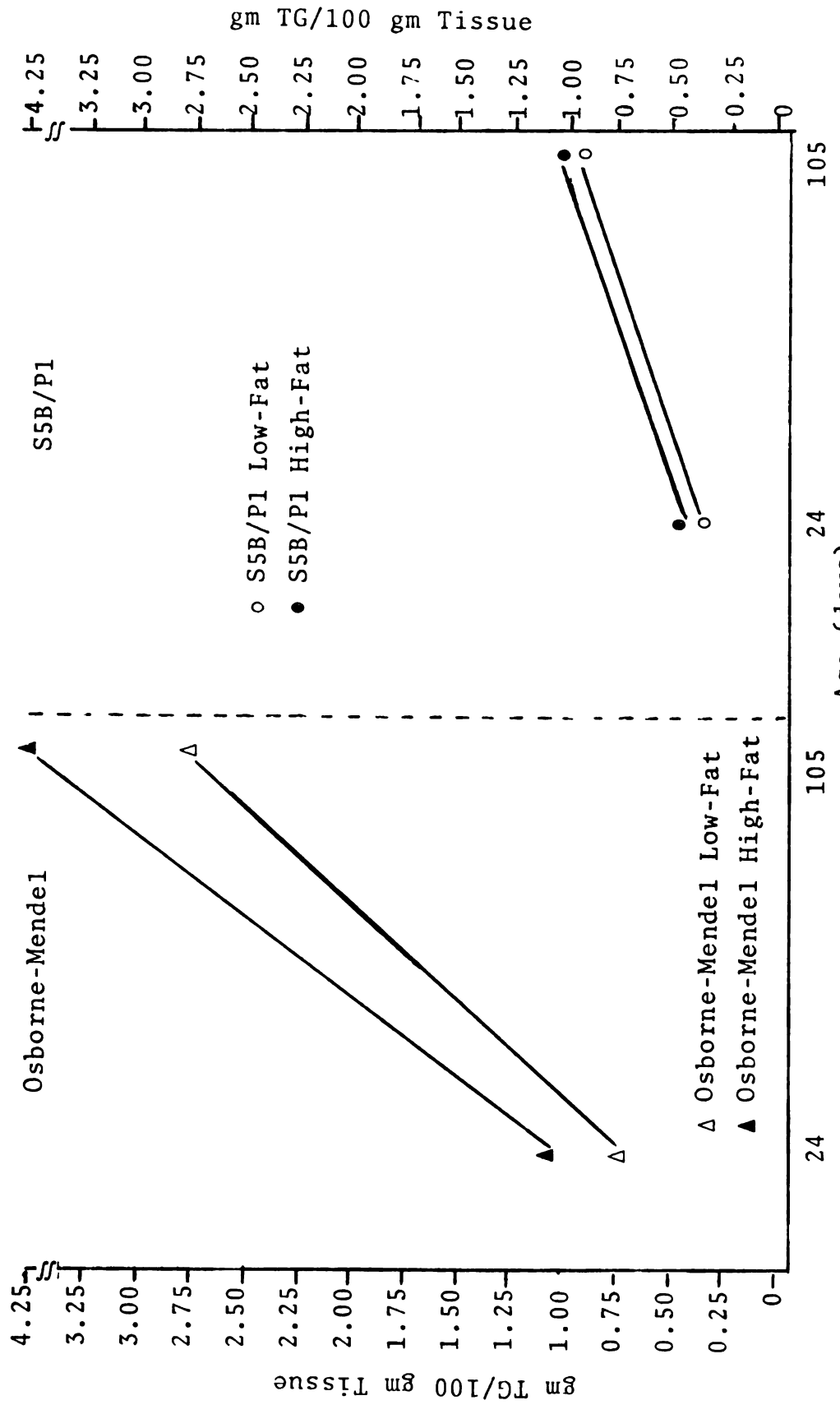


FIGURE 9.--Effect of a Low-Fat or High-Fat Diet on Gastrocnemius Muscle Triglyceride Content in Osborne-Mendel and S5B/P1 Rats.

RESULTS AND DISCUSSION

Body Weights

Data on mean body weights (in grams) of S5B/P1 and Osborne-Mendel rats used in this study are given in Tables 3 and 4 for ages 24 and 105 days, respectively. In all comparative groups of S5B/P1 and Osborne-Mendel rats, a significant increase in body weight ($P<0.01$) occurred with age. The mean gains in weight of Osborne-Mendel and S5B/P1 rats were 446 and 238 grams, respectively. In general, this represented a 5 to 6- fold increase in body weight for both strains.

A main effect of strain was also observed on body weight. All groups treated identically were significantly different ($P<0.01$) between Osborne-Mendel and S5B/P1 rats. At 24 days of age, the Osborne-Mendel rats weighed an average of approximately 28 grams more than the S5B/P1 rats. At 105 days of age, the Osborne-Mendel rats exceeded the S5B/P1 rats in weight by almost 240 grams.

Within the Osborne-Mendel and S5B/P1 strains, the high fat diet produced no significant difference in weight at 24 days of age. There was also no significant difference in weight in the S5B/P1 strain at 105 days, although

those animals fed the high fat diet were slightly heavier (Figure 1).

In contrast, at 105 days of age, a significant difference ($P<0.01$) in weight due to diet was observed in the Osborne-Mendel strain. Rats fed the high fat diet weighed 589 grams while those fed the low fat diet weighed 460 grams (Figure 1).

The data for rats fed the high fat diet are similar to those reported by Schemmel et al., (1970). These researchers found that the high fat diet exaggerated body weight in the Osborne-Mendel strain while having relatively little effect on the S5B/P1 strain. Schemmel et al., (1970) reported greater body weights in Osborne-Mendel rats fed the high fat diet than weights recorded in our study. This probably reflects the fat content (60%) of the diet used in their study. Our high fat diet contained only 44% fat. The Osborne-Mendel strain is apparently very efficient in depositing body fat as concluded by Schemmel et al., (1972).

Gastrocnemius Muscle Weights

The wet weight of gastrocnemius muscle tissue in S5B/P1 and Osborne-Mendel rats significantly ($P<0.01$) increased with age for comparative groups (Tables 5 and 6). At 24 days of age, the average tissue weight in the Osborne-Mendel strain was 0.31 grams, and 0.21 grams in the S5B/P1 strain. At 105 days of age, the average tissue weight was

2.58 and 1.67 grams in the Osborne-Mendel and S5B/P1 strains, respectively. In general, muscle weight increased 8-fold. Thus, a weight gain of 2.27 and 1.46 grams in gastrocnemius tissue weight of Osborne-Mendel and S5B/P1 rats, respectively, occurred with age.

At 24 days of age, there was no significant difference in tissue weight in comparative groups between S5B/P1 and Osborne-Mendel rats. At 105 days, a significant strain difference was apparent. The mean gastrocnemius tissue weight in the Osborne-Mendel strain was 0.90 grams greater than in the S5B/P1 strain ($P<0.01$, Figure 2). There were no significant effects of diet, supplementation, or litter size.

At 24 days, muscle-to-body weight was similar for all groups of rats in the S5B/P1 and Osborne-Mendel strains. However, at 105 days of age, the mean muscle to body weight was 0.51 to 0.63 gm/100 grams of body weight for all groups except the high fat fed, supplemented and non-supplemented Osborne-Mendel rats. Their mean muscle-to-body weight decreased with increased age and weight. The values for this group ranged from 0.40 to 0.45 gm/100 grams of body weight. This relative decrease in gastrocnemius weights expressed as gm/100 gm body weight was due to the increase in body fat accumulated by the Osborne-Mendel rats fed the high fat diet.

Gastrocnemius Muscle DNA

There was a significant ($P<0.01$) increase in DNA in gastrocnemius muscle of S5B/Pl and Osborne-Mendel rats from 24 to 105 days of age (Tables 7 and 8). The mean total DNA content in the muscle tissue of the S5B/Pl rats was 138 ug and 410 ug DNA at 24 and 105 days of age, respectively. The mean total DNA content of gastrocnemius muscle tissue in the Osborne-Mendel rats was 210 ug and 635 ug DNA at 24 and 105 days of age. The S5B/Pl and Osborne-Mendel strains gained 272 and 425 ug DNA from 24 to 105 days of age, respectively. A 3-fold increase was seen in both strains.

A mean significant ($P<0.01$) strain difference was also observed. For comparative groups, the Osborne-Mendel rats exceeded the S5B/Pl rats by a mean of 72 ug DNA at 24 days of age. At 105 days, this difference had reached a value of 224 ug DNA (Figure 3).

Enesco and Leblond (1962) reported a statistically significant increase in DNA during postnatal growth in gastrocnemius muscle. Although no explanation was given, the authors noted that the increase was due to an increase in the number of nuclei. That DNA increases during postnatal development in skeletal muscle has been confirmed by other researchers (Enesco and Puddy, 1964; Winick and Noble, 1965 and 1967; Cheek, 1975).

Supplementation did not produce a significant main effect in gastrocnemius muscle DNA content. It did, however, increase the DNA content in muscle of all supplemented

animals when compared to those non-supplemented. When grouped together, those animals supplemented had 360 ug DNA while those non-supplemented had 336 ug DNA.

The main effects of diet and litter were negligible, although there were significant interactions of both variables with supplementation. The high fat diet resulted in a greater ($P<0.01$) DNA content in supplemented animals, but the low fat diet produced a greater DNA content in those animals non-supplemented (Figure 4).

The remaining significant first-order interaction ($P<0.05$) for gastrocnemius muscle DNA content involved supplementation and litter size. The mean effect of supplementation in litters of six was to increase DNA by 46 ug DNA above that in non-supplemented animals. The mean effect of supplementation in litters of three was not significant. That is, non-supplemented and supplemented animals differed by only 1.3 ug DNA (Figure 5).

Gastrocnemius Muscle Protein

Mean values for the various treatment groups of S5B/P1 and Osborne-Mendel rats are given in Tables 9 and 10. A significant ($P<0.01$) age effect was seen in both strains. The S5B/P1 rats averaged 42 mg of tissue protein at 24 days of age and 370 mg protein at 105 days of age. At 24 days of age, mean protein in gastrocnemius muscle of Osborne-Mendel rats was 57 mg and increased with age so that at 105 days it was 583 mg ($P<0.01$). Thus, from weaning until 105

days of age, the S5B/P1 and Osborne-Mendel rats gained 328 and 526 mg protein, respectively. An increase of 8-fold, as was seen with gastrocnemius tissue weight, was observed in both strains.

The mean strain effect between groups treated identically in the S5B/P1 and Osborne-Mendel strains at 24 days of age was not significant. However, at 105 days of age, a significant difference ($P<0.01$) between strains existed. That is, Osborne-Mendel rats exceeded S5B/P1 rats by a mean difference of 213 mg protein (Figure 6). Based on tissue weight, the main effects of diet, supplementation, and litter size were negligible on tissue protein, although there was a significant interaction for diet and supplementation. Tissue protein was greater ($P<0.01$) in the supplemented, low-fat fed animals, whereas those non-supplemented animals had more tissue protein when fed the high fat diet ($P<0.01$, Figure 7).

With respect to the protein:DNA ratio (Tables 13 and 14), an increase in the amount of protein associated with DNA approached, if not exceeded, a 1:1 ratio with advancing age. Thus, the growth in gastrocnemius muscle after weaning was attributable, to an extent, to an increase in cell size.

The percent protein of muscle weight (Tables 15 and 16), was relatively constant between strains at 24 days of age. Protein averaged 19% of muscle weight at weaning for the S5B/P1 and Osborne-Mendel strains. At 105 days of

age, protein averaged 21% and 22% for the S5B/P1 and Osborne-Mendel strains, respectively. A slight increase with age was observed, but the values were within the normal range for percent protein of body weight.

Schemmel et al., (1973) reported that rats fed a high fat diet had more body protein at weaning than those fed grain. The results from the present study would seem to indicate that when a specific muscle tissue is examined, feeding a high fat diet causes little or no increase in protein when compared to rats fed a semi-purified diet low in fat.

Gastrocnemius Muscle Triglycerides

Data are presented in Tables 11 and 12 on the triglycerides content of gastrocnemius muscle in S5B/P1 and Osborne-Mendel rats. There was a significant statistical difference ($P<0.01$) between groups treated identically for Osborne-Mendel and S5B/P1 rats at 24 and 105 days of age. The S5B/P1 rats had a mean average muscle triglycerides content of 0.34 gm/100 gm tissue at 24 days. At 105 days, the average triglycerides content was 0.96 gm/100 gm tissue. A 3-fold increase with age was seen in this strain.

When all Osborne-Mendel rats were grouped together, they had an average triglycerides content of 0.83 gm/100 gm tissue at 24 days, but 3.5 gm/100 gm tissue at 105 days in

gastrocnemius muscle. In this strain, a 4-fold increase in tissue triglycerides content was seen.

There was a significant ($P<0.01$) main effect of strain difference at 24 and 105 days of age. The Osborne-Mendel rats had a mean average of 0.49 gm/100 gm tissue and 2.54 gm/100 gm tissue greater triglycerides content at 24 and 105 days, respectively, than the S5B/P1 rats (Figure 8).

At 24 days of age, although Osborne-Mendel rats fed the high fat diet had a greater muscle triglycerides content (0.86 gm/100 gm) than those fed the low fat diet (0.73 mg/100 gm), this difference was not significant. However, at 105 days of age, Osborne-Mendel rats fed the high fat diet had 4.25 gm/100 gm tissue triglycerides content compared to 2.75 gm/100 gm tissue triglycerides content for Osborne-Mendel rats fed the low fat diet. Thus, diet significantly ($P<0.01$) affected gastrocnemius muscle triglycerides content in these rats at 105 days of age (Figure 9).

Diet had no significant effect on muscle triglycerides content in the S5B/P1 strain. Pups fed the high fat diet had a slightly greater triglycerides content in gastrocnemius muscle than those fed the low fat diet (Figure 9).

Osborne-Mendel rats fed a high fat diet have a propensity towards obesity that is not seen in the S5B/P1 strain (Schemmel et al., 1970). When these strains were fed a high fat diet, Osborne-Mendel and S5B/P1 rats had 40%

and 14% body fat, respectively. In our study, the triglycerides content of gastrocnemius muscle in both strains followed the general pattern observed in total body fat. That is, the triglycerides content in gastrocnemius muscle in the Osborne-Mendel strain was greater than the triglycerides content in the S5B/Pl strain. The high fat diet had little or no effect on the triglycerides content of the S5B/Pl strain.

SUMMARY AND CONCLUSIONS

The effects of overfeeding nursing rats on body weights, gastrocnemius muscle weights, and DNA, protein, and triglycerides content of the muscle were examined in two strains of rats, Osborne-Mendel and S5B/P1. Age, strain, and diet were the predominate effects that significantly affected the measured variables. Supplementation and litter size had negligible main effects, but there were some significant interactions of these parameters with age, strain, and diet.

Body weights of comparative groups of Osborne-Mendel and S5B/P1 rats given identical treatments significantly differed at 24 and 105 days of age. Within the 105 day old Osborne-Mendel rats, the high fat diet produced a significantly heavier animal. The high fat diet had little effect on treatment groups within the S5B/P1 strain.

The gastrocnemius muscle wet weight did not differ between strains at 24 days of age. At 105 days, however, the gastrocnemius muscle weighed significantly more in the Osborne-Mendel strain. Gastrocnemius muscle weights increased 8-fold during the experimental period. Diet made little or no difference on muscle weight.

DNA content of gastrocnemius muscle significantly differed at 24 and 105 days between the Osborne-Mendel and S5B/Pl strains. Between 24 and 105 days, a 3-fold increase in DNA occurred within each strain. Although not significant, supplementation did increase the DNA content of gastrocnemius muscle in each strain. Feeding a high fat diet did not increase DNA content for either strain.

Protein content of gastrocnemius muscle was not significantly different between strains at weaning. At 105 days of age, comparative groups of Osborne-Mendel and S5B/Pl rats significantly differed. An 8-fold increase, as witnessed with muscle weights, occurred with age.

The triglycerides content of gastrocnemius muscle differed significantly between Osborne-Mendel and S5B/Pl groups treated identically at 24 and 105 days of age. The triglycerides content in gastrocnemius muscle in Osborne-Mendel rats fed the high fat diet significantly differed from those animals fed the low fat diet at 105 days of age. This difference probably reflected the greater body fat content of these animals.

SUGGESTIONS FOR FURTHER STUDY

Schemmel et al., (1970) concluded that the Osborne-Mendel strain of rats had a greater propensity towards obesity than the S5B/P1 strain when fed a high fat diet. This conclusion was based on the fact that body weight and body fat were significantly greater in the Osborne-Mendel strain than the S5B/P1 strain.

The results of our study indicate that this pattern of extreme difference also occurs within body tissues. Analysis of gastrocnemius muscle indicated that in all parameters measured (DNA, protein, and triglycerides), the Osborne-Mendel strain exceeded the S5B/P1 strain.

What controls the rate of cell division and enlargement in these strains? Studies investigating the rate of uptake of labeled molecules into tissues are needed. In addition, activity levels of enzymes controlling key steps in DNA and protein synthesis should be investigated. The fact that the triglycerides content of gastrocnemius muscle in the S5B/P1 strain was significantly less than that in the Osborne-Mendel strain suggests that either triglycerides were unavailable for uptake or that the mechanisms controlling their uptake were not functioning.

Fibers in skeletal muscle are multinucleated, so the amount of DNA, as determined in our study, is not a good indicator of the number of fibers present. It would be interesting to histologically determine if the growth in number of fibers in skeletal muscle of these strains differ. Are there more muscle fibers in the Osborne-Mendel strain? If so, do they account for the greater DNA and protein content of muscle in this strain?

The Osborne-Mendel strain grows at a faster rate than the S5B/Pl strain. Is this the result of an impairment of the endocrine system in the S5B/Pl strain? It would be worthwhile to measure growth hormone and insulin levels in these animals during the suckling period. Perhaps the S5B/Pl rat is born with a malfunctioning endocrine system which does not allow it to grow at a comparable rate to the Osborne-Mendel rat.

APPENDICES

APPENDIX A

DETERMINATION OF DEOXYRIBONUCLEIC ACID

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DETERMINATION OF DEOXYRIBONUCLEIC ACID

Reagents

Deionized Water

Because interfering substances may affect the sensitivity of this procedure, only deionized water was used in preparing reagents. Distilled water was passed through a mixed bed ion exchanger (Illinois Water Treatment Co., Rockford, Illinois) adjusted to a flow rate of approximately 10 ml per minute.

1 Pellet/Liter Sodium Hydroxide

This solution was prepared by dissolving 1 pellet of sodium hydroxide (NaOH, J.T. Baker Chemical Company, Phillipsburg, New Jersey) in 1 liter of deionized water.

10% Trichloracetic Acid

This solution was prepared by dissolving 100 gm of trichloroacetic acid (CCl_3.COOH, Mallinckrodt Chemical Works, St. Louis, Missouri) in 1 liter of deionized water.

Deoxyribonucleic Standard

Type 1, Calf Thymus, highly polymerized deoxyribonucleic acid (Sigma Chemical Co., St. Louis, Missouri) served as the standard. Ten mg of this product was weighed on a Mettler analytical balance, shredded, and placed into a 100 ml volumetric flask containing 50 ml of a sodium hydroxide solution of one pellet per liter. The deoxyribonucleic acid was dissolved by stirring with a glass rod and shaking. Fifty ml of 10% trichloracetic acid was added to the flask, forming a white precipitate. While stirring, the flask was heated until the white precipitate disappeared. Care was taken to avoid boiling the solution. The flask was cooled, stoppered, and stored at 4° C in a refrigerator. The final concentration of the standard solution was 100 ug/ml.

Diphenylamine Solution

This solution containing glacial acetic, sulfuric acid, and diphenylamine was made fresh just prior to use. It was prepared by dissolving 1.5 gm diphenylamine [$(C_6H_5)_2NH$, Mallinckrodt Chemical Works, St. Louis, Missouri] in 100 ml glacial acetic acid ($CH_3.COOH$, Fisher Scientific, Fair Lawn, New Jersey). To this, 1.5 ml concentrated sulfuric acid (H_2SO_4, Fisher Scientific, Fair Lawn, New Jersey) was added. The final solution was clear and kept in the dark until use.

Acetaldehyde Solution

This solution was made by weighing 1.6 gm of acetaldehyde ((C_2H_4) Mallinckrodt Chemical Works, St. Louis, Missouri) into 100 ml of deionized water for a final concentration of 16 mg/ml. The solution was stored at 4° C.

Diphenylamine-Acetaldehyde Solution

This solution was prepared by mixing 0.1 ml of the acetaldehyde solution per 20 ml of diphenylamine solution.

20% Trichloracetic Acid

This solution was prepared by dissolving 200 gm of trichloracetic acid (CCL_3.PPH, Mallinckrodt Chemical Works, St. Louis, Missouri) in 1 liter of deionized water.

5% Trichloroacetic Acid

This solution was prepared by dissolving 50 gm of trichloroacetic acid (CCL_3.COOH, Mallinckrodt Chemical Works, St. Louis, Missouri) in 1 liter of deionized water.

SUMMARY OF PROCEDURES

For Tissue Samples:

1. On ice, add 1 ml of cold freshly prepared homogenate to 5 ml of cold 20% TCA.
2. Mix and centrifuge 20 minutes at 5000 rpm.
3. Discard supernatant.
4. Add at room temperature 6 ml of 5% TCA to the pellet and heat for 30 minutes at 90° C. Cool for 10 minutes and centrifuge as before.
5. Use 2 ml aliquots from this supernatant for the assay.
6. To the 2 ml volume of supernatant, add 4 ml of the diphenylamine-acetaldehyde solution. Vortex for 15 seconds.
7. Incubate at room temperature for 16-20 hours.
8. Read at 700nm and at 595nm.
9. Subtract the 700nm readings from the 595nm readings.

For Standards:

1. Use protocol on page 26 to set up standards.
2. Treat with the diphenylamine-acetaldehyde solution.
3. Incubate at room temperature for 16-20 hours.
4. Read at 700nm and at 595nm.
5. Subtract the 700nm readings from the 595nm readings.

APPENDIX B

DETERMINATION OF PROTEIN

APPENDIX B

DETERMINATION OF PROTEIN

Reagents

Deionized Water

Because changes in the quality of distilled water may have affected the sensitivity of Lowry's method, only deionized water was used. Distilled water was passed through a mixed bed ion exchanger (Illinois Water Treatment Company, Rockford, Illinois) adjusted to a flow rate of approximately 10 ml per minute.

1N Sodium Hydroxide

This solution was prepared by dissolving 40 gm of sodium hydroxide pellets (NaOH, J.T. Baker Chemical Company, Phillipsburg, New Jersey) in 1 liter of deionized water.

10% Trichloroacetic Acid

This solution was prepared by dissolving 100 gm trichloroacetic acid (CCl_3.COOH, Mallinckrodt Chemical Works, St. Louis, Missouri) in 1 liter of deionized water.

Bovine Albumin Standard

Crystalline bovine albumin (Grand Island Biological Company, Grand Island, New York) served as the standard. On a Mettler analytical balance, 250 mg of this product was weighed. This amount was placed in 50 ml volumetric flask and filled to volume with deionized water. Because of foam formation upon mixing, this product was always stored at least 24 hours prior to use. The standard solution was stored in polyethylene bottles in a refrigerator at 4° C.

Phenol Reagent

This reagent containing lithium sulfate ($Li_2SO_4.H_2O$), sodium tungstate ($Na_2WO_4.2H_2O$), sodium molybdate ($Na_2MoO_4.2H_2O$), phosphoric acid (H_3PO_4), hydrochloric acid (HCL) and bromine (Br) was purchased from Fisher Scientific, Fair Lawn, New Jersey as a 2N solution. A 1N solution was obtained by diluting equal parts of the reagent with deionized water.

Cupric Sulfate Solution

This solution was prepared by placing 0.5 gm cupric sulfate crystals ($CuSO_4.5H_2O$, Mallinckrodt Chemical Works, St. Louis, Missouri) in a 100 ml volumetric flask and filling to volume with deionized water.

Sodium Carbonate-Sodium Tartrate Solution

The preparation of this alkaline buffer solution required dissolving 20 gm sodium carbonate (Na_2CO_3, J.T.

J.T. Baker Chemical Company, Phillipsburg, New Jersey) and 0.2 gm sodium tartrate $(Na_2C_4H_4)_6.2H_2O$, J.T. Baker Chemical Company, Phillipsburg, New Jersey) in a volumetric flask that was filled to volume with deionized water.

Sodium Carbonate-Sodium Tartrate: Cupric Sulfate Solution

This solution was prepared by mixing 50 ml sodium carbonate-sodium tartrate solution with 1 ml of cupric sulfate solution.

SUMMARY OF PROCEDURE

For Tissue Samples:

1. On ice, add 0.25 ml of cold freshly prepared homogenate to 5 ml of cold 10% TCA.
2. Mix and centrifuge for 20 minutes at 5000 rpm.
3. Decant supernatant.
4. At room temperature, add 10 ml of NaOH to pellet and dissolve pellet by vortexing.
5. Use 0.5 aliquots from this solution for the assay.
6. To the 0.5 ml aliquots, add 0.5 ml deionized water. Vortex.
7. Add 5 ml of the 2% Na_2CO_3-0.02% $Na_2C_4H_4O_6.2H_2O$: 0.05% $CuSO_4.5H_2O$ solution to each test tube while mixing. Wait 10 minutes.
8. Add 0.5 ml of the 1N Folin-Ciocalteau phenol reagent. Vortex. Wait 30 minutes.
9. Read at wavelength 740nm.

For Standards:

1. Use the protocol on page 29 to set up standards.
2. Repeat steps 7-9 given in the procedures for tissue samples.

APPENDIX C

FLUOROMETRIC DETERMINATION OF TRIGLYCERIDES

APPENDIX C

FLUOROMETRIC DETERMINATION OF TRIGLYCERIDES

Reagents

Listed below are reagents needed for the determination of triglycerides. These reagents may be bought from Technicon Instruments Corporation ready made or they may be prepared according to the directions given.

80% Isopropanol--Technicon No. T01-0474-56

Preparation: Place 200 ml of distilled water, q.s. in a one liter stoppered graduate cylinder. Add 800 ml of isopropanol and shake gently. Remove stopper to relieve pressure. Add distilled water to 1000 ml mark, if necessary.

0.80M KOH--Technicon No. T01-0473-56

Preparation: Place 500 ml of distilled water, q.s. in a one liter volumetric flask. Add 45 gm of potassium hydroxide and shake until completely dissolved. Dilute to volume with distilled water.

Periodate Reagent--Technicon No. T01-0372-56

Preparation: Place 500 ml of distilled water, q.s. in a one liter volumetric flask. Slowly add 115 ml of

glacial acetic acid and shake. Prepare in hood. Add 5.4 gm sodium periodate and shake until completely dissolved. Dilute to volume with distilled water.

Ammonium Acetate, 2M--Technicon No. T21-0373-56

Preparation: Place 800 ml of distilled water, q.s. in a beaker. Add 154 gm of ammonium acetate and stir until completely dissolved. Adjust the pH to 6.0 with 2N HCL. Dilute to one liter with distilled water.

Working Acetylacetone Reagent

Preparation: Mix 7.5 ml of 2,4-Pentaneodion (Technicon No. T01-0379-15) with 25 ml of 99% isopropanol and place in a one liter volumetric flask. Dilute to volume with 2M ammonium acetate. This reagent must be prepared fresh daily.

99% Isopropanol--Technicon No. T01-0064-19

It is strongly recommended that this reagent be purchased from Technicon Instruments Corporation as their product is distilled to eliminate contaminants which may produce fluorescence when combined with the reagent system. If Technicon's isopropanol is not bought, the product must be treated with sodium borohydride and redistilled.

Zeolite Mixture--Technicon No. T11-0375-20

Preparation: Grind 200 gm of zeolite to a fine powder in a blender, place in a shallow tray, and heat overnight at 100° C. Cool zeolite. Add 20 gm of Lloyd

reagent, 10 gm of powdered cupric sulfate, and 20 gm of powdered calcium hydroxide to the cooled zeolite in a 500 ml stoppered bottle. Shake the bottle until the contents are thoroughly mixed. Stopper tightly.

Stock Standard--Technicon No. T23-0374-15

Preparation: Place 80 ml of 99% isopropanol in a 100 ml volumetric flask. Add 1000 mg triolein and shake until dissolved. Dilute to volume with 99% isopropanol. Label with date and store in an amber glass bottle in the refrigerator at 2° C to 8° C. Standard is good for three months.

SUMMARY OF EXTRACTION PROCEDURE

For Tissue Samples:

1. Place 9.5 ml of 99% isopropanol in a 15 ml corex centrifuge tube. Pipette 0.5 ml of sample into the isopropanol.

2. Parafilm the tube and shake vigorously for 30 seconds on a vortex mixer.

3. Add 2 gm of zeolite mixture to the isopropanol extract. Re-parafilm the tube and vortex another 30 seconds.

4. Allow samples to stand for 30 minutes. During this period invert the tubes once every ten minutes and gently resuspend the mixture.

5. Centrifuge the tubes and carefully decant the supernatant into screw-capped culture tubes with Teflon-lined caps for storage until analysis.

For Standards:

1. Place 9.0 ml of 99% isopropanol and 0.5 ml of water into a 15 ml corex centrifuge tube. Add 0.5 ml of appropriate standard.

2. Repeat steps 2-5 as outlined for tissue samples.

APPENDIX D

YATES' METHOD FOR FACTORIAL ANALYSIS

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Yates' analysis of variance computes the effects for a 2^K factorial experiment, i.e., one with K factors each at two levels. This factorial analysis gives mean differences of the responses of the dependent variable to the different treatments (main effects) and the effects of changes in the level of each treatment on the responses of the others (interactions).

For computation, the total of the responses for a particular treatment combination is used. The first half of the treatment combination column is obtained by subtracting the first from the second response in a pair. The next column is obtained by repeating the addition and subtraction process for the values in the preceding column. This process proceeds K times for a 2^K experiment.

The accuracy of the "addition and subtraction process" is subjected to the sums-of-squares check. The following formulas are used.

1. $SS_{TC} = \sum^{\#TC} (TC_{total})^2 \; / \; \#/TC - (GT)^2 \; / \; TP$

   "TC" is treatment combination; "GT" is grand total; and "TP" is total population.

2. $SS_{TC} = \Sigma Q^2 \; / \; TP$

   "Q's" are all values except the first item in the K^{th} column of additions and subtractions.

The calculations in the table are correct if the two SS_{TC} values are equal.

The mean square experimental error (MS_E) is obtained by using the following formula:

$$MS_E = \left[\sum^{\#TP} Y_i^2 - \sum^{\#TC} (TC_{totals})^2 \; / \; n \right] \Big/ df$$

"Y" is the individual value of subjects in the total population; "n" is the number in the group; and "df" is the degrees of freedom.

The formula for calculating the standard error (SE) follows:

$$SE = \pm\sqrt{MS_E \; / \; n}$$

The significance level at which the test is to be performed is set and the minimum significant difference (MSD) is calculated using the following formula:

$$MSD = CV.\sqrt{MS_E / \; n}$$

"CV" is the critical value taken from Student's t-table.

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