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Influence of dietary fat, meal pattern and length of feeding on cholesterol metabolism, cardiac stress-strain response and morphology in maturing male rats

by

Chor-San Heng

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GLOSSARY

LF - low fat diet, contains 2% safflower oil as calories

SO - high fat diet, contains 38% safflower oil as calories

BT - high fat diet, contains 38% beef tallow as calories

AL - ad libitum feeding, 24 hours feeding out of a 24 hour period

MF - meal feeding, 3 hours feeding out of a 24 hour period

30 - 30 days feeding length, corresponding to age 2 months

60 - 60 days feeding length, corresponding to age 3 months

90 - 90 days feeding length, corresponding to age 4 months

DPS - digitonine precipitable steroids, mostly cholesterol

dpm - disintegrations per minute

LSD - least significant difference

$\pm$SEM - $\pm$standard error of the mean

CSS - composite stress-strain curve

ANOVA - analysis of variance

INTRODUCTION

The National Research Council and the American Medical Association recommended that individuals at risk from coronary heart disease reduce their total fat intake, increase their intake of polyunsaturated fat, and reduce saturated fat intake in order to reduce their serum cholesterol levels.[1] However, the mechanisms whereby polyunsaturated fats decrease serum cholesterol concentrations are under dispute. Available evidence points to accelerated cholesterol turnover in conjunction with redistribution of the total body cholesterol pool during polyunsaturated fat consumption (Bloomfield, 1964; Grundy and Ahrens, 1970). The action of polyunsaturated fats appears to be multi-faceted. The magnitude of hypocholesteremia produced by polyunsaturated fat intake could result from simultaneous changes in the metabolism of cholesterol, triglycerides, bile acids and lipoproteins.

The consequences of prolonged and high level intakes of polyunsaturated fats on apparently healthy mature or on young populations are not well documented. Recent findings indicate that polyunsaturated fat itself may pose risks to health. The risk seems to arise mostly from the high proportion of linoleic acids in most vegetable oils (50-80%). It is known that at least 2-3% of the total calories consumed should be derived from linoleic acid to prevent essential fatty acid deficiencies in both man and rat. However, the dietary level which may produce detrimental

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[1]Diet and Coronary Heart Disease. A joint statement by the Food and Nutrition Board, Division of Biology and Agriculture, National Academy of Sciences - National Research Council, and the Council on Foods and Nutrition, American Medical Association, July, 1972.

effects is not known. Part of this problem stems from insufficient knowledge of the overall physiological functions of linoleic acid. One example is the relationship of linoleic acid to prostaglandin synthesis, and the latter's involvement in coronary heart disease. Linoleic acid intake at 15-16% of total calories appears to be optimal for preventing platelet aggregation and producing maximal reduction of serum lipids, when dietary cholesterol intake averages 300-400 mg per day (Vergroesen, 1976). Other findings indicate that rats raised on safflower oil as 40% of calories (approximately 32% linoleic acid), have higher mortality rates during respiratory infection when compared to animals fed beef tallow at similar levels (Dupont et al., 1975). The fatty-acid composition of certain tissues, namely, liver, adipose and adrenals reflect dietary fat intake (Egwin and Kummerow, 1972). Animals fed polyunsaturated fats accumulate large amounts of unsaturated fatty acids in some of these tissues (Reeves, 1971). Furthermore, there is evidence of membrane structure alteration when linoleic acid replaces other fatty acids (Elson and Shrago, 1975). In addition unsaturated fats are susceptible to oxidation. There are indications that the resulting by-products, e.g., peroxides, are carcinogenic in some animal tissues. During exposure to X-ray irradiation, animals previously fed polyunsaturated fatty acids did not survive as well as controls (Yatvin et al., 1975).

In previous work in our laboratory serum cholesterol levels increased in adult rats when the feeding period was restricted to 8 hours in a 24 hour period (Reeves, 1971; Carlson, 1975). This elevation was more apparent when the experimental period was extended from 10 to 30 days. Meal frequency was therefore a major variable in the present study.

The present study was conducted in two sections. Section I consisted of experiment 1, while Section II consisted of experiments 2 and 3. The objective of the first experiment was to assess metabolic changes in the cholesterol pathway in maturing rats using the following dietary variables: 1) meal frequency, 2) dietary fat level and saturation, 3) duration of feeding (30 vs. 60 vs. 90 days). The effects of these variables on serum cholesterol concentrations in animals undergoing rapid development were explored. These feeding periods corresponded to developmental periods ending at ages 2, 3 and 4 months or the equivalent of 5, 7 1/2 and 10 years in humans. It was estimated that a dietary fat concentration of 20% by weight or 38% of calories was equivalent to the average fat intake of western populations. Safflower oil and beef tallow were chosen based on their occurrence in human diets, the high level of polyunsaturated fatty acids in safflower oil, the high level of digestibility and saturated fatty acids in beef tallow.

Radiotracers were used to monitor relative rates of cholesterol biosynthesis, degradation, reabsorption, excretion and transport in the rapidly equilibrating pools of serum, liver and intestines.

A second experiment was designed to study cardiac mechanical function in animals treated similarly to those of experiment 1. The objective of this experiment was to study the causes of increased cardiac fragility in safflower oil fed rats, which had been one of the subjective observations in experiment 1. The assumption was made that cardiac fragility would alter cardiac mechanical performance and this alteration would be reflected in structural changes, specifically in elastin, collagen and the

intercellular matrix. A stress-strain response of cardiac tissue was measured to assess changes in cardiac distensibility with dietary variations. Concurrently, a third experiment was designed to examine the ultrastructures of myocardial cells, in order to aid the interpretation of functional alterations. In experiment 3 only one time period (90 days) was used from the design employed in experiment 1.

SECTION I. CHOLESTEROL METABOLISM

Review of Literature

Introduction

Cholesterol is a sterol found in every tissue of the animal. It is the most abundant sterol in the animal kingdom. In the body, it serves four basic functions: 1) it is a structural component of cell membranes, 2) it stabilizes the structure of plasma lipoproteins, 3) it is a precursor of bile acids and their metabolites, and 4) it acts as a precursor of steroid hormones and vitamin D.

Cholesterol production is related to the needs of the organism, and in this is conditioned to some extent by nutritional, endocrine and neurohumoral influences. Cholesterogenesis is heightened during active tissue growth as in fetal brain, intestinal mucosa and certain tumors (Bortz, 1973). The diseases that are implicated in malfunction of cholesterol metabolism are numerous, and include xantheremia, cardio-vascular diseases and familial hypercholesteremia. In addition, abnormal development of brain and myelin sheath has been associated with cholesterol deprivation in infancy. Consequently, these defects have attracted investigators to the study of the regulations of cholesterol metabolism.

Maintenance of cholesterol homeostasis

In the intact animal, total body cholesterol is regulated at four loci: absorption, synthesis, degradation (conversion to bile acids) and excretion (neutral steroids and degradative products). There are two routes of input into body cholesterol pool, i.e., via dietary intake and endogenous synthesis from acetate. Cholesterol is removed from the body by

excretion via bile which includes acidic and neutral steroids, and as neutral steroids from the intestinal mucosa. Cholesterol homeostasis is maintained by an intricate balance between input versus output mechanisms. However, different species of animals vary in the interaction of these compensatory mechanisms. In humans, cholesterol absorption was reduced to 50% when cholesterol intake was moderate (540 mg per day), and declined to 25-30% when intake was high, 3 g/day (Quintao et al., 1971). In the rat and dog, cholesterol absorption was about 85-90% during moderate intake of 1% by weight of diet (Wilson, 1968). On the other hand, the rabbit showed no restraint towards amount of cholesterol absorbed. Feeding cholesterol to rabbits led to marked increases in plasma and tissue cholesterol levels (Ho and Taylor, 1968).

In most animals, cholesterol accumulation in the tissues is offset normally by either enhanced excretion of cholesterol as neutral and acidic steroids in the bile, or by feedback inhibition of cholesterol synthesis, or by decreased cholesterol absorption. In man, because of limited ability to convert cholesterol into bile acids, excessive absorption of exogenous cholesterol was curtailed by lowered ability to absorb dietary cholesterol, as well as by excretion of large quantities of neutral steroids (Quintao et al., 1971). In rats (Wilson, 1964), dogs (Ho and Taylor, 1968) and squirrel monkeys (Lofland et al., 1971), expansion of body cholesterol pool due to high dietary cholesterol intakes was prevented by increased bile acid excretion. In rats after 9 months of cholesterol feeding, only the liver showed signs of increased cholesterol content. In addition, plasma hypercholesterolemia was not observed. Cholesterol content in other tissues, e.g., adrenal, brain, lungs, spleen, heart, skin, small intestine,

colon, aorta, kidney, adipose tissue and skeletal muscle remained fairly constant. Under this same condition, liver cholesterogenesis was completely (>90%) suppressed. In contrast, small intestinal cholesterogenesis decreased only slightly. In humans, depending on the metabolic states of the individual tested, cholesterol absorption averaged 63% (Nestel et al., 1973) for obese subjects, compared to 50% for normalized subjects (Quintao et al., 1971). Cholesterol synthesis per day in obese subjects was approximately twice that in lean subjects (Nestel et al., 1973).

Body cholesterol pools In general, the exchange rates between tissue and plasma cholesterol vary with the type of tissue. The turnover of plasma cholesterol has been studied in several species by administration of either labeled cholesterol or a labeled biosynthetic precursor, followed by sequential determinations of plasma cholesterol specific activities over several weeks. In most studies, it had been a consistent feature that the semilogarithmic plot of cholesterol specific activity against time described an initial curved portion followed by a straight line (Dietschy and Wilson, 1970). The first detailed analysis of the disappearance curve of radioactive cholesterol was made by Goodman and Noble (1968). Their work, based on 10 human subjects, led to the initial concept of two cholesterol pools, A and B (Figure 1). In the formulation of the two cholesterol pool model, it was assumed that the escape of cholesterol from Pool B to Pool A was negligible. The entry, however, of cholesterol from Pool A to Pool B was important. The two pool model was substantiated by work from other investigators (Nestel et al., 1969; Grundy et al., 1969). Wilson (1970 later modified the two pool model to three by the addition of a nonexchangeable pool, C, based on work with squirrel monkeys

and baboons (Figure 1).

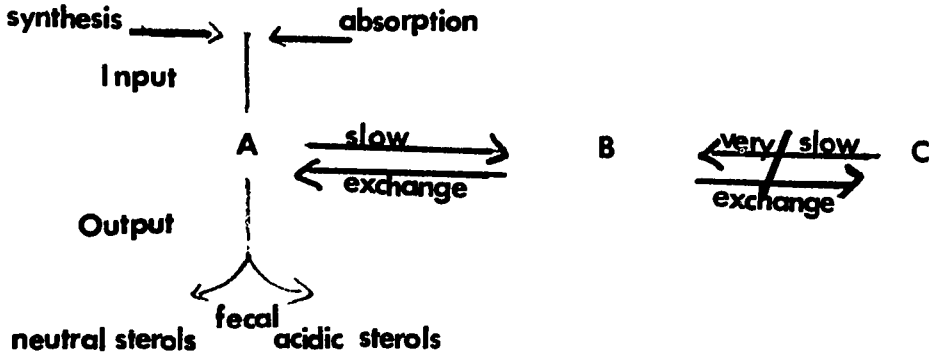

Figure 1. The three pool model of Wilson (1970). In this model cholesterol comprised of one nonexchangeable or very slow exchanging pool (C) and two exchangeable pools--a rapidly exchanging pool (A) and a slowly exchanging pool (B). Input into pool A occurred via endogenous synthesis from acetate and absorption of dietary cholesterol. Net output from the system occurred via the feces as neutral and acidic sterols. Each of these routes of input and output were subjected to regulations.

Hepatic cholesterogenesis

Liver is the prime locus of endogenous cholesterol synthesis, with the small intestine ranking next in importance. In the rat, both organs contribute about 80% of the total endogenous source (Dietschy and Wilson, 1970). Under most physiological and nutritional changes, for example in fasting, cholesterol feeding, fat feeding, bile salts feeding, the liver is more sensitive to changes than the small intestine. The liver is the key site of bile acid and cholesterol production, controls the enterohepatic flow of bile and therefore cholesterol absorption, synthesizes

triglycerides and lipoproteins, and finally is a source of plasma cholesterol (Hotta and Chaikoff, 1955; Siperstein and Fagan, 1966).

Control of hepatic synthesis The rate of hepatic cholesterol synthesis is regulated by three main variables: dietary cholesterol intake, enterohepatic bile acid circulation and fasting.

Dietary cholesterol Hepatic cholesterol synthesis was noticed to be under negative feedback control by the amount of dietary cholesterol intake in rats, squirrel monkeys and man (Dietschy and Wilson, 1968; Siperstein, 1970; Siperstein and Guest, 1960). In rats feeding cholesterol led to a feedback inhibition of 80-96%, while in monkeys and man, suppressions were less apparent at 60-80% and 50%, respectively (Ho and Taylor, 1970). The rate of cholesterogenesis also varied inversely with the amount of cholesterol in the liver (Gould et al., 1953; Frantz et al., 1954; Shapiro and Rodwell, 1971). In rat, the conversion of acetate to cholesterol was reduced to 5% of maxima after prolonged feeding of cholesterol at levels of 1-5% in the diet by weight (Tomkins et al., 1953; Dietschy and Siperstein, 1967). Cholesterol feeding had, however, no effect on acetate conversion to CO_2 or to fatty acids (Dietschy and Wilson, 1970).

Workers have noted that the inhibition of hepatic cholesterogenesis occurred as early as 4 hours after cholesterol feeding, coinciding with increased hepatic cholesterol content. The suppression was most apparent after 12 hours of cholesterol feeding (Shapiro and Rodwell, 1971; Sakakida et al., 1963). On the other hand, the restitution of normal levels of hepatic synthesis following discontinuance of cholesterol feeding was related to the duration of cholesterol feeding. Taylor et al. (1956), for

example, showed that resumption of normal rates of hepatic cholesterogenesis required 24 hours of cholesterol deprivation, if cholesterol feeding had been 24 hours. However, when cholesterol intake was extended to 15 days, recovery to normal hepatic sterolgenesis required about 2 months on a cholesterol free diet.

The infusion of either chylomicrons or serum from hypercholesteremic subjects suppressed hepatic cholesterogenesis (Sakakida et al., 1963; Tanabe et al., 1972). This indicated that hepatic sterolgenesis was influenced by dietary cholesterol present in lymphatic channels and in lipoproteins.

The conversion of beta-hydroxy-beta-methyl-glutaryl CoA (HMG-CoA) to mevalonate has been identified as the rate limiting step in cholesterol biosynthesis (Gould and Popjak, 1957; Siperstein, 1970). The activity of the regulating enzyme of this step, HMG-CoA reductase, correlated well with hepatic cholesterol synthesis under various physiological and nutritional influences, such as cholestyramine feeding, cholesterol feeding, fasting, fat feeding, diurnal variations (Shapiro and Rodwell, 1972; Dietschy and Brown, 1974). Under these conditions cholesterol synthetic capacity was varied over a hundredfold by dietary modifications. In each instance the correlation in magnitude and direction between cholesterol synthesis and reductase activity was evident.

Evidence is conflicting regarding the active form of cholesterol required for product inhibition of HMG-CoA reductase. Siperstein (1970) proposed a lipoprotein form of cholesterol, rather than cholesterol itself, as the effective feedback inhibitor based on reports in the literature that showed 1) chylomicra, when infused through the systemic

circulation, reduced hepatic cholesterogenesis and 2) removal of chylomicra by thoracic fistula stimulated hepatic cholesterogenesis. Findings by others with cultured cells showed that when LDL and VLDL were added to the medium, cholesterol synthesis was depressed via reduction of the reductase activity (Brown and Goldstein, 1974; Kirsten and Watson, 1974). On the other hand, HDL did not suppress reductase activity in human fibroblasts (Brown and Goldstein, 1974). It appeared that the effectiveness of LDL and VLDL in suppressing HMG-CoA reductase activity was afforded by apolipoprotein B, which is present in both LDL and VLDL, but absent in HDL. Brown and Goldstein (1974) proposed that the molecular mechanism of LDL suppression involved firstly, the attachment of LDL to the cell membrane, secondly, the degradation of the protein moiety, thirdly, the transfer of the cholesterol moiety across the membrane and fourthly, the inhibition of the reductase activity by cholesterol.

The mechanism of action of cholesterol and other steroids on the inhibition of HMG-CoA reductase activity is not fully understood. Siperstein and Fagan (1966) suggested that feedback control by cholesterol involves end product rather than mere suppression of reductase synthesis or genetic repression based on the following evidence: 1) the existence of a feedback inhibitor, 2) the swiftness of inhibitory action, and 3) the occurrence of feedback early in the cholesterol synthetic pathway. However, in later review, McNamara et al. (1972) emphasized that sterol inhibition of reductase activity was affected primarily by decreasing the reductase content. Recent evidence points to both modulation of existing reductase activity and changes in the quantity of reductase protein as

essential for cholesterol inhibition of HMG-CoA reductase (Higgins and Rudney, 1973).

Higgins and Rudney (1973) proposed a biphasic model for the regulation of rat liver HMG-CoA reductase by dietary cholesterol which involved an initial reduction of reductase activity (4 hours after cholesterol feeding), followed by a slower decrease of reductase quantity (8 hours after cholesterol feeding).

The mechanism by which cholesterol modulates the activity of reductase is not clear. Direct allosteric inhibition of reductase appeared unlikely for cholesterol, because cholesterol-rich lipoproteins from either plasma or liver did not always inhibit the reductase activity of liver microsomes from mice (Kandutsch and Packie, 1970) or rats (Shapiro and Rodwell, 1971; Hamprecht et al., 1971) or cell free extracts of human fibroblasts (Brown and Goldstein, 1974). In addition, the reciprocal relationship of cholesterol synthesis and hepatic content was not always demonstrated (Bortz and Steele, 1973; Bortz, 1973). Finally, in vitro preparations of liver homogenates on partially purified HMG-CoA reductase failed to demonstrate reduction in cholesterol synthesis when cholesterol as suspension and emulsion was added to the medium (Linn, 1967; Kawachi and Rodney, 1970).

Sterols may function indirectly, activating or inducing synthesis of reductase inhibitors. However, no inhibitors of reductase activity had been isolated from tissue homogenates of cholesterol-fed animals (Shapiro and Rodwell, 1971) or from extracts of cultured cells grown in cholesterol-rich media (Brown and Goldstein 1974). Since modulation of hepatic cholesterol activity occurred early (4 hours after feeding 1% cholesterol

diet) in the in vivo regulation by cholesterol (Higgins and Rudney, 1973), inhibitors might be transitory, acting only until a decrease in synthesis is initiated (Rodwell et al., 1976).

Reduction of reductase activity may also be mediated through cAMP and MgATP-dependent inactivation systems (Beg et al., 1973). When cAMP was added to liver slices or homogenates, the rate of cholesterol synthesis was decreased (Raskin et al., 1974). Furthermore, reductase in microsomes isolated from cAMP-treated homogenates had decreased activity (Beg et al., 1973). However, the physiological significance of the in vitro inhibition of cholesterol synthesis and reductase activity was questioned by Raskin et al. (1974). In their work, the minimum concentrations of cAMP required for an observable in vitro effect were 3-4 orders of magnitude greater than physiological concentrations. A rapid decrease in reductase activity was observed when isolated microsomes were incubated with Mg-ATP prior to reductase assay (Beg et al., 1973). The mechanism of activation and inactivation of HMG-CoA reductase activity may be similar to that for acetyl-CoA carboxylase in which phosphorylation inactivates the enzyme and diphosphorylation activates it (Carlson and Kim, 1973).

Regulation of the amount of HMG-CoA reductase synthesized involved the interaction of cholesterol with chromatin (Davidson and Gould, 1973). Rat liver chromatin in the DNA acidic protein fraction was associated with free and esterified cholesterol. Feeding cholesterol increased chromatin-bound cholesterol while decreasing the ratio of free to esterified cholesterol associated with chromatin.

A study by Kirsten and Watson (1974) with HTC cells showed that when LDL was added to cells grown in free LDL medium, reductase activity was

rapidly reduced. However, when actinomycin D was added with LDL, reductase activity was unaffected. These results suggested that depression of reductase synthesis by LDL occurred at a site subsequent to RNA transcription. It is possible that cholesterol regulates HMG-CoA reductase synthesis by interfering at the level of DNA transcription.

Thus although the process of cholesterogenesis seemed to exhibit physiological symptoms of product feedback control, biochemical characteristics failed to completely identify the specifics of this process.

Fasting Fasting up to 24 hours resulted in a marked decrease in hepatic cholesterol synthesis in several species, e.g., rat, monkeys, man (Tomkins and Chaikoff, 1952; Dietschy and Wilson, 1968; Weis and Dietschy, 1969). The decreased hepatic cholesterogenesis was associated with 1) decreased enzymatic activity of HMG-CoA reductase (Bucher et al., 1960), 2) decreased enzymatic synthesis (Regen et al., 1966), 3) increased cholesterol content, particularly the esterified cholesterol content of hepatic microsomes. This last observation supports the concept of direct inhibition of HMG-CoA reductase (Tsai and Dyer, 1973). In addition, evidence of a partial block between squalene and cholesterol during fasting has also been reported (Bucher et al., 1960).

Enterohepatic circulation of bile acids The initial observations that biliary diversion resulted in an increased rate of hepatic and small intestinal cholesterogenesis led to the speculation that certain components in the enterohepatic bile circulation had a controlling effect on sterol synthesis in these organs (Dietschy and Siperstein, 1965). In general, any dietary or surgical manipulations that result in depletion

of enterohepatic circulation of bile acids could lead to augmented rates of cholesterogenesis in the liver. Examples are external biliary fistulation in rats (Myant and Eder, 1961), ileal bypass in monkeys (Moutafis and Myant, 1968), and feeding of cholestyramine, a bile acid sequestrant, to swine (Schneider et al., 1966) or rats (Huff et al., 1963). Conversely, expansion of the bile acid pool by feeding bile acids or their derivatives could lead to decreased endogenous cholesterol synthesis. Beher and Baker (1959) showed that feeding bile salts to rats resulted in decreased conversion of acetate to cholesterol. More recently, Grundy et al. (1966) obtained similar results in humans, using steroid balance techniques.

The mechanism by which bile acids or bile salts influence hepatic cholesterogenesis may be affected through HMG-CoA reductase and cholesterol absorption. Hamprecht et al. (1971) showed that cholic acid feeding prevented the diurnal rise of HMG-CoA reductase activity, while Barth et al. (1973) reported reduction of HMG-CoA reductase synthesis with bile salt feeding.

Increased hepatic cholesterogenesis following biliary drainage could be secondary to decreased cholesterol absorption. This could be achieved through the release of negative feedback inhibition of cholesterol on hepatic synthesis.

Small intestine cholesterogenesis The small intestine ranks next in importance to the liver in synthesizing endogenous cholesterol. Cholesterol synthesis in the small intestine, compared to liver, is less susceptible to feedback inhibition by dietary cholesterol. This effect has been reported for several animals including rats (Dietschy, 1968), baboons (Wilson, 1972) and man (Dietschy and Gamel, 1971). The

primary functional contribution of cholesterol synthesis in the small intestine is geared to epithelial turnover. However, during cholesterol ingestion when hepatic cholesterogenesis is suppressed, the small intestine contributes 50-80% of endogenous cholesterol in the rat (McIntyre and Isselbacher, 1973; Chevallier and Lutton, 1973).

Cholesterol synthesis occurs throughout the whole length of the small intestine. However, the terminal ileal regions appear to be most active in rats (Dietschy and Siperstein, 1965) and in man (Dietschy and Gamel, 1971). The rate of cholesterogenesis in the intestine varies with the tissue layer. The crypt cells, where mitosis is most active, have the highest rate of cholesterogenesis compared to the villi and muscle cells (Wilson, 1968).

Control of small intestine cholesterol synthesis Small intestine cholesterogenesis, like that in liver, is influenced by dietary cholesterol, by fasting and by bile acid flow in the enterohepatic circulation. The rate limiting steps and enzymes controlling small intestine cholesterogenesis are similar to those found in hepatic synthesis.

Dietary cholesterol In rats, dogs and monkeys dietary cholesterol decreased cholesterogenesis in the small intestine only slightly or not at all (Dietschy and Wilson, 1968; Gould et al., 1953). The HMG-CoA reductase activity of intestinal tissue was not suppressed by dietary cholesterol. However, feeding rats cholesterol plus taurocholate or taurochenodeoxycholate did suppress reductase activity. It has been suggested that the inability of cholesterol alone to suppress intestinal reductase activity could be attributable to failure to reach its site of action within the intestinal cells (Shefer et al., 1973). It could also

imply that intestinal HMG-CoA reductase is an isozyme of hepatic HMG-CoA reductase, with therefore a higher Km for cholesterol inhibition.

Fasting In rats, food deprivation for 48 hours did not affect intestinal cholesterogenesis (Dietschy and Siperstein, 1967). In baboons, however, an 18-20 hour fast stimulated cholesterol synthesis slightly, while a 48 hour fast in man reduced intestinal cholesterogenesis at the distal duodenum by 50% (Dietschy and Gamel, 1971). It is therefore difficult to infer the effects of fasting on intestinal cholesterogenesis from the limited work which has been done with the various conditions under which experiments were done.

Enterohepatic bile circulation In rat and man the intestine responded to bile acid diversion with increased cholesterol synthesis throughout the bowel, while infusion of bile caused a marked decrease of sterol formation (Dietschy and Siperstein, 1965). Biliary diversion or feeding of B-sitosterol enhanced HMG-CoA reductase in rat intestinal crypt cells. On the other hand, feeding taurocholate and taurochenodeoxycholate decreased HMG-CoA reductase activity (Shefer et al., 1973). The inhibitory activity of bile salts seemed to depend on the presence of a 3 or 7α-hydroxy group and a carboxyl group at the terminal side chain. Inhibition was abolished when the 6 or 12 position was hydroxylated. Taurine conjugates of bile acids were as effective as free bile acids, while glycine conjugates were only half as effective as other conjugates (Hatanaka et al., 1972).

The mechanism of bile acid control on intestinal cholesterogenesis is not well developed. Cholesterol synthesis at any section of the intestine is related to bile acid concentration in the lumen at the corresponding section. Ho and Taylor (1970) suggested that regulatory

mechanisms involve HMG-CoA repression at the genetic level rather than competitive or allosteric inhibition. This argument was based on the following evidence: 1) a latent period required for intestinal inhibition after infusion of bile salts into the intact animal, 2) absence of feedback inhibition by the addition of bile salts in vivo, and 3) the sensitivity of Vmax but not Km of HMG-CoA reductase to bile salts.

The reciprocal relationship between lumen bile concentration and intestinal cholesterogenesis is dependent only on the former. When rats were fed tomatine, a compound which precipitates cholesterol and makes it unavailable for absorption, the rate of synthesis in the intestine increased, in spite of unchanged luminal bile acid turnover and level (Cayen, 1971).

Rate-limiting steps in cholesterol synthesis

About 26 steps have been identified in the synthesis of cholesterol from acetate (Popjak and Cornforth, 1960; Frantz and Shroepfer, 1967).

$$18 \text{ acetyl CoA} \rightarrow \text{cholesterol} + 9\ CO_2$$

The initial chemical reactions in this sequence are part of pathways common to other metabolic end products or initiating substrates such as glycolysis, tricarboxylic acid cycle and fatty acid synthesis. The fourth step whereby HMG-CoA is converted to mevalonate is the primary rate controlling step of cholesterogenesis.

Though mevalonate is the product of a committed step, which leads primarily to cholesterol synthesis, it is also the precursor of several nonsteroidal products. These quantitatively minor but physiologically important products include the polyisoprenol side chains of ubiquinone and dolichol, which are lipid carriers in glycoprotein synthesis (Bloch, 1976).

Furthermore, recent work by Bhavani and Short (1973) strongly suggests a branching of the main pathway at a point just prior to squalene leading to the estrogen-related equilin and equilenine directly rather than via cholesterol.

Other rate limiting steps of cholesterol synthesis which are of secondary importance include the conversion of acetyl CoA → HMG-CoA, mevalonate → cholesterol, and squalene → cholesterol.

Acetyl CoA → HMG-CoA The findings of Lane et al. (1973) indicated that beta-ketoacyl thiolase and HMG-CoA synthetase occurred in the cytoplasm as well as in mitochondria. Synthetase and thiolase activities decreased following prolonged cholesterol feeding (White and Rodney, 1970; Beg and Gibson, 1973). These changes, however, are slow and incomplete, thereby suggesting that the thiolase and synthetase enzymes function as potential loci for an adaptive, secondary mechanism of regulation.

Mevalonate → cholesterol Prolonged cholesterol feeding depressed the conversion of mevalonate into farnesyl pyrophosphate as well as the subsequent conversion of farnesyl pyrophosphate into squalene (Gould and Swyryd, 1966). In addition, fasting decreased the activities of pyrophosphomevalonate decarboxylase, isopentenyl pyrophosphate isomerase, dimethylallyltransferase and squalene synthetase (Slakey et al., 1972). Although these changes were pronounced, they were not as rapid or extensive as were changes in cholesterol synthesis or reductase activity.

Involvement of sterol-carrier protein (SCP) between squalene → cholesterol Based on recent studies by Ritter and Dempsey (1971), Scallen et al. (1971) and Tai and Bloch (1972), 2-3 noncatalytic proteins have been implicated to act in the course of squalene → cholesterol.

These proteins are referred to as Sterol Carrier Proteins (SCP) or Soluble Protein Factor (SPF). So far, squalene epoxidase and 7-dehydro-cholesterol reductase are the only enzymes which have responded to these proteins. The SCP preparation isolated by Ritter and Dempsey (1971) mimicked the action of human high density apolipoprotein. In contrast, SCP isolated by Scallen et al. (1971) possessed the properties of low-density lipoprotein (LDL). The soluble carrier protein appeared to function as a substrate carrier, stimulating interactions of dispersed water-insoluble intermediates with membrane-associated enzymes. The exact mode of action of these protein factors is unclear. Their effects on the regulation of cholesterol synthesis is presently under investigation.

Cholesterol absorption, degradation and excretion

Cholesterol absorption, degradation and excretion has been reviewed extensively by Dietschy and Wilson (1970). Consequently only summaries of the topics are given here.

Cholesterol absorption is controlled by several factors. These include the physical form of dietary cholesterol, the pool size of bile acids, the permeability status of the lumen mucosal membrane, secretion and activity of the hydrolyzing enzymes like pancreatic esterase and cholesterol esterase, and finally the luminal concentrations of fatty acids, glycerides, and phospholipids derived from dietary fats (Dietschy and Wilson, 1970). According to Kim and Ivy (1952) and Wilson (1962), dietary fat may enhance exogenous cholesterol absorption by 1) stimulating bile flow to the intestinal lumen, resulting in increased cholesterol solubilization into micelles and thus increased rates of chylomicron forma-

tion and 2) increasing supplies of amphipathic substances like monoglycerides and free fatty acids which are essential for micelle formation.

The rate limiting step of cholesterol absorption may occur at the movement of chylomicrons from mucosa into lymphatic circulation. Bile acids and lipoproteins have been implicated to be essential for the transfer (Sylven and Borgstrom, 1968).

Cholesterol degradation to bile acids occurs primarily in the liver (Harold et al., 1955). Free form cholesterol is the preferential substrate for bile acid synthesis (McGovern and Quackenbush, 1973b). The bile acids formed are subsequently conjugated with glycine or taurine, forming primary bile acids. In the distal intestinal tract, the primary bile acids are deconjugated by bacterial action (bacteriodes and bifidobacterise to secondary bile acids) which are either reconjugated and reabsorbed, or excreted as acidic steroids in the feces (Dietschy and Wilson, 1970).

The control of cholesterol degradation or bile acid synthesis is under feedback inhibition by bile acids in rat, monkey and man (Dietschy and Wilson, 1970). In rats, bile acid excretion increased with bile duct cannulation (Kay and Entenman, 1961), cholestyramine feeding (Johansson, 1970), and decreased with bile acid infusion into the small intestine after bile duct cannulation (Shefer et al., 1970). Short term cholesterol feeding in rats led to enhanced bile acid synthesis (Takeuchi et al., 1974). Feedback control by bile acid and substrate induction by cholesterol are affected by 7 α-hydroxylase, which is the major rate-limiting enzyme of bile acid synthetic pathway (Shefer et al., 1970; Takeuchi et al., 1974).

Cholesterol excretion takes place in two forms, as acidic steroids and neutral steroids in the feces. Acidic steroids are derived from bile acids, while neutral steroids (containing cholesterol and its derivatives such as coprostanol and coprostanone) are derived from bile and intestinal epithelial cells. The proportion of acidic to neutral steroids excreted varies with the type of diets consumed. Diets high in polyunsaturated fats (McGovern and Quackenbush, 1973a), cholesterol (McGovern and Quackenbush, 1973b), and fiber (Kritchevsky et al., 1974) increased total cholesterol excretion, with greater excretion as acidic than neutral steroids.

Meal pattern and cholesterol metabolism

Recent work in our laboratory has shown that meal feeding, in contrast to ad libitum feeding, may increase serum cholesterol concentrations. Adult male rats were depleted to 60% of their original weight. Depletion was followed by a 10- or 30-day realimentation regimen with diets containing 2 or 40% of kcal as fat (Reeves and Arnrich, 1974; Carlson, 1975). Irrespective of types of diets tested, the authors found that adult rats consuming their food calories in an 8-hour meal, showed higher serum cholesterol concentrations compared to rats consuming their food calories ad libitum. Similarly, increases in serum cholesterol level with meal feeding have been observed in chickens allowed access to food for 2 1-hour periods per day (Cohn et al., 1961), in monkeys fed 2-3 1-hour periods per day (Gopalan et al., 1962) and in humans consuming 3 meals per day (Cohn, 1964).

Not all investigations have come to the same inference. Okey and coworkers (1960) failed to observe differences in mean serum cholesterol

concentrations between rats fed 3 hours per day and rats fed ad libitum. In a recent study, Wadhwa et al. (1973) also failed to distinguish an effect of meal pattern on serum cholesterol levels in humans.

The effect of meal pattern on serum cholesterol levels appears to vary with length of feeding period. Reeves and Arnrich (1974) observed greater differences in serum cholesterol concentrations between meal fed and ad libitum fed rats, at 30- compared to 10-days of feeding. Gopalan et al. (1962), working with monkeys, reported bigger differences between the two meal patterns at 8 compared to 4 weeks of feeding. In contrast, chicks fed 2 1-hour periods had elevated serum cholesterol levels over ad libitum fed birds only during the first 3-6 weeks. The difference disappeared when feeding was extended to 10 weeks. In summary, the effects of meal feeding on serum cholesterol levels appear to vary with species, age, feeding length and number of meals per day.

One factor contributing to increased serum cholesterol concentration with meal feeding could be plasma free fatty acid levels during fasting. Increased free fatty acids in plasma stimulate triglyceride secretion by the liver (Prigge and Grande, 1973; Wadhwa et al., 1973). In the rat, VLDL are secreted by the liver and function to circulate triglyceride to other tissues. Cholesterol and phospholipids are required to stabilize the structures of VLDL. Increased serum cholesterol concentration observed with meal feeding may be a consequence of elevated serum triglyceride formation in response to free fatty acid concentrations (Goh and Heimberg, 1973).

The influence of meal pattern on other parameters of cholesterol metabolism, such as synthesis, excretion, degradation, have gained little

attention. Recent work with adult rats failed to show changes in rate of hepatic cholesterogenesis with meal feeding (Carlson, 1975). Other investigators working with young rats reported increased (Dupont and Lewis, 1963; Dupont, 1965) hepatic cholesterol synthesis with meal feeding.

Few data are available which relate the influence of frequency of feeding to cholesterol degradation and excretion. Bobek et al. (1973) compared rats fed for 2 hours versus ad libitum feeding. Recoveries of 4-^{14}-C-cholesterol, four days after administration, in liver, serum and small intestine revealed little variation between the two meal patterns. In addition, cholesterol degradation to bile acids and excretion of neutral steroids were identical between the meal patterns. Recent work by Carlson (1975) confirmed some of Bohek's results. Hepatic and small intestine cholesterogenesis, bile acid formation and excretion, and neutral steroid excretion varied little between ad libitum and meal feeding.

Age and cholesterol metabolism

Our literature search yielded limited information about effects of maturation or aging on cholesterol metabolism in rats. Data for growing rats were obtained primarily from one laboratory (Dupont, 1966; Dupont et al., 1972). In one study maturation and aging had a marked effect on cholesterol biosynthesis in response to dietary fat level and type (Dupont, 1966). Male weanling rats were fed test diets containing 2 or 42% of calories as fat coming predominantly from either corn oil or beef tallow. Rats were fed these diets until they were 3 or 4 1/2 months of

age. In general, rate of cholesterogenesis at 3 months exceeded those of 4 1/2 months old rats, regardless of the dietary variables. At 3 months of age, corn oil and beef tallow caused 29 or 3-fold increases, respectively, in hepatic cholesterogenesis compared to the low fat diet. At 4 1/2 months of age, differences between low fat diet and either corn oil or beef tallow diets were less dramatic, but evident.

In another study Dupont et al. (1972) found little reduction of cholesterol synthesis from acetate when 3 and 18 months old rats were compared. However, an oscillating effect in cholesterogenesis rates with maxima at 3 and 12 months and minima at 6 and 18 months was evident. With advancing age, fecal neutral steroid excretion decreased. Carcass acidic steroid contents were much higher in 18-months compared to 6 months old rats. Serum cholesterol level was doubled between 12 and 18 months of age. In general increase in serum cholesterol concentration is consistent with advancing age, regardless of diet. Changes in hepatic cholesterogenesis are not so predictable with age, and these changes are influenced by diets.

Dietary fat and cholesterol metabolism

Dietary fat level Several investigators have demonstrated an increase in fasted serum cholesterol levels following dietary treatments with high- compared to low-fat containing diets (Prigge and Grande, 1973; Hill, 1960). Prigge and Grande (1973) compared diets containing 11 or 40% fat from either coconut oil, olive oil or sunflower oil in dogs. Increasing fat level was accompanied by elevated levels of serum cholesterol, phospholipids and postprandial serum free fatty acids (FFA). A positive correlation of FFA to serum lipids was observed. The authors suggested that

alterations in serum FFA associated with high fat feeding might be involved in the mechanism whereby dietary fat influenced serum cholesterol levels in the dog.

Hill et al. (1960) tested the effects of diets containing 15% corn oil, Wesson oil, Snowdrift or lard to a zero fat diet in rats. Regardless of fat source, hepatic cholesterogenesis from ^{14}C-acetate was elevated with fat feeding, 12 hours after inhibition of hepatic lipogenesis. Under conditions of controlled feedings, Bortz (1967), Goldfarb and Pitot (1972) and Carlson (1975) reported similar findings with fat feeding in young and adult rats.

Fat feeding effects on hepatic cholesterogenesis appear to be exerted prior to the formation of mevalonate and result in increased amounts of HMG-CoA reductase (Goldfarb and Pitot, 1972).

Elevated intracellular levels of acetyl CoA and fatty acyl CoA, as a result of decreased lipogenesis, leads to increased availability of substrates for cholesterol and ketone body formation (Bortz, 1967). Increased hepatic cholesterol synthesis required HMG-CoA reductase synthesis before accelerated cholesterol formation was noted.

In rats dietary fat feeding decreased hepatic bile acid content but increased cholesterol content (Bortz, 1967; Goldfarb and Pitot, 1972).

Dietary fat saturation Merrill (1959) observed enhanced rates of hepatic cholesterogenesis with feeding linoleic acid versus coconut oil. Similarly, Wood and Migocovsky (1958) reported enhancing effects on

hepatic cholesterol biosynthesis with polyunsaturated and unsaturated fatty acids. Thus, rape-seed oil, corn oil, erucic acid and oleic acids stimulated greater rates of cholesterol synthesis than coconut oil.

More recent reports by other laboratories have arrived at the same conclusion (Boyd, 1962; Tria et al., 1971; Dupont et al., 1972). In contrast, Carlson (1975) and Dupont et al. (1975) failed to demonstrate differences in cholesterol biosynthetic rates with safflower oil and beef tallow feeding. Several theories have been proposed to explain the stimulatory effect of polyunsaturated fats on cholesterol synthesis. Increased cholesterogenesis may be related to increased bile acid formation, since the half-life of bile salt (cholate) was reduced in animals fed safflower oil (McGovern and Quackenbush, 1973c) and in humans fed corn oil.

A decrease in cholesterogenesis in the small intestine with polyunsaturated fat feeding has been reported by several laboratories (Cayen, 1971; Chevallier and Lutton, 1973; Carlson, 1975). In all these studies, depressed intestinal cholesterogenesis was attributed to increased intestinal cholesterol concentration, indicative of feedback control.

In general, most studies with rat liver perfusates showed no quantitative difference in bile acids production between saturated and polyunsaturated fat feeding. In another study with rats given oral infusions of either linoleate or oleate, no variation in bile acid excretion

between treatments was observed (Iritani and Nogi, 1974). In contrast, other investigators had reported that animals responded to polyunsaturated fat feeding with increasing bile acid formation. Substitution of polyunsaturated ruminant fat for regular fat (Nestel et al., 1969; 1973) or sunflower seed oil for butterfat (Antonis and Bersohn, 1962) in normalipic men, led to elevated bile acid excretion in the excreta. In adult rats, elevated bile acid formation and excretion occurred with safflower oil compared to beef tallow feeding.

The result of polyunaturated fat feeding is associated with shortened bile acid half life resulting in increased bile acid formation and excretion. McGovern and Quackenbush (1973c) obtained direct evidence of bile acid synthesis in liver via biliary cannulation. The authors reported 13% increase in bile acid production and 8% decrease in bile acid secretion in liver with safflower oil compared to beef tallow feeding in young rats.

Excretion of neutral steroids may be elevated with polyunsaturated fat feeding. However excretion of this metabolite is usually less dramatic compared to bile acid excretion. Increased neutral steroid excretion has been reported for corn oil and safflower oil feeding (Connor et al., 1969; Moore et al., 1968).

Methods and Materials

Experiment 1 was designed to study the effects of the following variables on cholesterol metabolism:

(1) dietary fat level: 2 vs. 38% of calories from fat.

(2) saturation of dietary fat: beef tallow (BT) vs. safflower oil (SO).

(3) frequency of feeding: 24 vs. 3 hours of food availability.

(4) length of feeding: 30 vs. 60 vs. 90 days.

A double label system using ^{3}H-acetate and 4-^{14}C-cholesterol was used to assess distribution, synthesis, degradation and excretion of cholesterol.

Design of experiment

Eighteen dietary treatments were used as shown in Figure 2. Each treatment group consisted of 10 rats. Littermates in sets of two were used. They were assigned to the same diet and length of feeding, but to two different feeding patterns. Three experimental diets were tested. Two of the diets contained 38% of the calories from fat (SO or BT). The percentages of linoleic acid in these fats varied between 79 (SO) and 4 (BT). In the remaining diet 2% of the calories was from fat, given as safflower oil (LF). The rest of the calories were met by increasing the carbohydrate and protein components. All diets were isocaloric with respect to protein, mineral mix and nonnutritive fibers. Two feeding patterns were used. Rats were given food jars for either 24 (AL) or 3 (MF)

hours out of a 24 hour period. Animals were placed on these dietary regimens for 30, 60 or 90 days.

For each animal the following parameters were measured:

(1) food intake, body weight gain and food efficiency.

(2) wet weight of liver, spleen, epididymal fat pads, kidneys, small and large intestines (with contents), heart and stomach (without contents).

(3) total lipid of liver.

(1) and (2) provide information on growth pattern of maturing rats with the dietary variables.

(4) cholesterol concentration for serum, liver, and small intestine. These parameters estimate distribution in designated sites.

(5) ^{14}C and ^{3}H-cholesterol as digitonin precipitable steroids (DPS) of serum. These data estimate the contribution of cholesterol by the liver and small intestine to the circulating pool.

(6) ^{3}H in DPS of liver and small intestine. These counts represent newly synthesized cholesterol in the major sites of synthesis.

(7) ^{14}C in DPS of liver and small intestine. These counts represent the nondegraded cholesterol.

(8) ^{14}C-counts in saponifiable lipid of liver, small intestine, large intestinal content and feces. These counts represent bile acid metabolites of cholesterol lost from body pools.

(9) plasma triglycerides.

(10) plasma thyroxine.

Animal treatment

Weanling male rats of the Wistar strain[1] were raised on a modified Steenbock XVII ration (Table 1). This diet was supplemented weekly with 15 g lean ground beef, 20 g raw carrots and 10 g fresh cabbage. In addition vitamins A and D were given orally once a week in 50 mg corn oil containing 165 mcg retinol equivalents as retinyl palmitate and 1.25 mcg vitamin D_3. When rats weighed 100±5 g (30±3 days old), they were transferred to an experimental room with a reversed lighting schedule. Dark hours were set between 9 AM and 7 PM. Room temperature was maintained at 24±2° and relative humidity at approximately 40%. Animals were housed individually in suspended wire mesh cages, with free access to distilled water. Cages were changed once a week, and water checked for turbidity. Skin and respiratory problems were monitored periodically. This care procedure was taken to provide clean and sanitary living conditions for the animals throughout the length of the study, thus reducing the risk of infection and animal loss, both of which would complicate an aging study.

A day before the experiment, animals were fasted overnight (5 PM-9 AM). Assignment to treatment groups was made randomly using a lot system. Each set of two littermates was assigned to the same diet and length of feeding, but two different feeding frequencies. Rats were fed and weighed by 9 AM, i.e., at the beginning of the dark cycle. Food jars of rats on the 3 hr feeding schedule (MF) were removed by 12 PM. Concurrent with their diet, animals received daily supplements of vitamins in separate cups. Food intake and body weight gain were recorded every other

[1]Iowa State University, Dept. of Food and Nutrition stock colony.

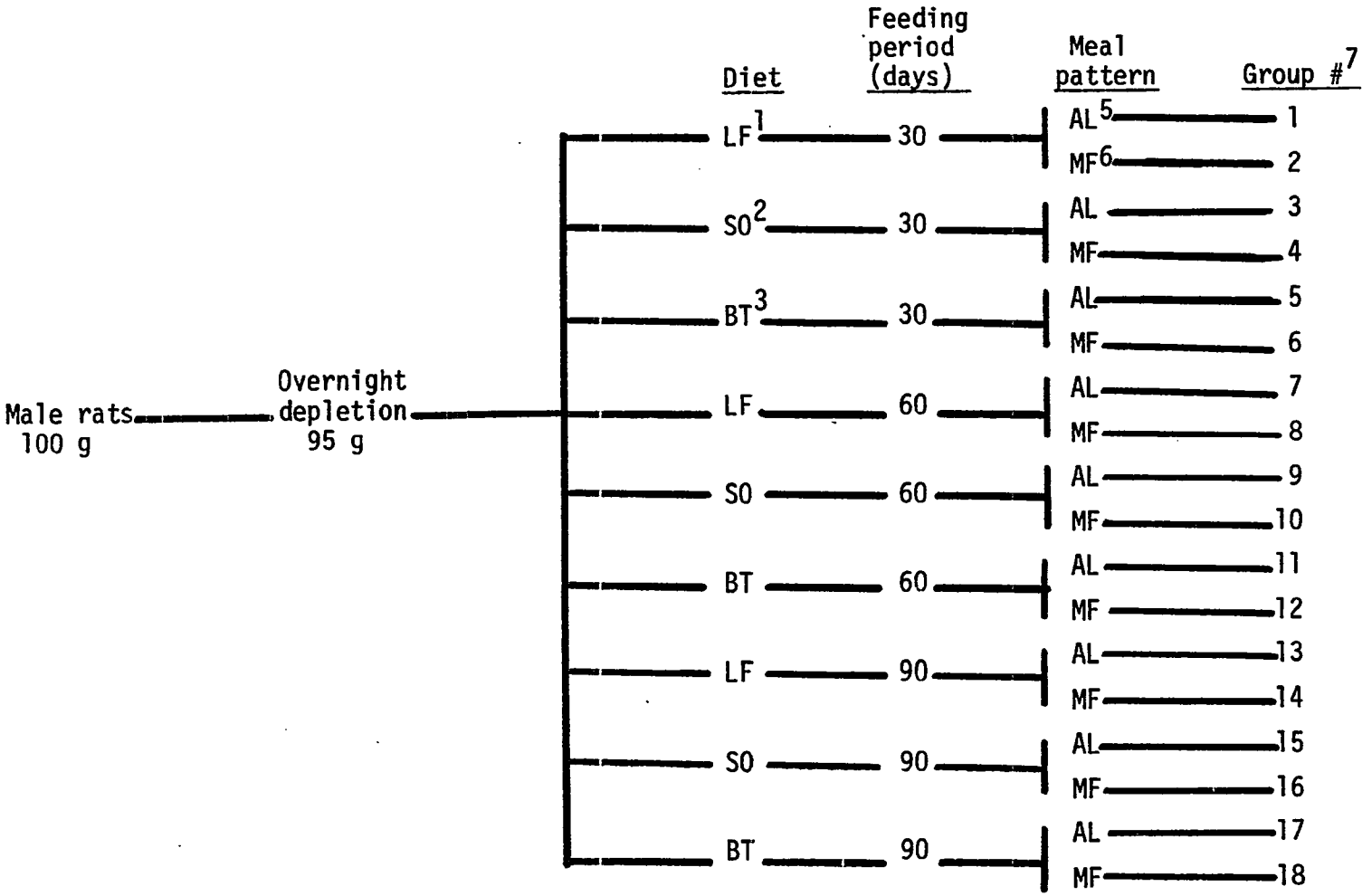

Figure 2. Schematic design of experiment 1: 1 = 2% safflower oil as calories; 2 = 38% safflower oil as calories; 3 = 38% beef tallow as calories; 4 = littermates in sets of two; 5 = ad libitum (24 hr feeding out of a 24-hr period); 6 = meal fed (3 hr feeding out of a 24-hr period); 7 = 10 rats per group.

day. A food efficiency ratio of body weight gain to food consumed in kcal was calculated for each diet.

Diets

Three experimental diets were tested. Composition of each diet is given in Table 2. Percentages of calories from corn starch, casein and fat are listed in Table 2. Protein, salt mix, nonnutritive fiber were equalized on the basis of calories.

Fat was the only component altered in this study. Dietary fat provided 2 (LF) or 38 (SO or BT) % of calories. Fat calories were furnished by beef tallow (BT) or safflower oil (LF and SO). Fatty acid composition of safflower oil, beef tallow and the Steenbock XVII ration are shown in Table 3.

Protein level for the test diets was approximately 21% of calories. This was provided by vitamin free casein which contained approximately 91.4% of protein by assay. DL-methionine was supplemented at the level of 1.5 g/100 g casein. Cholesterol at 0.02 mg/100 g diet was added to LF and SO diets to compensate for the cholesterol level present in BT.

Daily supplements of fat- and water-soluble vitamins were given in addition to the experimental diets. Tables 4 and 5 show the composition and daily allowances of the water- and fat-soluble vitamins. Fat soluble vitamins included vitamins A, D and E. Both A and D were furnished by 50 mg cod-liver oil. Vitamin E, as DL-αtocopherol acetate, was diluted with corn oil to provide a daily dosage of 1 mg DL-α-tocopherol acetate. Water soluble vitamins were measured using a calibrated 1/4 teaspoon that closely approximated 500 mg. Both fat- and water-soluble vitamins were given together in a cup. They were usually prepared 1/2 hour before feeding.

Table 1. Stock ration for male rats: modified Steenbock XVII (1974-75)

Dietary components	% by weight
Corn meal[a]	48.3
Linseed meal[b]	13.8
Skim milk[c]	10.3
Wheat germ[d]	8.6
Brewers yeast[a]	8.6
Casein, high protein[a]	4.3
Corn oil[e]	3.5
Alfalfa meal[f]	1.7
NaCl (iodized)[g]	0.4
$CaCO_3$ + trace elements[h]	0.4
Corn oil + vitamin D_3[i]	0.1

[a]Teklad, Madison, Wisconsin.

[b]Froning and Deppe Elevator, Ames, Iowa.

[c]Des Moines Cooperative Dairy, Des Moines, Iowa.

[d]General Mills, Inc., Minneapolis, Minnesota.

[e]Mazola, Best Foods Division Corn Products Co., New York.

[f]National Alfalfa, Lexington, Nebraska.

[g]Morton salt, local grocer.

[h]Matheson Coleman & Bell Division, Matheson Company, Inc., Norwood, Ohio.

[i]Crystalline vitamin D_3 (cholecalciferol) diluted to 2,000 IU (50 mcg/kg diet) with corn oil.

Table 2. Composition of experimental diets based on weights and calories

Dietary components	Diets		
	LF	SO	BT
	g/100 g diet		
Cornstarch[a]	71.5	46.1	46.1
Casein, vitamin free[b]	22	27	27
Safflower oil[c]	1	20	0
Beef tallow[d]	0	0	20
Cholesterol[e]	0.02	0.02	0
Salt mix[a] (William & Briggs)	3.5	4.3	4.3
Nonnutritive fiber[a] (cellulose)	2.0	2.5	2.5
Dl-methionine[f]	0.33	0.40	0.40
	Kcal/100 Kcal		
Cornstarch	77	41	41
Fat	2	38	38
Casein protein	21	21	21

[a]Clinton Corn Products, Clinton, Iowa.

[b]91.3% protein, Teklad Test Diets, Madison, Wisconsin.

[c]Pacific Vegetable Oil Corp., Richmond California (α-tocopherol added as anti-oxidant at 0.1% by weight).

[d]Oscar Mayer, Madison, Wisconsin.

[e]J. T. Baker Chemical Co., Phillisburg, New Jersey (added at level of 1 mg/1 g BT).

[f]1.5 g/100 g casein; Teklad Test Diets, Madison, Wisconsin.

Table 3. Fatty acid pattern of stock ration, beef tallow and safflower oil by gas liquid chromatography

Diets or ingredients	% fatty acids						
	14.0	16.0	16:1	18:0	18:1	18:2	18:3
Stock ration[a]	0.6	17.8	2.1	2.7	21.7	49.4	5.7
Safflower oil	neg	6.1	0.0	2.1	12.8	79.0	0.0
Beef tallow	3.1	23.1	3.5	20.2	43.5	3.8	1.1

[a]Steenbock XVII, 1975. Lipid extracted by Folch et al. method (1957).

Diet preparation A standard procedure was set up to reduce variations between diet preparations. Diet was prepared in 15 kg batches in a 20 kg stainless steel mixer. Nonnutritive fiber, William-Briggs salt mix and casein were mixed for 3 minutes at low speed. Cholesterol and methionine in an oil mixture were added next. Cholesterol was first ground in a mortar, then combined with methionine in some oil (5 - 10 mls). Methionine does not dissolve in oil but was introduced to the dry ingredients in this way to reduce loss during transfer and stirring in the mixer. Following the addition of cholesterol and methionine, the remaining oil (or melted BT) was added. The ingredients were mixed for 3 minutes at low speed. Corn starch was added next. The final mixture was mixed for 5 minutes at medium speed or until no clumps were visible. The temperature of the fresh diet was measured. Each diet was stored in Nalgene containers and refrigerated at 4°.

Beef tallow was melted at 40°, weighed and allowed to cool before it was added to the dry ingredients.

Vitamin preparation A mixture of water soluble vitamins was usually prepared in 2,000 g batches. The vitamins were weighed and ground with about 30 g dextrin in a mortar for 20 minutes or until the mixture was homogeneous. The mixture was transferred to a large stainless steel mixing bowl and mixed with the remaining dextrin for 1 hour. The vitamin mixture was stored in amber jars at 4° or at -20° if more than one week's storage was required. Vitamin A and D were given in cod liver oil. A calibrated dropper which delivered about 50 mg in 2 drops was used. This amount provided 85 IU of vitamin A and 8.5 IU of vitamin D per rat per day.

Table 4. Composition of water soluble vitamin mixture for growing rats

Vitamin	dose composition/rat/day
Thiamin[a]	40 mcg
Riboflavin	60 mcg
Pyridoxine HCl	40 mcg
Ca-Pantothenate	100 mcg
Nicotinic acid	500 mcg
Folic acid	8 mcg
Biotin mixture[b]	1 mcg
B_{12}[c]	0.72 mcg
L-ascorbic acid	1 mg
Meso-Inositol	10 mg
Para-aminobenzoic acid (USP XIV, PABA)	10 mg
Choline Cl	5 mg
Dextrin to make 500 mg[d]	-
	500 mg = 1 dose

[a]All vitamins obtained from General Biochemicals, Inc., Chagrin Falls, Ohio (known as Teklad, Madison, Wisconsin since 1975).

[b]Biotin mixture prepared by mixing 1 mg biotin with 99 mg dextrin.

[c]B_{12} in mannitol at 0.1 mg B_{12}/100 mg of mixture.

[d]Teklad, Madison, Wisconsin.

Table 5. Fat soluble vitamins for growing rats

Vitamin	dose/day/rat
A	85 IU in 50 mg cod liver oil[a]
D	8.5 U
DL-αtoxopherol[b]	1 mg in 50 mg corn oil[c]

[a]Squibbs cod liver oil USP (50 mg/day).

[b]General Biochemicals, Inc., Chagrin Falls, Ohio.

[c]Mazola, Best Foods Division Corn Products Co., New York.

Vitamin E was provided as DL-α-tocopherol. One mg per rat per day was administered to the growing rat. This was diluted with corn oil so that 50 mg contained the daily allowance. The solution was stored in amber medicinal bottles at 4°.

Feeding frequencies (meal patterns)

Two meal schedules were used. Rats were either allowed access to a given diet for 24 hours (AL) or for only 3 hours (MF) out of a 24 hour period. Food jars for AL rats were given at 9 AM and replaced with fresh food jars at the same time two days later. Meal-fed rats had access to food from 9AM-12PM each day. Body weight change and food consumed were calculated on a two day basis.

Lengths of feeding

Three feeding periods were used. Rats were raised on a given diet for 30, 60 or 90 days. Rats were killed on the morning of the 31st, 61st and 91st day of the experiment.

Isotope injections

Six days prior to sacrifice (i.e., 25th, 55th and 85th), rats were given a single IP injection of 4-^{14}C-cholesterol[1] (2.5 μC/.5 ml saline) just before feeding. Feces were collected hereon for the next 6 days. On the day the rats were killed they were allowed access to food for 1 hour (9-10 AM). Subsequently a single IP injection of ^{3}H-acetate[1] (50 μC/.5 ml saline) was administered. Two and a half hours later, rats were

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[1]Amersham/Searle, Arlington Heights, Illinois.

anesthetized with an IP injection of Na Pentobarbital[1] (25 mg/100 g b.w.) according to the procedure of Carlson (1975).

Tissue collection

Blood was obtained from the right ventricle by heart puncture and stored in cold 10 ml heparinized tubes (140 IU Na heparin/ml). Blood was spun in the cold in a clinical centrifuge for 20 minutes at 2000 rpm. Plasma was stored in tightly screwed 2 dram vials at -20°.

Immediately after the removal of blood, the liver was excised, rinsed in cold distilled water, dried on filter paper and weighed. It was then wrapped in aluminum foil, quick-frozen in liquid nitrogen, packaged in sealed plastic pouches and stored at -20°. The stomach (contents washed out), spleen, small and large intestines with contents, epididymal fat pads, kidneys and heart were removed in the order listed. The viscera were weighed and stored as described for liver. Mysenteric fat adhering to the intestines were carefully trimmed before weighing.

Feces collected earlier were sealed in plastic pouches and stored at -20°.

Radioactive counting and quench corrections

An automatic dispenser was used to dispense 10 ml scintillation cocktail (spectrafluor butyl PBD[2] in toluene) to each sample. Two samples were prepared for each parameter. Samples were counted in duplicate for 15 or 20 minutes, depending on activity. Liquid scintillation counters[3]

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[1]Nembutal sodium, Abbott Laboratories, Chicago, Illinois.

[2]Amersham/Searle Corporation, Arlington Heights, Illinois.

[3]Packard Instrument Company, Downers Grove, Illinois.

(Packard Tri Card) model 3320 and 2405 (automated ^{14}C and ^{3}H settings) were used. Channels and gains were set for ^{14}C and ^{3}H countings. ^{14}C spillover into ^{3}H channel was about 8.5% and 2% for models 3320 and 2405 respectively. ^{14}C and ^{3}H efficiencies for double label counting were 63 and 48% for model 3320 and 65 and 58% for model 2405.

Loss in counting efficiency due to quenching (methanol, color agents, traces of PPT, traces of phase separation, etc.) was corrected for by using both internal and external standardizations. ^{3}H and ^{14}C-toluene[1] were used as internal standards. The third channel of the instrument was used for external standard counts for each sample. A plot of count efficiency against either count rate in dpm (model 3320) or count ratio (model 2405) was obtained from a series of ^{3}H and ^{14}C standards with known quantities of methanol. Methanol was added in amounts of 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 4, 6 or 8 mls for each isotope series. In all instances, 10 ml butyl PBD in toluene was added as cocktail. Since methanol was used to solubilize cholesterol in a toluene base cocktail, it was also used as the quenching agent for the standard curves. Each instrument has its characteristic curves. From the count rate or count ratio, a corresponding count efficiency factor was determined, which was used for calculating the dpm for each isotope in a sample:

$$\text{count efficiency} = \frac{\text{cpm}}{\text{dpm}}$$

$$\text{dpm} = \frac{\text{cpm}}{\text{count efficiency}}$$

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[1]Amersham/Searle Corporation, Arlington Heights, Illinois.

Variations between individual instruments were 5% for ^{14}C and 10% for ^{3}H. Therefore, whenever possible samples measuring the same parameter were counted on the same instrument. The counting difference was taken into consideration during calculation.

Chemical Analysis

Lipid extraction

The method of Folch et al. (1957), as modified by Stadler (1969), was used to delipidate the liver (L), small intestine (SI), epididymal fat pads (EF) and heart (H). A diagram of the extraction procedure is shown in Figure 3. In general, a weighed amount of tissue was minced finely on a cold watch glass and homogenized in $CHCl_3$:CH_3OH (1:1) for 5 minutes. (In the case of SI, the whole organ with contents was thoroughly minced and mixed before a representative 4 g sample was taken.) A homogenizer[1] with a 50 ml stainless steel cup was used. The homogenate was centrifuged[2] at -4° for 12 minutes at 12,000 rpm. The supernatant was decanted into a 50 ml round bottom glass tube containing 10 ml (12 ml for SI) of cold 0.2 M $MgCl_2$. The precipitate was reextracted with 12 ml (20 ml for SI) $CHCl_3$:CH_3OH (2:1) with 3 minutes homogenization and 30 minutes centrifugation. The second supernatant was combined with the first, and the mixture was shaken vigorously for at least 1 minute. The mixture was stored overnight at 4° for complete phase separation.

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[1]Lourdes multi-mix homogenizer, Brooklyn, New York.

[2]Refrigerated centrifuge, model HR1, International Equipment Co., Boston, Massachusetts.

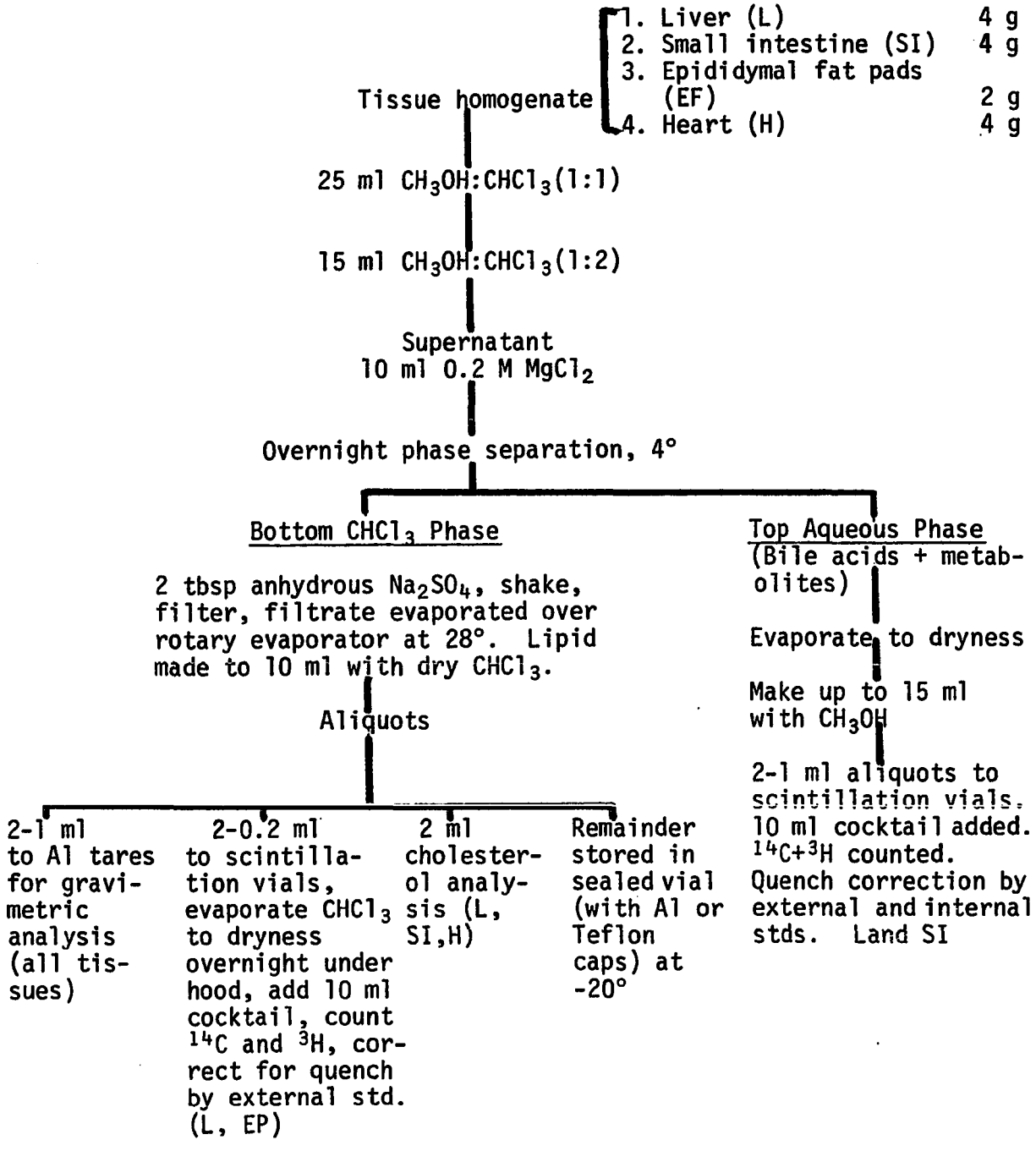

Figure 3. Schematic diagram of lipid extraction procedure using the method of Folch et al. (1957) as modified by Stadler (1969).

^{14}C-conjugated acid steroid The upper aqueous phase contained most of the bile acids, while the lower chloroform layer carried most of the lipids. For SI, a second extraction of the lower phase with 5 ml $MgCl_2$ was necessary to remove most of the bile acids. Occasionally, when cloudiness appeared in the chloroform layer, a few drops of methanol were added to clear the solution. For H and EP, the aqueous phase was discarded. For L and SI, the aqueous phase was transferred to scintillation vials, evaporated to dryness at 70° in a steam bath and made to 15 ml with methanol. Two 1 ml aliquots were used for ^{14}C and ^{3}H counting.

Neutral lipids and cholesterol The chloroform layer was shaken vigorously for 1 minute with 2 tablespoons of anhydrous Na_2SO_4. The slurry was filtered, and the filtrate evaporated over a rotary evaporator immersed in a water bath at 28°. The residue was a yellowish, oily layer. This was made to 10 ml with dry chloroform. From this the following aliquots were removed for various analyses:

(1) two 1 ml aliquots for gravimetric quantitation of total lipid

(2) two 0.2 ml fractions for total ^{14}C and ^{3}H counts

(3) one 2 ml for cholesterol analysis

The remaining extract was stored at -20° in a tightly sealed vial with Teflon- or aluminum-lined caps.

Cholesterol analysis Cholesterol was determined according to a modified procedure of Sperry and Webb (1950). Free and total cholesterol were determined as digitonin precipitable sterols (DPS). A scheme for cholesterol analysis is shown in Figure 4. A 2 ml lipid extract was pipetted into a 25 volumetric flask, evaporated to dryness under N_2 and made to volume with acetone (redistilled)-ethanol (95%) (1:1). Eight 3 ml

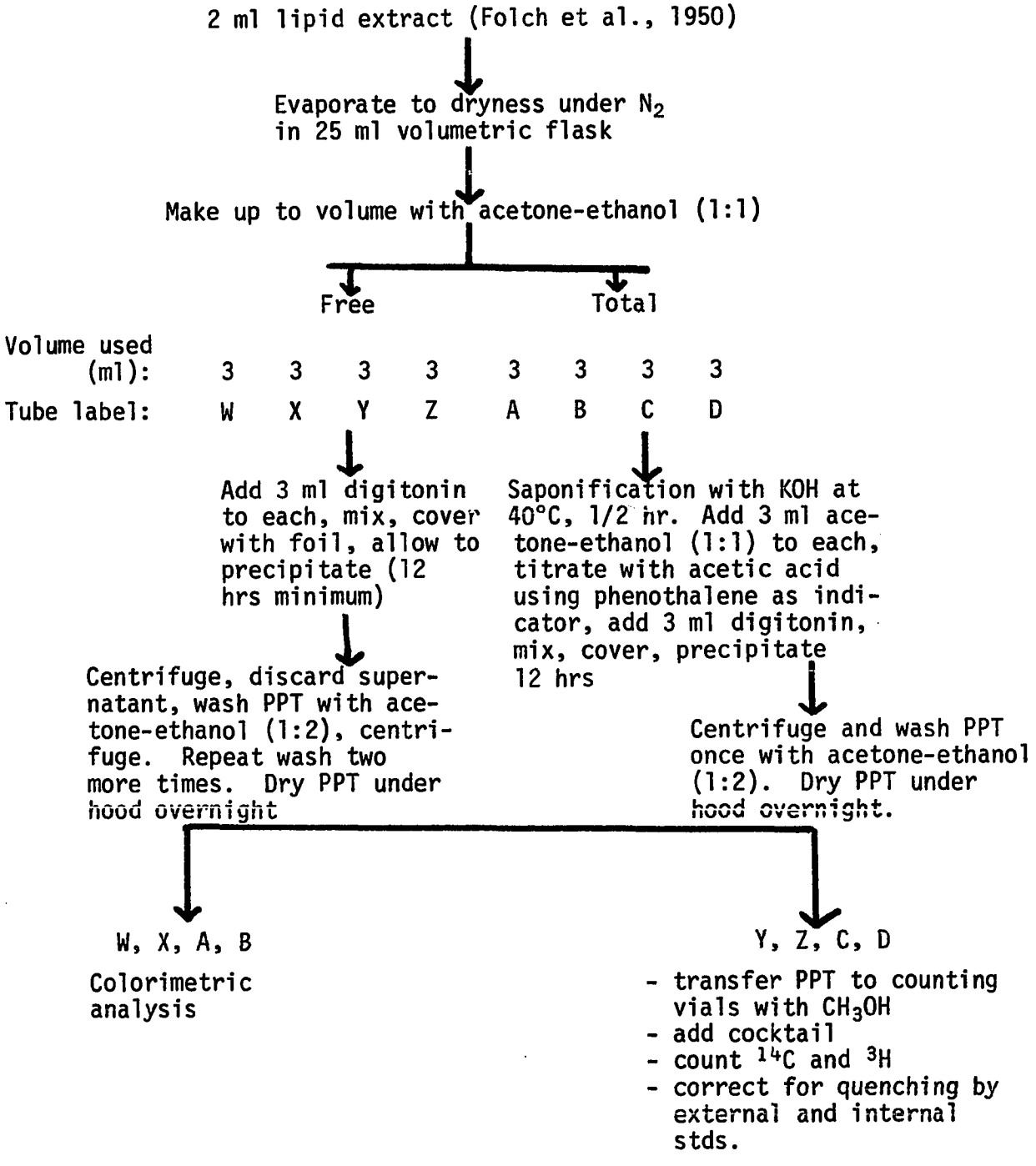

Figure 4. Cholesterol analysis by the modified method of Sperry and Webb (1950) for serum, liver, small intestines and heart

aliquots were transferred to 15 ml centrifuge test tubes. Tubes were marked w-z for free and A-D for total cholesterol. DPS from Y, Z, C, D were transferred with methanol to scintillation vials and counted for ^{14}C and ^{3}H. The remaining samples were used for colorimetric analysis of cholesterol on a spectrophotometer[1] with a digital readout hookup.[2]

For serum or plasma aliquots (0.6-1 ml), lipid was extracted by the method of Sperry and Brand (1955). The aliquots were extracted with 10 ml acetone-ethanol (1:1), shaken vigorously and warmed in boiling water. The precipitate was filtered and the cooled filtrate made to 25 ml with acetone-ethanol (1:1). The rest of the procedure was as described above.

Feces and large intestinal contents

Feces and large intestinal contents were washed into a 50 ml stainless steel homogenizing cup. Distilled water was added to fill 3/4 of the cup. The mixture was homogenized for 5 minutes at high speed, or until a homogenous mixture was attained. The homogenate was made to 200 ml with distilled water. Two 5 ml aliquots were pipetted into aluminum tares and frozen at -20°. They were lyophilized[3] at -15° for 4-6 hrs. Dry material from each tare was weighed and transferred to a 50 ml round bottom tube. Twenty ml of 1 N NaOH in 90% ethanol was added. Samples were shaken vigorously for 1 minute. The tubes were stoppered and heated at 45° for 1 hr in a water bath. They were left at room temperature overnight.

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[1]Beckman D.U. spectrophotometer, model 2400, Beckman Instrument Inc., Fullerton, California.

[2]Update Inc., Madison, Wisconsin.

[3]Virtes lyophilizer, Gardiner, New York.

Neutral ^{14}C-steroid

Contents were transferred to a 250 ml round bottom flask with 10 ml distilled water. To each sample 50 ml petroleum ether (a mixture of Skelly A and B (1:1), bp 30-60°) was added. The bottle was closed with a #6 rubber stopper wrapped in aluminum foil. It was shaken vigorously for 1 minute and held in the cold for 20 minutes for phase separation. The upper phase was transferred to a 200 ml beaker. The lower phase was re-extracted twice with 50 ml petroleum ether. The three petroleum ether fractions were combined and washed with 10 ml 1 N NaOH in 50% ethanol. The sample was evaporated to near dryness in a water bath (70°), transferred to a scintillation vial and evaporated to dryness. Ten ml cocktail was added. Each sample was counted for 20 minutes at settings for ^{14}C counting only. Quenching was corrected by both internal and external standardizations. Counts for ^{3}H were not determined.

Acid ^{14}C-steroid

The aqueous phase was acidified to pH 2 with concentrated HCl. Litmus paper was used to determine pH range. Following the procedure of Grundy et al. (1965) as modified by Carlson (1975) the acidified layer was extracted with 75 ml $CHCl_3$-CH_3OH (2:1) without saponification. Phase separation was obtained by allowing the sample to stand at 4° for 30 minutes. The lower phase was transferred to a 150 ml flask. The aqueous phase was reextracted two more times with 50 ml dry chloroform. The three extracts were combined and evaporated to near dryness, transferred to a 15 ml volumetric flask, and evaporated to dryness. It was made to volume with methanol. Two 1 ml fractions were removed for ^{14}C-counting.

Plasma triglycerides

Plasma triglyceride level was measured by the semi-automated procedure of Kessler and Lederer (1965). A Technicon auto analyzer[1] N-70 was used. Five tenths ml plasma was extracted with 9.5 ml isopropanol in the presence of a slurry containing zeolite, copper lime, and Lloyd reagent. The mixture was centrifuged, and the supernatant containing the lipid extract was sampled into an air-segmented alcoholic KOH solution. Saponification of triglycerides to glycerol occurred on stream in a 50° heating bath. Periodate reagent containing glacial acetic acid, sodium periodate and distilled water was added to oxidize the glycerol to formaldehyde. This was followed by condensation with diacetylacetone and ammonia to yield a fluorescent product, 3,5-diacetyl-1, 4-dihydrolutidine. Both oxidation and condensation steps were carried out in the bath. After heating, the reaction mixture entered the fluorometer where air was automatically removed and the fluorescence activated. The fluorometer was connected to a recorder. Two sets of standards containing 50, 100, 200 and 300 mg triolein/dl isopropanol were monitored before and after sample runs. Blanks using KOH in isopropanol were run concurrently. Three aliquots per plasma sample were run. The second value was taken as the actual triglyceride value. Values one and three were discarded as they contained carry over contaminants from previous samples. A standard curve relating peak heights to mg triglyceride/dl blood was constructed from the standards.

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

Samples were run at a rate of 40/hr.

Plasma thyroxine and liothyronine

Plasma thyroxine (T-4) and liothyronine (T-3) were measured using Thyopac-4[1] and Thyopac-3[2] kits. Both procedures provided assessment of thyroid function by the quantitative measurement of the relative saturation of the thyroxine binding proteins. The main binding protein is thyroxine binding globulin (TBG) which binds both T-3 and T-4. Thyroxine binding prealbumin (TBPA) binds mainly T-4, while albumin binds both T-4 and T-3.

T-4 Thyopac-4 assay

Principle A tracer quantity of L-thyroxine I-125 (^{125}T-4) is added to TBG. T-4 in the sample when added to a TBG-^{125}T-4 reagent will compete with ^{125}T-4 for the binding sites on TBG. As more T-4 is added, competition for available TBG sites increases with the result that a proportionally smaller amount of ^{125}T-4 is bound to TBG. The amount of ^{125}T-4 bound to TBG or the amount of ^{125}T-4 can be related to the amount of T-4 added to the TBG-^{125}T-4 amount in standard samples. ^{125}T-TBG is separated from ^{125}free T-4 by absorbent granules.

Procedure One ml 95% or absolute ethanol was added to 0.5 ml unknown plasma in an extraction tube. The tube was stoppered and shaken briskly, mixed for 2 minutes by rotation and centrifuged for 5 minutes at

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[1]Thyopac™-4 kit, codes IM.64 and IM.641, Amersham/Searle, Arlington Heights, Illinois.

[2]Thyopac™-3 kit, codes IM.62 and IM.621, Amersham/Searle, Arlington Heights, Illinois.

2000 g. One-half ml of supernatant was transferred to a Thyopac-4 vial containing 50 μC L-thyroxine I-125, absorbent granules suspended in buffer and human TBG. The mixture was rotated at ambient temperature for 30 minutes. Granules were allowed to settle for 2 minutes. Two 1 ml aliquots were transferred to plastic 25 x 17 mm tubes and counted in a solid scintillation counter[1] for I-125. The quantitation of each sample was achieved by including in each run, two standard reference sera with predetermined Thyopac-4 values. A linear relationship existed between the T-4 concentration of the sample and the inverse of the count rate of the sample. Hence, only two standards (one high and one low) were required to establish a linear plot.

T-3 Thyopac assay

Principle A modified Hamolsky (1957) procedure was used. Hamolsky added a constant amount of ^{125}T-3 to whole blood samples to saturate the protein binding sites. The red blood cells served as secondary binding receptors. The mixture was incubated and the red blood cells were washed and counted for radioactivity. Thus using a known quantity of ^{125}T-3, and an adsorbent granule as secondary binding sites, the ability of the serum to bind the labeled T-3 was an indication of the degree of saturation of the protein binding sites by T-4. Consequently an assessment of thyroid status could be made.

Procedure A 0.1 ml aliquot of serum was added to a Thyopac-3 vial. Each vial contained 0.5 μC liothyronine I-125, buffer and adsorbent granules. The content of the vial was mixed in a rotator for 10 minutes

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[1]New England Nuclear solid scintillation counter, Boston, Mass.

and allowed to settle for 2 minutes at ambient temperature. Two 1 ml aliquots were transferred to plastic tubes and counted in a well type solid scintillation counter.

Calculations and Statistical Analysis

Since all rats were injected with an equal dosage of ^{14}C-cholesterol and ^{3}H-acetate (2.5 μC and 50 μC, respectively), adjustment for body weight differences was made. Radioactive data were calculated on the basis of a dosage of 0.5 μC/100 g body weight for ^{14}C-cholesterol and 10 μC/100 g body weight for ^{3}H-acetate, respectively.

Group means, standard errors of means for groups, and simple manipulative conversions of the data were performed on a Monroe 1350 calculator. Unweighted analyses of variance were used for treatments and residual, and for fat, meal pattern, length of experimental time tested against residual. The statistical analysis system (SAS) developed by Barr and Goodnight (1971) was used. Data were run on an IBM 360. Differences between groups were calculated using the student T test, LSD or one way ANOVA on either a programmed Cannola 167P or Wang 600 calculator. Effects of $p<0.05$ were taken as significant.

Results

The data were categorized into the following groups: 1) growth rate, 2) tissue lipid levels, 3) cholesterol metabolism, 4) serum hormone levels and 5) subjective observations. The reader is referred to the Procedure Section for parameters measured, and to Figure 2 for the experimental design.

Assessment of growth rate was based on in vivo measurements of body weight, caloric intake, food efficiency, and from organ weights.

Tissue lipid concentrations pertained to hepatic lipid content and plasma triglycerides (90-day treatment period only).

Cholesterol metabolism was studied by measuring distribution, synthesis, degradation (bile acids formation) and excretion of neutral steroids and cholesterol metabolites. An IP injection of 2.5 μC of 4-^{14}C-cholesterol was given six days prior to sacrifice. Tissue cholesterol was assessed as digitonin precipitable steroids (DPS). In most rat tissues DPS measures primarily cholesterol, while precursors, e.g., lanosterol, constitute a small fraction. In the intestinal mucosa, however, the DPS fraction contains certain plant sterols (campesterol, β-sitosterol as well as cholesterol precurors, lathosterol and 7-dehydrocholesterol (McIntyre et al., 1971).

The following interpretations have been given to the data:

1. Recovery of ^{14}C-DPS in conjunction with total DPS fraction present in plasma, liver and small intestine (plus contents) to indicate tissue cholesterol retention.

2. Recovery of ^{3}H-DPS in liver and small intestine with contents do indicate relative synthesis of cholesterol from ^{3}H-acetate in the major synthetic sites over a 2 1/2 hour period. In the rat, liver and small intestine contribute about 80% and 10%, respectively, to endogenous cholesterogenesis (Dietschy and Siperstein, 1967).
3. Recovery of ^{14}C-acid steroids to indicate bile acids formation and excretion in liver, small intestine (plus contents) and feces plus large intestinal contents. Thus, relative rates of cholesterol degradation could be estimated. In addition, recovery of ^{14}C-neutral steroids in feces plus large intestinal contents allowed estimation of relative rates of neutral steroid excretion (mostly cholesterol). Data on cholesterol metabolism pertained only to acute changes occurring in the rapidly equilibrating pools of plasma, liver and intestines.

Serum hormone measured was thyroxine, which was monitored for the 90-day treatment period only.

Subjective observations included physical conditions of the animals throughout the experiment (e.g., infection and general constitution), as well as gross morphology of organs at the time of death.

In the following sections, the terms aging and maturation were used interchangeably. Likewise, the terms "30-, 60-, 90-day feeding periods" were not distinguished from the terms "2-, 3- and 4-month old animals." With the design used, effects of extended feeding cannot be distinguished from effects of maturation. Similarly, the effects of maturation cannot

be isolated from effects of aging in this study. Though these animals were maturing, based on their chronological ages, they were, however, not fed a nonpurified (stock) diet. The possibility exists that detrimental changes, induced by semi-purified though nutritionally adequate diets, may have occurred earlier and with greater frequencies here than would have occurred with nonpurified control diets.

Growth Rate

Mean body weights, food intakes and food efficiencies are shown in Table 6. Mean organ weights are shown in Table 7.

Body weight

Initial body weights, preexperimental and weights following overnight fast were statistically similar by design. Analysis by least square difference for all groups showed that body weight means varying by more than 41- and 54-g were significantly different at $p<.05$ and $p<.01$, respectively.

Body weight differences were significant ($p<.001$) for fat level, fat saturation, age and meal pattern. Body weight increase resulting from raising dietary fat level was greater with BT than SO as source of fat. Body weight increase was also greater with AL- than MF- feeding ($p<.05$). Between 2 to 3 months of age, LF- fed animals on AL feeding schedule weighed as much as SO- or BT- fed animals on similar feeding frequencies. This similarity disappeared at 4 months of age, when body weight increase slowed down in both the LF- and SO- fed animals, but not in BT fed animals ($p<.01$). Conversely, meal fed animals on diet LF showed consistently

lower body weights than meal fed animals on the high fat diets. In general, decreased caloric intake with meal feeding decreased body weights ($p<.001$); however the magnitude of decrease depended on the type of diet. Based on diet-meal pattern interaction analysis, the difference in body weights between the two meal patterns with diet LF was twice as large as the difference between patterns with diets SO or BT, throughout the experiment. Finally, lower body weights with SO compared to BT feeding were apparent regardless of age and meal pattern ($p<.001$).

Food intake and food efficiency

Food intake was calculated as the amount of food in kcal consumed per day. There were significant differences for dietary fat level ($p<.05$), when BT was used as fat source. Energy value of food consumed by animals on diet LF was less than that on diet BT, due in part to reduced food intake of the meal fed animals. Animals on diet BT tended to consume more food than animals on diet SO. Food intakes of animals between ages 2-4 months were not decreased. Range values for both age groups were small, between 53-74 kcal. Group mean values for all treatment groups were between 65-74 kcal.

Food efficiency was calculated as body weight gain per 100 kcal consumed. Food efficiencies were not influenced by fat level, fat saturation and meal pattern. However, food efficiencies were significantly decreased between ages 2-4 months ($p<.001$). Decrease in food efficiencies was observed regardless of diets (Table 6).

Table 6. Body weights, food intakes and food efficiencies (FE)

	2 months 30 days			3 months 60 days			4 months 90 days		
	Body weight g	Food intake kcal	FE g/kcal	Body weight g	Food intake kcal	FE g/kcal	Body weight g	Food intake kcal	FE g/kcal
LF-AL	312±19[a]	78±2	8±1	428±9	76±	4±.3	505±13	76±2	4±.3
LF-MF	231±22	53±2	10±1	307±10	60±3	6±.4	393±19	61±3	4±.2
SO-AL	302±7	79±2	9±1	430±13	66±2	5±.3	487±26	72±3	3±.2
SO-MF	257±8	60±2	10±1	389±6	63±2	6±.4	441±23	69±3	3±.2
BT-AL	312±12	76±3	9±1	448±9	69±4	6±.3	583±14	74±3	3±.2
BT-MF	287±9	68±3	8±1	412±11	70±3	5±.4	496±22	73±3	4±.4

[a]Mean ± SEM.

Liver Liver weight increased with elevated dietary fat level ($p<.001$), with increase in age ($p<.001$) and with AL versus MF feeding ($p<.001$). Increase in liver weight with dietary fat level was equally effective with either source of fat. Group mean values for SO and BT groups were almost identical, 12.0 vs. 13.3 g, respectively. With LF and SO feeding, near maximal increases in liver weights were achieved by 3 months of age (60 days feeding). Conversely, BT-fed animals showed an almost linear increase in mean liver weights between the ages of 2-4 months. Differences in liver weight between the feeding patterns were apparent with diet LF compared to diet SO. During the first 3 months of age, lower weights of BT-fed rats did not respond to meal pattern variations.

Kidneys Kidney weights were elevated when dietary fat level was increased ($p<.001$), when fat saturation was increased ($p<.01$) and when the animals aged ($p<.001$). Conversely, kidney weights were decreased with meal feeding ($p<.001$). Variable mean value for groups LF, SO and BT were 2.6, 3.0 and 3.2 g, respectively. In addition, kidney weights at ages 2, 3 and 4 months were 2.3, 3.0, 3.3. Variable mean values for the meal patterns were 3.1 and 2.6 g for AL- and MF-feeding, respectively.

Small and large intestine (plus contents) Weights for small and large intestines were approximations of tissue weights only, since intestinal contents were included. Weights varied with amount of food consumed prior to autopsy. Food intake was not controlled in these animals; therefore, it could not be assumed that intestinal contents would be similar for all dietary treatments. Consequently, weights of these tis-

Table 7. Weights of liver (L), heart (H), small intestine with contents (SI), large intestine with contents (C), kidney (K), spleen (SP), epididymal fat pads (EF) and stomach without contents (ST)

	2 months 30 days				3 months 60 days				4 months 90 days			
	L	H	SI	C	L	H	SI	C	L	H	SI	C
LF-AL	11±.6[a]	1.2±.1	8±.3	5±.1	14±.3	1.4±.1	9±.4	5±.2	14±.5	1.6±.1	10±.4	6±.4
LF-MF	8±.3	.8±.1	7±.3	3±.2	10±.6	1.2±.1	8±.3	4±.3	11±.7	1.2±.1	9±.5	5±.3
SO-AL	11±.4	1.2±.1	8±.3	5±.1	15±.6	1.4±.1	10±.3	6±.2	15±.7	1.5±.1	9±.3	6±.3
SO-MF	10±.4	1.0±.1	8±.4	4±.1	13±.4	1.3±.1	10±.2	5±.2	13±.9	1.4±.1	9±.4	5±.2
BT-AL	11±.5	1.2±.1	10±.4	5±.3	13±.5	1.5±.1	10±.3	6±.3	17±.6	1.5±.1	11±.4	6±.3
BT-MF	11±.4	1.1±.1	10±.4	5±.2	13±.5	1.4±.1	10±.3	6±.3	15±.1	1.4±.1	10±.3	6±.2
	K	SP	EF	ST	K	SP	EF	ST	K	SP	EF	ST
LF-AL	2.6±.1	.74±.07	2.7±.2	1.4±.1	3.3±.1	.81±.14	4.7±.2	1.8±.1	3.6±.1	1.0±.07	6.6±.5	1.9±.1
LF-MF	1.7±.1	.60±.04	1.3±.1	1.4±.1	2.3±.1	.80±.10	2.8±.3	1.7±.1	2.6±.1	.86±.05	4.2±.5	2.1±.1
SO-AL	2.5±.1	.79±.04	2.6±.3	1.4±.1	3.3±.3	.0±.07	5.6±.3	1.9±.1	3.4±.1	.82±.07	8.9±.6	1.9±.1
SO-MF	2.1±.1	.67±.04	2.5±.2	1.6±.1	2.9±.1	.81±.04	4.8±.5	1.9±.1	3.0±.1	.73±.03	5.7±.9	2.0±.1
BT-AL	2.6±.1	.89±.03	3.7±.3	1.6±.1	3.3±.1	.95±.03	6.7±.3	1.8±.1	3.6±.1	.98±.04	13.4±.6	2.3±.1
BT-MF	2.4±.1	.85±.08	3.0±.2	1.8±.1	3.1±.1	.93±.03	5.3±.5	1.9±.1	3.5±.1	1.1±.04	9.7±.1	2.3±.1

[a]Mean ± SEM.

Table 7. (Continued)

ANOVA	L	H	SI	LI	K	SP	EF	ST
Diet	p<.001	p<.001	p<.001	p<.001	p<.001	NS	p<.001	p<.001
Age	p<.001	NS	p<.001	p<.001	p<.001	NS	p<.001	p<.001
Meal pattern	p<.001	p<.001	p<.01	p<.001	p<.001	NS	p<.001	NS
Diet x age	NS	NS	NS	NS	NS	NS	p<.01	NS
Diet x meal pattern	p<.01	NS	NS	NS	p<.001	NS	NS	NS
Pattern x day	NS	NS	NS	p<.02	NS	NS	p<.02	NS
Diet x age x pattern	NS	NS	NS	NS	NS	NS	NS	NS
Fat saturation	NS	NS	p<.01	p<.001	p<.01	NS	NS	p<.01
Fat level	p<.001	p<.001	p<.001	p<.001	p<.001	NS	p<.001	p<.001

sues will not be discussed. However, data for intestinal weights plus contents were analyzed statistically (Table 7).

Spleen The spleen did not respond to dietary manipulations or to aging. It appeared that this organ had reached maximal weight in 2 months old animals, that is, after the first 30-day period of the experiment (Table 7). Among dietary regimens, range values were between 0.60-1.1 g, with group mean values between 0.68-1.0 g.

Stomach (minus contents) Stomach weights were increased with fat feeding, regardless of fat source and with increasing age ($p<.001$, Table 7). Safflower oil feeding decreased stomach weights when compared to beef tallow feeding ($p<.01$). Meal pattern as a variable did not influence stomach weights.

Epididymal fat pads Weight of epididymal fat pads (EF), representative of total adipose tissue mass, was increased when dietary fat level was elevated ($p<.001$, Table 7). Increase of EF weights with fat feeding was independent of fat source. Beef tallow, however, invoked a greater increase in EF weights than safflower oil. Between the ages of 2-4 months, EF weights increased ($p<.001$). Meal feeding decreased EF weights ($p<.001$) with the greatest difference between patterns in the LF group.

Heart Cardiac weights were elevated with an increase in dietary fat level ($p<.001$, Table 7), regardless of fat source. On the contrary, variation in dietary fat saturation had no effect on cardiac weights. Cardiac weights tended to increase with age. Meal feeding, however, decreased cardiac weights ($p<.001$) due in part to a proportional decrease in body weights.

Tissue Lipids

Hepatic total lipid content

Hepatic total lipid content was calculated based on 100 g of wet tissue. In general, hepatic lipid content increased when dietary fat level was elevated. This increase was apparent only when safflower oil ($p<.01$) was used as fat source. Hepatic lipid contents between groups LF and BT were not statistically different at all ages. Livers from SO-fed animals accumulated more lipid than livers from BT-fed animals ($p<.05$). Increased hepatic lipid content, in all groups, was most pronounced at age 3 months (60 days feeding period), decreasing slightly at age 4 months. There was an overall age related increase in total lipid content of the liver when all dietary groups were considered ($p<.02$). Livers of meal-fed rats contained less lipid than did those of ad libitum fed rats ($p<.02$). This decrease was observed for all periods of this experiment (Table 8).

Plasma triglycerides

Plasma triglycerides in mg/dl of blood was measured only in the 4-month old groups (after 90-days of treatment). The combination of small group mean differences (range 236-265 mg/dl) and large standard deviations within groups (range ±15-25) failed to give significant differences with fat level, fat saturation, and meal pattern. Group mean values were 248, 236, and 220 mg/dl for LF-, SO- and BT-fed animals, and 234 and 231 mg/dl for ad libitum and meal fed animals, respectively (Table 9).

Table 8. Hepatic total lipid content in g/100 g wet tissue

	g/100 g wet tissue		
	2 months 30 days	3 months 60 days	4 months 90 days
LF-AL	3.5±.3[a]	4.3±.2	3.2±.4
MF	3.6±.5	4.0±.2	2.9±.3
SO-AL	5.5±.4	6.9±.6	6.4±.5
MF	4.7±.3	5.8±.5	4.8±.5
BT-AL	3.7±.3	5.0±.4	4.3±.5
MF	3.1±.4	4.3±.3	4.4±.4
Fat level	p<.01		
Fat saturation	p<.05		
Age	p<.02		
Meal pattern	p<.02		
Diet x age	NS		
Diet x meal pattern	NS		
Age x meal pattern	NS		
Diet x age x meal pattern	NS		

[a]Mean ± SEM.

Table 9. Plasma triglyceride level (mg/dl), after a 1 hr meal

			4 months 90 days
LF-AL			249±48[a]
MF			265±43
SO-AL			238±38
MF			237±50
BT-AL			242±55
MF			236±51
LF	257		
SO	238		
BT	244		
AL	243		
MF	246		
Fat level		NS	
Fat saturation		NS	
Meal pattern		NS	
Interactions were all		NS	

[a]Mean ± SEM.

Cholesterol Metabolism

Plasma cholesterol concentrations

Plasma cholesterol concentrations in mg/dl of blood were uniformly low for all dietary treatment groups (Table 10, Figure 5). The range of mean values was narrow, between 35 and 52 mg/dl. Mean values of most groups were between 40 and 50 mg/dl. Consequently, plasma cholesterol concentrations were not significantly affected by any of the variables used. In general, with each dietary treatment, plasma cholesterol concentrations tended to increase with age. The increase was small, however, about 23% on the average.

With most dietary treatments, approximately half of plasma cholesterol was in the free form. Group mean percentages of free cholesterol with the three dietary regimens were 43, 45 and 46% for diet LF, SO and BT, respectively. In general, significance was not obtained for any of the variables used.

Plasma ^{14}C-cholesterol

Plasma ^{14}C-cholesterol was assessed as ^{14}C counts in digitonin precipitable steroids (^{14}C-DPS). In general, plasma ^{14}C-DPS was not influenced by dietary fat level, fat saturation, age and meal pattern due in part to high variations within groups. Large values for all dietary regimens were between 378-666 dpm x 10^3, with group mean values 412-640 dpm x 10^3.

Plasma ^{3}H-cholesterol

Plasma ^{3}H-cholesterol was assessed as ^{3}H-digitonin precipitable steroids (^{3}H-DPS). Plasma ^{3}H-DPS counts were too low to be regarded with

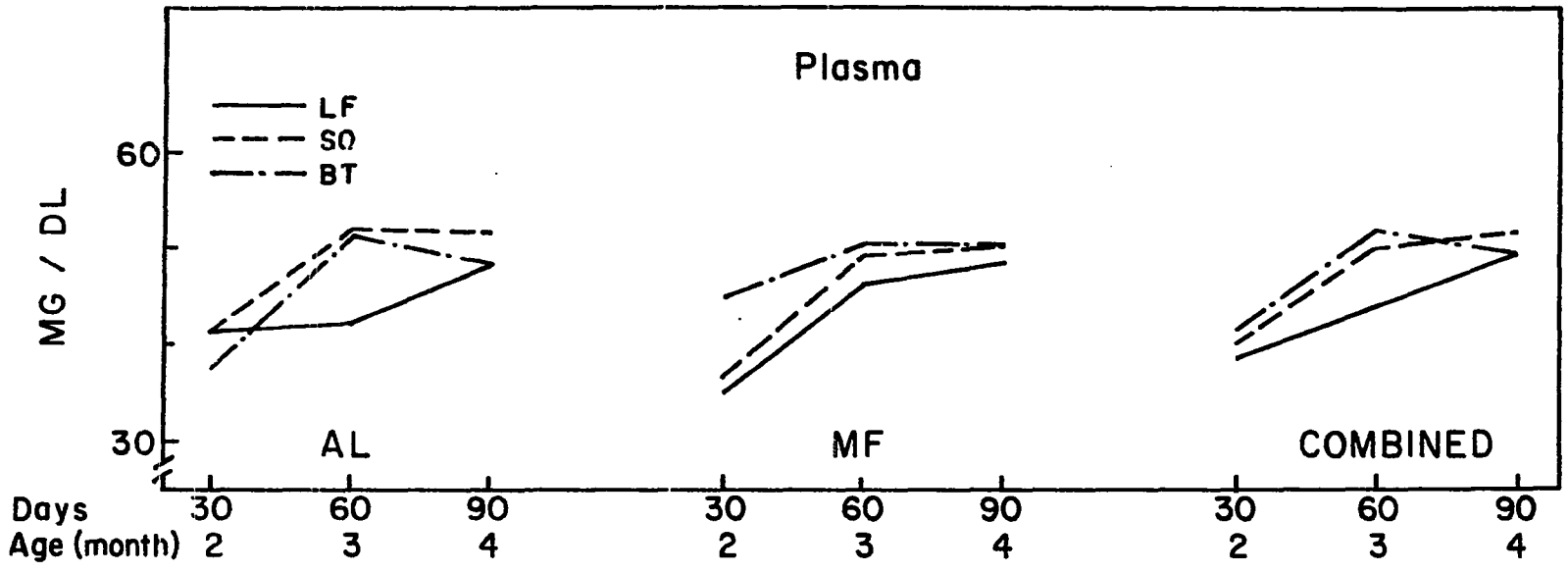


Figure 5. Plasma cholesterol concentration (mg/dl blood). Cholesterol was measured as digitonin precipitable steroids (DPS).

confidence. Counts in some groups were 2 to 3 cpm over background, after correction for ^{14}C-quenching. Consequently, these data will not be discussed. Since plasma ^{3}H-DPS is an estimate of newly synthesized cholesterol transported out of the liver (Dupont et al., 1972), the low counts obtained with plasma ^{3}H-DPS indicate that the 2 1/2 hours time period used for studying cholesterol synthesis from ^{3}H-acetate was too short for maximal hepatic cholesterogenesis and cholesterol transport into the plasma.

Hepatic cholesterol concentrations

Hepatic cholesterol concentrations were expressed as mg cholesterol per g of wet tissue (mg/g), and as total mg cholesterol in the whole tissue (total mg). In general, SO fed animals had more cholesterol per g liver and in the whole liver than did either LF- or BT-fed rats ($p<.05$, Table 10). Increased hepatic cholesterol with polyunsaturated fat feeding was observed as early as age 2 months (30 days feeding), and persisted until the termination of the study at age 4 months. Hepatic cholesterol concentrations of LF- and BT-fed animals were relatively similar, whether expressed on a mg per g or total mg basis. Hepatic cholesterol levels tended to increase between ages 2-4 months ($p<.05$). These changes were correlated in part with liver weight. Meal feeding did not appear to influence hepatic cholesterol concentration (mg/g). However, when values for total liver cholesterol content were compared, meal feeding caused consistently lower hepatic cholesterol levels than did the ad libitum regimen ($p<.05$). For example, overall means were 36 and 46 mg for MF and AL feeding, respectively.

Table 10. Plasma, hepatic and small intestine plus contents (SI) cholesterol concentrations in mg cholesterol per g of wet tissue and total mg cholesterol in whole tissue; cholesterol was assessed as digitonin precipitable steroids (DPS)

	2 months 30 days					3 months 60 days		
	Serum	Liver		SI		Serum	Liver	
	Total mg/dl	mg/g	Total mg	mg/g	Total mg	Total mg/dl	mg/g	Total mg
LF-AL	41±2[a]	2.2±.1	39±2	2.3±.2	21±2	43±3	1.8±.1	26±2
LF-MF	35±4	2.0±.1	28±2	2.6±.2	19±2	46±3	1.8±.1	18±2
SO-AL	41±2	3.1±.1	66±2	3.3±.2	28±3	52±3	3.6±.3	55±5
SO-MF	36±2	3.4±.3	44±2	3.1±.2	25±3	48±3	2.8±.3	36±2
BT-AL	38±2	1.9±.1	40±3	2.2±.1	23±2	52±2	2.3±.3	30±3
BT-MF	45±4	2.4±.1	43±2	2.0±.1	20±1	50±2	1.9±.2	25±3

ANOVA

	Serum	Liver		SI	
		mg/g	Total mg	mg/g	Total mg
Fat level	NS	$p<.05$	$p<.05$	NS	NS
Fat saturation	NS	$p<.05$	$p<.01$	$p<.05$	$p<.05$
Age	NS	$p<.05$	$p<.05$	$p<.05$	NS
Meal pattern	NS	NS	$p<.05$	NS	NS
Diet x age	NS	NS	NS	NS	NS
Diet x meal pattern	NS	NS	NS	NS	NS
Age x meal pattern	NS	NS	NS	NS	NS
Diet x age x meal pattern	NS	NS	NS	NS	NS

[a]Mean ± SEM.

3 months 60 days		4 months 90 days				
SI		Serum	Liver		SI	
mg/g	Total mg	Total mg/dl	mg/g	Total mg	mg/g	Total mg
3.3±.2	30±4	48±3	2.9±.2	40±2	2.8±.3	28±2
3.1±.2	25±1	49±3	2.0±.1	22±1	2.9±.2	26±2
3.6±.2	37±4	51±3	4.0±.4	60±5	4.4±.3	39±3
3.8±.2	33±4	50±3	4.4±.4	57±6	4.2±.3	38±3
3.3±.2	33±3	49±4	3.0±.3	51±3	2.9±.3	32±2
2.8±.2	28±3	48±3	3.2±.4	48±3	2.8±.2	28±2

Small intestine cholesterol concentrations

The concentrations of cholesterol in small intestine plus contents were expressed as mg cholesterol per g of wet tissue and total mg cholesterol in the whole tissue. Intestinal contents contained dietary cholesterol, though dietary contribution was low (about 0.01 mg/g fat). Since food intake was not controlled prior to termination, intestinal contents, and thus amounts of dietary cholesterol, may have varied with the amount of food ingested. Consequently, small intestine cholesterol concentrations determined in this study were only estimates of actual cholesterol content in the small intestinal mucosa (Table 10).

In general, increasing dietary fat level increased small intestine cholesterol level only when safflower oil and not beef tallow was used as a source of fat ($p<.05$). Small intestine cholesterol was not influenced by age when calculated as mg cholesterol per whole organ. However, based on unit tissue weight, small intestine cholesterol levels increased with age ($p<.05$), and maximal increases occurred between ages 3-4 months. Meal pattern, as a variable, did not appear to affect cholesterol levels of small intestine based on either concentration or total content.

Cholesterol synthesis

Hepatic ^{3}H-cholesterol Hepatic ^{3}H-cholesterol was assessed as ^{3}H counts in digitonin precipitable steroids (^{3}H-DPS). Hepatic cholesterogenesis was elevated when dietary fat level was increased ($p<.001$). Diet SO was as effective as diet BT in enhancing hepatic cholesterogenesis when compared to diet LF. Variable mean values for groups LF, SO and BT were 142, 247 and 243 dpm x 10^{3}, respectively. Hepatic cholesterogenesis with

Table 11. Cholesterol synthesis 2 1/2 hours after ^{3}H-acetate injection. ^{3}H-counts in cholesterol from liver and small intestine plus contents. Cholesterol was assessed by digitonin precipitation method. Counts are expressed in dpm x 10^{3} per organ

	2 months 30 days	4 months 60 days	6 months 90 days
		Liver	
LF-AL	79±10[a]	169±23	167±24
MF	90±10	74±19	276±45
SO-AL	157±25	322±30	332±65
MF	84±16	267±30	318±39
BT-AL	132±17	352±20	304±58
MF	67±10	240±39	358±91
		Small intestine + contents	
LF-AL	29±3	45±5	53±3
MF	18±2	25±1	35±3
SO-AL	15±3	21±2	32±5
MF	13±2	24±3	30±4
BT-AL	39±5	62±6	85±5
MF	28±3	43±6	56±6

[a]Mean ± SEM.

Table 12. Cholesterol synthesis 2 1/2 hours after ^{3}H-acetate injection. ^{3}H-counts in cholesterol from liver and small intestine plus contents. Cholesterol was assessed by digitonin precipitation method. Counts are expressed in dpm x 10^{3} per organ. Variable means, main effects and interactions

	Main effects	
	Liver	SI
Diet	p<.001	p<.001
LF	142	34
SO	247	22
BT	243	52
Fat level	p<.001	NS
LF	142	34
HF (SO + BT)	245	37
Fat saturation	NS	p<.001
SO	247	22
BT	243	52
Day Age	p<.001	p<.001
30 2	102	24
60 3	237	37
90 4	394	49
Meal pattern	NS	p<.001
AL	224	42
MF	197	30

Table 12. (Continued)

Interactions

	Liver	SI
Diet x age	NS	NS
Diet x meal pattern	NS	p<.001
Age x meal pattern	p<.01	NS
Diet x age x meal pattern	NS	NS

	2	3	4
LF	84	121	221
SO	121	294	356
BT	100	296	332

	2	3	4
LF	24	35	44
SO	14	23	31
BT	33	53	69

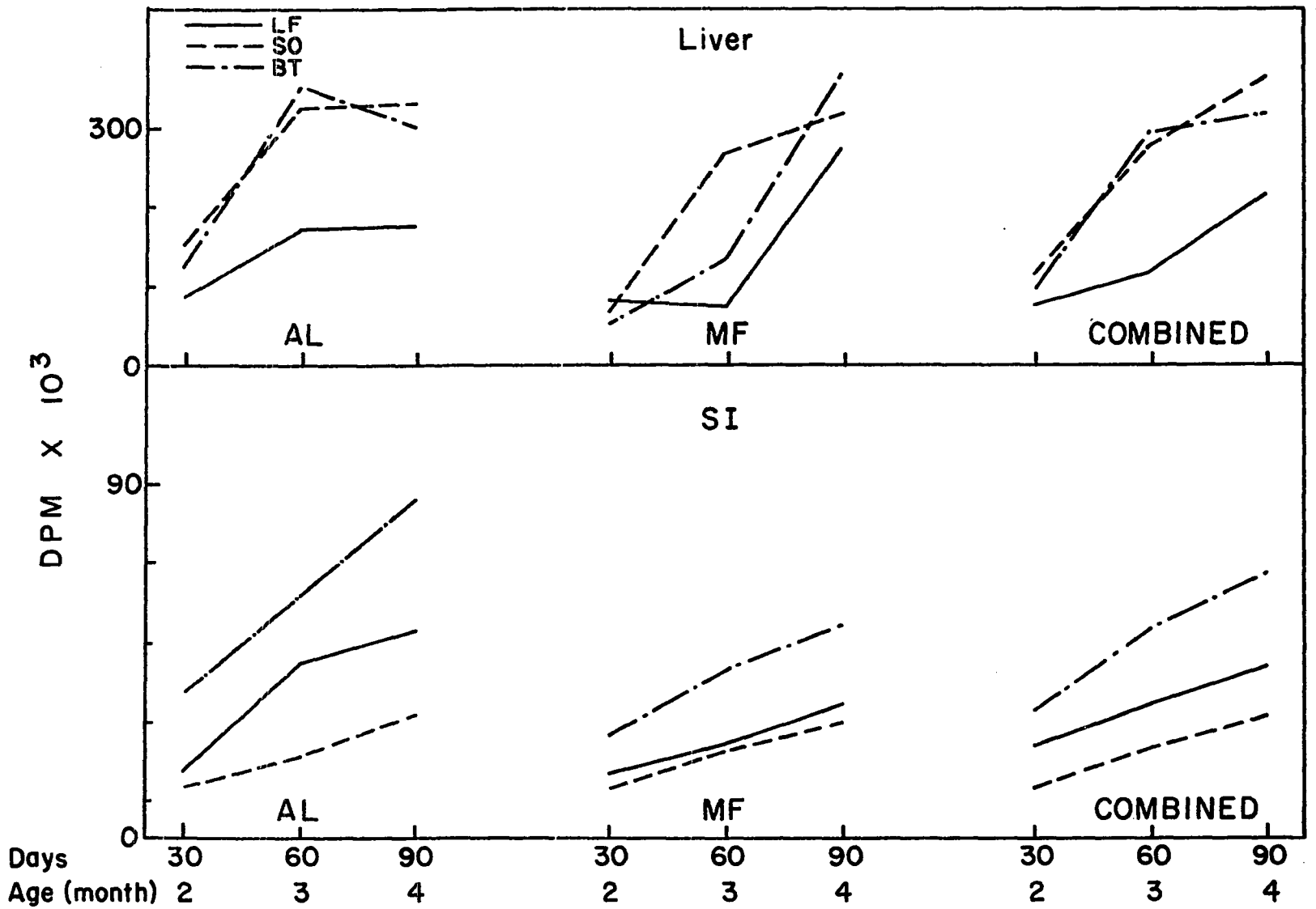
	AL	MF
LF	138	147
SO	271	223
BT	262	222

	AL	MF
LF	43	26
SO	23	22
BT	62	41

	2	3	4
AL	123	281	268
MF	81	194	318

	2	3	4
AL	28	43	57
MF	20	31	39

Figure 6. Cholesterol synthesis from ^{3}H-acetate injected 2 1/2 hours prior to termination. Cholesterol was assessed by digitonin precipitation method. Top figure shows total ^{3}H-counts in digitonin precipitable steroids (DPS) from liver. Bottom figure shows total ^{3}H-counts in DPS from small intestine plus contents. Counts are expressed DPM x 10^{3} per organ.


LF
SO
BT
Liver
300
0
AL
MF
COMBINED
DPM X 10^3
SI
90
0
AL
MF
COMBINED
Days
30
60
90
Age (month)
2
3
4

SO feeding was not different from that with BT (247 vs. 243 dpm x 10^3). Hepatic cholesterogenesis increased with aging ($p<.001$). The increase between ages 2-4 months was almost linear. For examples, the variable mean values for ages 2, 3 and 4 months were 102, 237 and 493 DPM x 10^3. Hepatic cholesterogenesis was not affected by meal feeding. However, at age 3 months, the difference between meal patterns was about 2 times greater than differences between patterns for ages 2 or 4 months (age x pattern interaction, Table 11). The big difference between patterns at age 3 months was due in part to unusually low hepatic cholesterogenesis rates in group LF-MF (Figure 6).

Small intestine ^{3}H-cholesterol Tritium labeled cholesterol in small intestine plus contents was assessed on the basis of ^{3}H counts in digitonin precipitable steroids (^{3}H-DPS). Unlike cholesterol synthesis from ^{3}H-acetate in liver, that in small intestine was higher with LF-feeding than with SO feeding ($p<.01$), but was lower than with BT feeding ($p<.001$). It follows that cholesterogenesis in the small intestine was affected by fat source. Tritium counts in intestinal DPS of BT fed rats exceeded those with the SO treatment by more than 100% ($p<.001$). Group mean values for groups LF, SO and BT were 34, 22 and 52 dpm x 10^3, respectively. Safflower oil feeding affected intestinal cholesterogenesis as early as 30 days on the diet. Intestinal cholesterogenesis was elevated with aging ($p<.001$). Meal feeding decreased intestinal cholesterogenesis ($p<.001$). Differences in cholesterogenesis between patterns were most apparent with diets LF and BT. There was no difference between patterns with diet SO. The magnitude of cholesterogenesis decrease with

meal feeding in groups LF and BT was approximately 1 1/2 times that in group SO (diet x meal pattern interaction, Table 12, Figure 6).

^{14}C-cholesterol retention Carbon-14 cholesterol in liver and small intestine was measured by counting ^{14}C in digitonin precipitable steroids (^{14}C-DPS).

Hepatic ^{14}C-cholesterol Hepatic ^{14}C-cholesterol increased when dietary fat level was elevated regardless of fat source ($p<.02$, Figure 7). Furthermore, ^{14}C-cholesterol recovered from SO fed groups was not statistically different from that of groups receiving BT. Group mean values were 428, 585 and 552 dpm x 10^3 for groups LF, SO and BT, respectively. The increase in hepatic ^{14}C-cholesterol with age ($p<.001$) was almost linear. Highest values occurred at age 4 months with every dietary treatment. Variable mean values at ages 2, 3 and 4 months were 400, 537 and 629 dpm x 10^3, respectively. Hepatic ^{14}C-cholesterol retention decreased with meal feeding in contrast to ad libitum feeding ($p<.001$) with variable mean values of 593 and 451 dpm x 10^3 for AL- and MF-treatments, respectively (Tables 13-14).

Small intestine plus contents ^{14}C-cholesterol At all age periods studied, retention of ^{14}C-cholesterol in the small intestine with diet LF was consistently lower than retention with SO ($p<.001$) and BT ($p<.01$). Variable mean values for groups LF, SO and BT were 29, 47 and 34 dpm x 10^3, respectively. While fat saturation had no influence on hepatic ^{14}C-cholesterol, counts in the small intestine were elevated with SO compared to BT feeding ($p<.001$, 47 vs. 34 dpm x 10^3, respectively). Again, this increase with SO feeding compared to BT feeding was observed throughout

Table 13. Cholesterol retention, ^{14}C-cholesterol in liver and small intestine plus contents. ^{14}C-cholesterol was assessed as ^{14}C-digitonin precipitable steroids (^{14}C-DPS). Counts are expressed as dpm x 10^3 per organ

	2 months 30 days	3 months 60 days	4 months 90 days
		Liver	
LF-AL	464±41[a]	478±115	588±86
MF	201±63	413±72	422±118
SO-AL	353±66	793±87	630±154
MF	422±62	580±66	689±64
BT-AL	398±71	607±35	1021±124
MF	436±65	347±51	424±98
		Small intestine + content	
LF-AL	24±3	42±4	51±6
MF	15±2	18±3	26±4
SO-AL	31±4	51±3	61±5
MF	29±3	50±3	57±5
BT-AL	29±2	48±4	55±8
MF	17±2	41±5	47±5

[a]Mean ± SEM.

Table 14. Cholesterol retention, total ^{14}C-DPS in liver and SI; variable means, main effects and interactions

		Main effects	
		Liver	SI
Diet		p<.02	p<.001
LF		428	29
SO		585	47
BT		552	34
Fat level		p<.001	p<.001
LF		428	29
HF (SO + BT)		569	41
Fat saturation		NS	p<.001
SO		585	47
BT		552	34
Day	Age	p<.001	p<.001
30	2	400	24
60	3	537	42
90	4	629	49
Meal pattern		p<.001	p<.001
AL		593	44
MF		451	33

Table 14. (Continued)

	Interactions	
	Liver	SI
Diet x age	NS	NS
Diet x meal pattern	p<.1	p<.01
Age x meal pattern	p<.01	NS
Diet x age x meal pattern	p<.05	NS

	2	3	4
LF	333	446	505
SO	410	686	659
BT	457	477	723

	2	3	4
LF	19	30	38
SO	30	51	59
BT	23	44	51

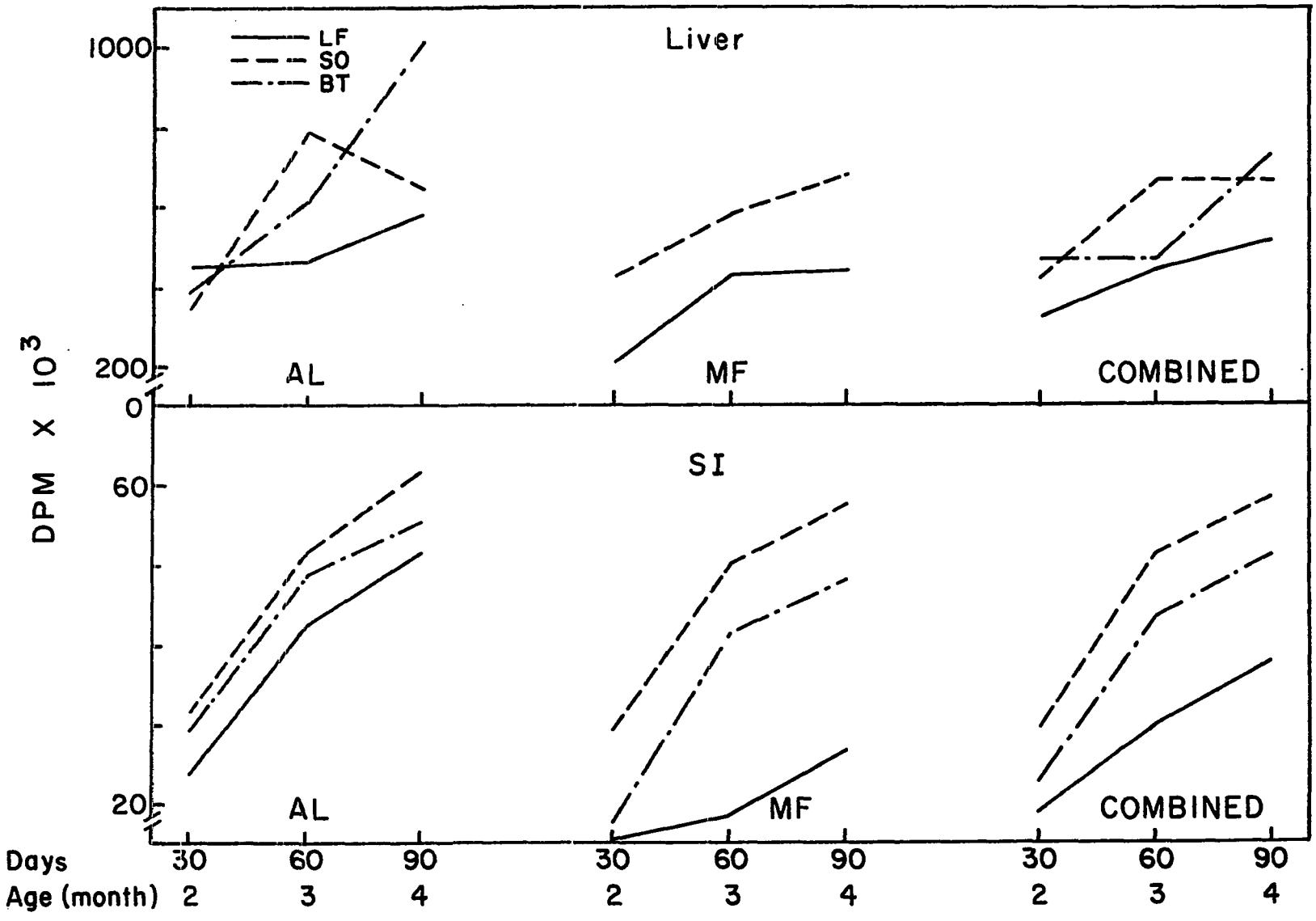
	AL	MF
LF	510	346
SO	592	579
BT	676	429

	AL	MF
LF	40	19
SO	48	45
BT	44	40

	2	3	4
AL	405	627	746
MF	395	447	512

	2	3	4
AL	45	47	56
MF	21	36	43

Figure 7. ^{14}C-cholesterol retention in liver and small intestine. Cholesterol was assessed by digitonin precipitation method. Top figure shows total ^{14}C-counts in digitonin precipitable steroids (DPS) from liver. Bottom figure shows ^{14}C-counts in DPS from small intestine plus contents. Counts are expressed as DPM x 10^3 per organ.


LF
SO
BT
Liver
1000
200
0
AL
MF
COMBINED
DPM X 10^3
SI
60
20
AL
MF
COMBINED
Days
30
60
90
Age (month)
2
3
4

the experiment. As in liver, ^{14}C-cholesterol retention in the small intestine increased with age ($p<.001$). Corresponding values increased by approximately 20% between ages 2 and 4 months in all dietary treatment groups. With meal pattern as a variable, small intestine ^{14}C-cholesterol retention increased with ad libitum feeding. Values were 44 and 33 dpm x 10^3 for AL and MF, respectively ($p<.001$). On a unit tissue weight basis, ^{14}C-cholesterol retention was also increased with AL over MF feeding ($p<.05$). Differences in ^{14}C-cholesterol between meal patterns in LF groups were two times those of either SO- or BT-fed rats ($p<.01$). The biggest difference between meal patterns occurred at age 2 months (Table 13).

Cholesterol degradation and excretion In analyzing ^{14}C derived from 4-^{14}C-cholesterol administered 6 days prior to termination, the assumption was made that none of the ^{14}C could be lost from the steroid ring compound during degradation to bile acids (Tables 15-16, Figure 8).

Hepatic ^{14}C-acid steroids Mean hepatic ^{14}C-counts in the acidic steroid fraction of group LF were lower than those of group SO ($p<.001$), but identical to those of BT fed rats. Variable mean values for these groups were 2.5, 3.4 and 2.4 dpm x 10^3 for LF, SO and BT, respectively. This implied that a high degree of polyunsaturation stimulated conversion of cholesterol to bile acids. The data indicated also that a low fat, high carbohydrate diet led to similar rates of acid steroid formation. These observations held true for each age period. Hepatic ^{14}C-acid steroid levels increased with age ($p<.001$). Decreased acid steroid content was also observed with meal feeding ($p<.001$). A decrease in counts was attributed to decreased liver size of meal fed animals only, since counts on a unit tissue weight basis between ad libitum and meal feeding

Table 15. Cholesterol degradation and excretion, ^{14}C-counts in acid steroids from liver, small intestine plus contents and feces. Counts are expressed in dpm x 10^3 per organ

	2 months 30 days	3 months 60 days	4 months 90 days
		Liver	
LF-AL	2.9±.4[a]	2.8±.4	3.4±.4
MF	1.9±.3	2.9±.5	1.3±.2
SO-AL	3.2±.4	5.2±.5	2.9±.6
MF	2.9±.4	3.8±.4	2.2±.3
BT-AL	2.2±.3	3.5±.6	2.0±.3
MF	2.6±.5	2.5±.5	1.6±.2
		Small intestine + contents	
LF-AL	9±2	8±1	16±2
MF	8±1	8±1	13±3
SO-AL	12±1	22±3	22±3
MF	10±1	25±5	25±2
BT-AL	14±2	10±1	17±1
MF	9±1	9±1	13±1
		Fecal + cecal contents	
LF-AL	30±6	42±5	52±3
MF	19±6	20±2	24±5
SO-AL	26±4	42±5	43±5
MF	34±3	45±5	24±7
BT-AL	29±3	40±6	39±9
MF	18±2	24±2	22±4

[a]Mean ± SEM.

Table 16. Cholesterol degradation and excretion, ^{14}C counts in acid steroids from liver, small intestine plus contents and feces. Counts are expressed in dpm x 10^3 per organ. Variable means, main effects and interactions

		Main effects		
		Liver	SI	Feces
Diet		p<.01	p<.001	NS
	LF	2.5	10	31
	SO	3.4	19	36
	BT	2.4	12	29
Fat level		NS	p<.001 (4.138)	NS
	LF	2.5	10	31
	HF (SO + BT)	2.9	16	33
Fat saturation		p<.001	p<.001	p<.01
	SO	3.4	19	36
	BT	2.4	12	29
Day	Age	p<.001	p<.001	p<.01
30	2	2.6	10	26
60	3	3.5	14	35
90	4	2.2	17	34
Meal pattern		p<.001	NS	p<.001
	AL	3.1	14	38
	MF	2.4	13	26

Table 16. (Continued)

	Interactions		
	Liver	SI	Feces
Diet x age	NS	p<.02	NS

	2	3	4
LF	2.3	2.8	2.4
SO	3.1	4.5	2.6
BT	2.4	3.0	1.8

	2	3	4
LF	9	8	14
SO	11	24	23
BT	11	10	15

	2	3	4
LF	25	31	38
SO	30	43	34
BT	24	32	31

Diet x meal pattern

	NS		NS		p<.05	
	AL	MF	AL	MF	AL	MF
LF	3.0	2.0	11	10	41	21
SO	3.8	3.0	18	20	37	34
BT	2.6	2.3	14	10	36	21

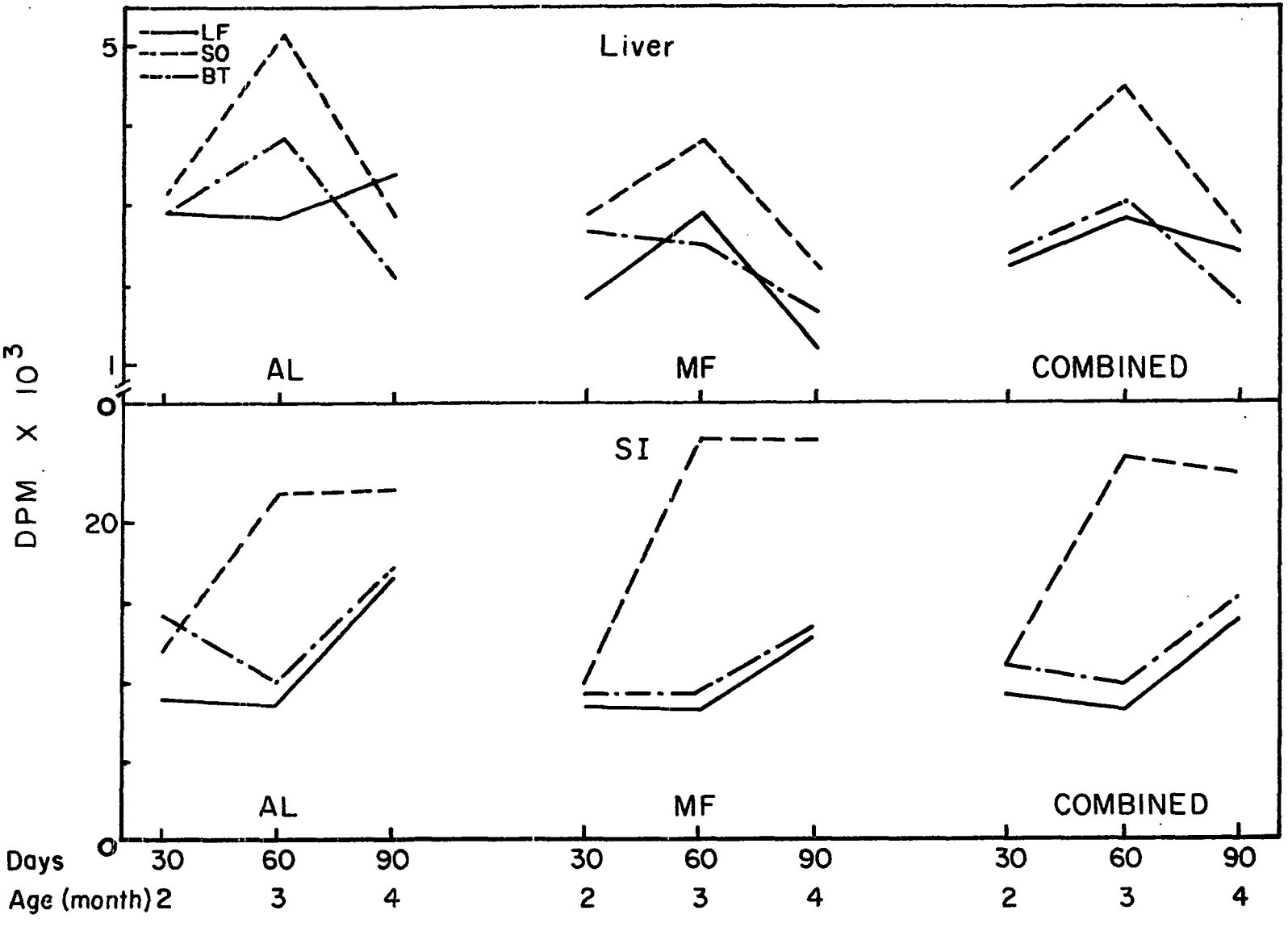
Age x meal pattern

	NS			NS			p<.05		
	2	3	4	2	3	4	2	3	4
AL	2.8	2.8	3.1	12	18	14	28	45	30
MF	3.8	2.5	1.7	14	9	17	41	24	24

Day x age x meal pattern

NS	NS	NS

Figure 8. Cholesterol degradation and excretion. Total ^{14}C-counts in acid steroids from liver, small intestine plus contents, and feces. Counts are expressed as dpm x 10^3 per organ.


LF
SO
BT
Liver
5
1
0
AL
MF
COMBINED
DPM X 10^3
SI
20
0
AL
MF
COMBINED
Days
30
60
90
Age (month) 2
3
4

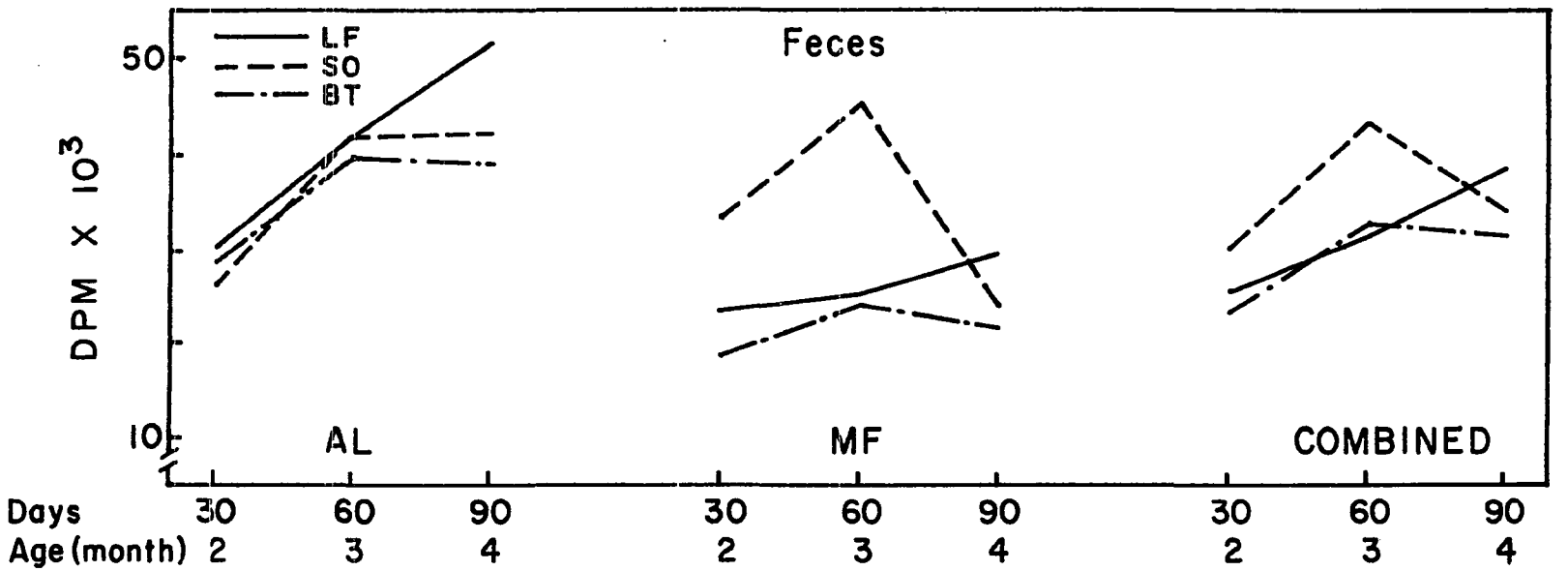


Figure 8. (Continued)

were not different (0.23 vs 0.20 dpm x 10^3,Tables 15-16, Figure 8).

Small intestine ^{14}C acid steroids As in the case of liver, small intestine ^{14}C acid steroid content was increased with SO feeding compared to LF or BT feedings ($p<.001$, Tables 15-16, Figure 8). In addition, ^{14}C acid steroid excretion in group LF was relatively similar to group BT. Variable mean values for groups LF, SO and BT were 10, 19 and 12 dpm x 10^3, respectively. Acidic steroid excretion, in general, increased between the ages of 2-4 months ($p<.001$). Variable mean values for these three periods were 10, 14, and 17 dpm x 10^3. Maximal increase with group SO was attained at age 3 months. Finally, ^{14}C-acidic steroid content in the intestine was not influenced by variations in meal pattern based on total ^{14}C-counts in the whole tissue (14 vs. 13 dpm x 10^3 for AL vs. MF) or ^{14}C counts per g of wet tissue (1.5 vs. 1.4 dpm x 10^3 for AL vs. MF).

Fecal ^{14}C-acid steroids Fecal ^{14}C acid steroids included acid steroids from large intestinal contents and feces. Fecal ^{14}C-acid steroid excretion was similar with LF and BT diets. Though the variable mean with SO exceeded those for LF and BT by 13 and 24%, respectively. only the SO-BT comparison was statistically significant ($p<.01$). Increased fecal acid steroid excretion with SO over BT was observed in all age groups studied. In general, fecal acid steroid excretion increased with age ($p<.01$), though the increase was nonlinear with plateaus at ages 3-4 months. With meal feeding, fecal acid steroid excretion decreased in meal fed rats (26 dpm x 10^3) in contrast to ad libitum fed animals (38 dpm x 10^3, $p<.01$, Tables 15-16, Figure 8).

Fecal ^{14}C-neutral steroids Due to technical difficulties with the scintillation counter, counting of fecal ^{14}C-neutral steroids was delayed

for 6 weeks. Consequently, phase separation and precipitation occurred in the scintillation cocktail, giving rise to erratic counts. Data for fecal ^{14}C-neutral steroids are therefore not presented.

Plasma hormone

Plasma thyroxine levels Plasma thyroxine levels were used to estimate thyroid status. Thyroxine levels were assessed only in the 4-months old animals. Three measurements were used in this study to estimate thyroid status: 1) T3 (triiodothyronine) levels, 2) T4 (tetraiodothyronine) levels, 3) T4/T3.

Plasma T3 levels were not influenced by dietary fat level and meal pattern, though values for SO-fed groups tended to be higher than those for BT groups ($p>.05$). Variable group mean values were 69, 75 and 66 thyopac units for groups LF, SO and BT, respectively (Table 18).

Plasma T4 levels were not influenced by any of the variables used. Range values were narrow, between 3.5-4.9 µg/dl plasma, and group mean values were between 3.8-4.9 µg/dl plasma.

Plasma T4/T3 value was also not influenced statistically by the variables used, attributable to both high standard deviations (about 20-50%) and small group differences. Range values for T4/T3 ratio were between 5-7, while group mean values were fairly similar among the three dietary groups, for example, 5.5, 6.5 and 6 for LF, SO and BT, respectively.

Table 17. Serum thyroxine levels

	4 months 90 days		
	T3[a]	T4[b]	T4/T3[c]
LF-AL	67±4[d]	4.3±.4	6±1
MF	70±5	3.6±.4	5±1
SO-AL	78±3	4.8±.4	6±1
MF	72±4	4.9±.9	7±2
BT-AL	65±3	4.7±.9	7±2
MF	67±3	3.5±.3	5±2
	T3	T4	T4/T3
Fat level	NS	NS	NS
Fat saturation	$p<.05$	NS	NS
Meal pattern	NS	NS	NS

[a]Triiodothyroxine in thyopac units.

[b]Tetraiodothyroxine in µg/dl plasma (thyroxine free acid form).

[c]Free thyopac index, estimate of thyroxine level in serum.

[d]Mean ± SEM.

Discussion

In the present study an experimental model was used suitable for monitoring acute alterations in cholesterol metabolism during maturation. Thirty day old male rats were challenged with semi-purified diets either low or high in fat and varying in degree of saturation of the fat source (SO or BT). These animals were killed after 30, 60 or 90 days on their respective regimens. The time periods corresponded to chronological ages of 2, 3 and 4 months. Rats were trained to eat their daily caloric intake in 3- or 24-hours out of a 24-hour period. Room lighting schedule was reversed, with a dark photo period between 9 am to 6 pm. Under these conditions maximal peak activity of HMG-CoA reductase, the rate limiting step in cholesterogenesis should have been reached at 2 pm for ad libitum fed rats, and 6 pm for meal-fed rats (Edwards et al., 1972). Recent reports by McNamara et al. (1972) indicated that the amplitude and phase changes in HMG-CoA reductase activity associated with the post-weaning period would have declined and stabilized to those of adult levels by day 30. Thus, it is assumed that data measured in this study were uncomplicated by post-weaning hormonal-induced reductase activity, rather that they were induced by the variables used in this experiment.

The animal model used here, however, had some limitations since it did not consider fully all differences in cholesterol metabolism, which may have resulted from the variables tested.

First, cholesterogenesis in meal fed groups might have been underestimated, since it may not have been determined at peak activity which

occurs around 6 pm. In the present study, rats, regardless of meal pattern, were killed between 12:30-2:30 pm, the time for peak cholesterogenesis in ad libitum feeding.

Secondly, ad libitum fed rats tended to consume most of their food during the first 8 hours of the dark period. Similar observations were made by Carlson (1975) with adult rats. This voluntary meal pattern could have affected activities of enzymes which follow diurnal rhythms, such as HMG-CoA reductase and 7-α-hydroxylase, the latter of which controls the first step in cholesterol degradation to bile acids.

Finally, assessments of rates of cholesterogenesis 2 1/2 hours after ^{3}H-acetate injection might not have measured maximal rates of cholesterogenesis. According to several investigators (Goldfarb and Pitot, 1972; Bortz, 1973), maximal rates of cholesterogenesis occurred 10-12 hours after acetate injection.

In spite of these limitations, certain changes were apparent in cholesterol metabolism resulting from the variables used in this study.

Growth Rates

Data on body weights, food intakes and food efficiencies with the three diets (LF, SO and BT) confirmed those collected previously in our laboratory (Reeves, 1971; Carlson, 1975). In general, beef tallow fed animals appeared to eat more and gain more weight than did rats raised on safflower-oil. However, food efficiencies between the two fat types were not different.

Certain tissue weights were sensitive to changes in dietary fat and/or to aging, for example, liver, epididymal fat pads, stomach (with contents removed), kidneys. The spleen, however, appeared to reach maximal weight by age 2 months, and did not respond to any of the variables used.

Plasma Cholesterol and Triglyceride Levels

Plasma cholesterol concentration did not alter with the dietary variables used in young, 2-4 months old rats. Failure to demonstrate differences with variations in dietary fat level and in fat saturation may be related to low plasma cholesterol values characteristic of young rats. Studies with humans had indicated that serum cholesterol concentrations of subjects with low cholesterol concentrations (around 150-180 mg/dl) did not respond to changes in dietary fat as dramatically as did those of patients with hypercholesteremia (Grundy et al., 1969). Lack of response of humans and animals with low serum cholesterol levels to changes in dietary fat may be due to more efficient cholesterol degradative and excretory mechanisms, or to faster cholesterol exchange between serum and the slowly exchanging pools such as muscle, adrenals, heart and others.

Plasma cholesterol concentrations of animals over the three age periods (2-4 months) averaged between 40-50 mg/dl. Dupont et al. (1972) reported a range of 50-80 mg/dl for Carworth-CFE rats between the ages of 3-6 months.

Plasma cholesterol concentrations tended to increase between ages 2-4 months (about 23%). Dupont et al. (1972, 1975) reported increases in serum cholesterol concentrations with age with the greatest increase after 9

months of age. Failure to bring out significant increases with age in our study may be related to the young age groups studied here. It could also be that young rats are more resistant to dietary variables compared to older rats.

Plasma cholesterol concentrations of rats fed a 3 hour meal were not different from rats fed continuously. This finding confirms that observed in humans (Wahdwa et al., 1973) and that in rats (Okey et al., 1960). This finding contradicts that reported with adult rats (Reeves and Arnrich, 1974; Carlson, 1975) and with monkeys (Gopalan et al., 1962). These authors found increased serum cholesterol concentrations with meal feeding, regardless of dietary types. Failure to demonstrate increased cholesterol concentration with meal feeding in this study could be related in part to the feeding habits of ad libitum fed animals, which consumed most of their food in 6-8 hours during the dark period. Consequently ad libitum fed controls may have responded physiologically like meal fed rats. Meal fed rats consumed in a 3 hour period about 75-88% of the food calories of ad libitum controls. In addition it is likely that young rats adapt readily to meal feeding, and that therefore the physiological response to differences in feeding pattern is small.

Increase in serum cholesterol concentration is transitory, and may disappear with prolonged feeding (Leveille and Hanson, 1965). Since plasma cholesterol concentrations at the start of the experiment were not measured, and since the earliest measurement came 30 days after initiation of the meal feeding regimen, the possibility exists that increased plasma cholesterol concentrations could have been obliterated by 30 days of feeding.

Data for plasma ^{14}C-cholesterol retention in the plasma pool after 6 days showed little difference in total counts between diets, meal patterns and age. These radiochemical data corresponded to chemical data on cholesterol concentration.

Plasma triglyceride levels averaged between 230-250 mg/dl in variable mean values for the three diet regimens. Significance was not obtained due to small group differences and large standard error of the means. Data from plasma triglyceride levels support data from plasma cholesterol concentrations, both of which showed neither increase nor decrease with variations in dietary fat level, saturation and meal pattern. The relationship between plasma triglyceride and cholesterol levels is not well documented, though alterations in circulating triglyceride levels have been implicated in subsequent changes in plasma cholesterol levels (Goh and Heimberg, 1973).

Plasma Thyroxine Level

Thyroid hormone affects hepatic cholesterogenesis and HMG-CoA reductase activity in rats and in humans (Fletcher, 1958; Gries et al., 1962). Hyperthyroidism may decrease plasma cholesterol levels, while hypothyroidism is often accompanied by hypercholesterolemia. It has been speculated that changes in plasma cholesterol concentrations and hepatic synthesis are results of changes in oxidative rates which overcompensate (hyperthyroidism) or undercompensate (hypothyroidism) for alteration in cholesterol synthesis. More specifically tri-iodothyramine has stimulated hepatic HMG-Co reductase in hypothyroid rats. In the present study, plasma thyroxine level, as estimated from either T_3 or T_4, was not

different for most dietary treatment groups when dietary fat level or fat saturation was varied. Though the plasma T_3 level was increased with safflower oil feeding (Table 18), this increase was not associated with changes in hepatic cholesterogenesis or plasma cholesterol. Plasma T_4/T_3 was not influenced by any of the variables used. Compared to T_3 and T_4 concentrations these ratios, purportedly, are better controlled parameters of thyroid status since they are less influenced by stress, drugs, and infections.

Cholesterol Synthesis

Effects of high fat diets on cholesterol synthesis appear to depend on the organ under consideration. Hepatic cholesterogenesis was enhanced with fat feeding, regardless of fat source. However, small intestinal cholesterogenesis was decreased with safflower oil and increased with beef tallow compared to the LF diet. Increased hepatic cholesterogenesis with fat feeding confirmed the findings of others (Linazasoro et al., 1958; Wood and Migocovsky, 1958). Under conditions of controlled feeding, other investigations have also reported elevated hepatic cholesterogenesis with fat feeding in young rats (Bortz, 1967; Goldfarb and Pitot, 1972) and in adult rats (Carlson, 1975). Increased rates of hepatic cholesterogenesis with high fat diets may be related to a concomitant inhibition of fatty acid synthesis which could result in elevated cellular levels of acetyl CoA for cholesterol synthesis (Hill et al., 1960; Bortz, 1967; Goldfarb and Pitot, 1972). More specifically, high fat diets caused accumulations of intrahepatic levels of fatty acetyl CoA , which inhibits acetyl CoA carboxylase and subsequently lipogenesis

(Bortz, 1963). Increased rates of hepatic cholesterogenesis may also be related to elevated cellular levels of NADPH. In adipose tissue of rats, high fat diets caused elevated production NADPH (Zaragoza, 1974). Since maximal HMG-CoA reductase activity occurred after preincubation with NADPH (Tormanen et al., 1975), it is conceivable that elevated hepatic cholesterogenesis with fat feeding can be attributed to increased HMG-CoA reductase activity. Supporting work comes from Goldfarb and Pitot (1972) who showed increased HMG-CoA reductase synthesis with fat feeding.

Changes in cholesterol biosynthesis in the small intestine with fat feeding depended on fat type. Safflower oil induced a decrease in cholesterogenesis in contrast to beef tallow. This finding confirms that of Carlson (1975) with adult rats. Reduced rates of cholesterogenesis in the small intestine with safflower oil feeding may be due to increased cellular cholesterol content, which causes feedback inhibition of HMG-CoA reductase activity (Shefer et al., 1973).

A number of publications indicated increased hepatic cholesterol synthesis with dietary intake of polyunsaturated fatty acids (Goldfarb and Pitot, 1972; Dupont et al., 1972). The present study with young rats failed to demonstrate differences due to degree of fat saturation. However, our results corroborate with those of Dupont et al. (1975) and Carlson (1975) who worked with adult rats and with dietary fats identical to those used in the present work. The failure to find a stimulation of cholesterogenesis with polyunsaturated fats may be related to the time lapse after tritiated acetate injection (2 1/2 hours). This period may not have been sufficiently long to detect differences due to fat types. Hepatic cholesterol synthesis, measured 10-12 hours after labeled acetate

administration indicated enhanced rates of synthesis, as well as maximal activity of HMG-CoA reductase (Bortz and Steele, 1973). Though 2 1/2 hours may have been too short to see effects on cholesterol synthetic rates due to diet, this time period was selected in favor of the maximum at 10 hours to avoid extensive exchange between tissues of the newly synthesized cholesterol.

Data from the present study indicated increased rates of hepatic and intestinal cholesterogenesis in rats matured between ages 2-4 months. Dupont et al. (1975) have reported increased tritiated alanine incorporation into cholesterol in liver in rats aged 9-12 months. Increased rates of cholesterogenesis with age in the present study may be attributable to increased organ weights over the three age periods.

In the present study meal pattern did not affect hepatic cholesterogenesis while small intestine cholesterogenesis decreased with meal feeding. Failure to demonstrate differences in hepatic cholesterogenesis may be due to the limitation of the experimental design. According to the literature, maximal rates of hepatic cholesterogenesis would have occurred later for meal fed compared to ad libitum fed groups. In contrast, rates of small intestinal cholesterol biosynthesis decreased with meal feeding, whether expressed on a unit tissue or whole tissue weight basis. Evidence is available that rates of cholesterol synthesis were unaffected with meal feeding in adult rats. No other reports have been found on this subject.

Cholesterol Degradation and Excretion

The consistent increase in bile acid contents of liver, small intestine and feces with safflower oil feeding was the most striking finding of cholesterol degradation to bile acids and subsequent bile acid excretion. At all three sites, the substitution of beef tallow as fat source produced almost identical levels of bile acids as did the low fat diet. Increased bile acid production and subsequent excretion were also shown by others in rats using either corn or safflower oil (Gordon et al., 1964; McGovern and Quackenbush, 1973a). Previous studies in our laboratory with adult rats indicated similar elevations of bile acid formation and excretion. The evidence was indirect, based on calculations involving bile acid specific activity (Carlson, 1975).

The mechanism whereby polyunsaturated fatty acids increase bile acid formation and excretion is only speculative at this present time. Increased cholesterol degradation to bile acids could be due to shortened half-life of bile acids with fat unsaturation (McGovern and Quackenbush, 1973c; Lindstedt et al., 1965; Moore et al., 1968). McGovern and Quackenbush (1973a) found that substitution of safflower oil for beef tallow increased cholesterol conversion to bile acids by 13.5% while the secretion of the acid steroid label from the liver was increased by 8.6%.

Other factors which could influence bile acid production are the plant sterols, such as β-sitosterol, present in safflower oil. Beta-sitosterol enhanced bile acid production by competing with cholesterol for absorption (Spritz et al., 1965). Thus negative feedback by cholesterol on hepatic cholesterogenesis would be removed. However, this factor may

be of minor importance in this study since exogenous cholesterol was not present, so that only reabsorption of endogenous cholesterol could have been affected.

The present study indicated increased bile acid formation and excretion with increase in age from 2-4 months. Meal-feeding decreased bile acid synthesis in liver, and decreased bile acid excretion in feces. These results contradict those of Carlson (1975) with adult rats, who failed to find a meal feeding effect.

Cholesterol Distribution

Data from these studies indicate that cholesterol content in the plasma-hepatic-small intestinal pool was influenced by level of dietary fat, though plasma cholesterol concentrations did not decrease with polyunsaturated fat feeding. Cholesterol content, as determined chemically, was elevated with safflower oil feeding in liver and small intestine. Beef-tallow feeding did not appear to invoke differences in cholesterol content in these tissues when compared to low fat feeding. In liver, radiochemical data on the distribution of ^{14}C-cholesterol label six days after injection did not support the chemical data. Measurements of ^{14}C-cholesterol in liver were similar with the two fats. This discrepancy between chemical and radiochemical interpretation could be attributable to relatively large individual variations with radiochemical methodology. In small intestine, measurements from cholesterol label verified increased cholesterol accumulation with high fat feeding particularly with safflower oil. Increased cholesterol accumulation in liver and small intestine with polyunsaturated fat intake was also reported in rats (Bloomfield, 1964).

In young rats between the ages of 2-4 months, cholesterol accumulation in the liver and small intestine increased. This observation was verified both chemically and radiochemically. Dupont et al. (1972) also reported increases in cholesterol content and ^{14}C-cholesterol label in the liver of young rats between 3-18 months of age. Accumulation of tissue cholesterol correlated to increases in organ and body size with aging.

Meal feeding decreased ^{14}C-cholesterol content in liver and small intestine. However, cholesterol contents, determined chemically, were not different between meal patterns in these sites. Similar findings were reported by Carlson (1975) with adult rats. In young rats, however, the inverse correlation between hepatic and serum cholesterol levels was not observed. This finding supports the study of Anderson and Fausch (1964) who showed decreased hepatic cholesterol concentrations, though plasma concentrations with ad libitum and meal fed swine were similar. This study, however, contradicts those of Reeves (1971), Carlson (1975) and McGovern and Quackenbush (1973a,b,c) who demonstrated reciprocal relationships between plasma and hepatic cholesterol levels.

Subjective Observation

Increased susceptibility of safflower oil fed rats to respiratory infection observed in this study confirms observations of Dupont et al. (1975) who reported high frequencies of chronic respiratory problems and subsequent high mortality in older rats (>7 months). These animals had been maintained from young on diets containing high levels of safflower oil. Other groups fed beef tallow or a mixed fat were less affected by respiratory problems. The investigators, however, did not report the

occurrence of skin and eye infections. The deleterious effects of safflower oil feeding may be related to excessive intercellular accumulation of linoleate derived from safflower oil, which contains approximately 79% linoleic acid. Linoleic acid is a precursor of homo-gamma-linoleate and arachidonic acid which are precursors of prostaglandins (PG). Injections of arachidonic acid into rabbits resulted in sudden death due to platelet aggregation mediated through certain prostaglandins (Silver, 1974).

Little information is available relating safflower oil feeding and tissue fragility. Increased intestinal and cardiac fragility could be related to alterations in mitochondrial membrane structure. Mitochondrial membranes of rats fed high levels of safflower oil or corn oil contained 85 or 53%, respectively, more unsaturated fatty acids than those of beef tallow fed rats. In addition, the activities of several membrane bound enzymes were related to the physical characteristics of the membranes. Cytochrome C oxidase, ATP phospholipase, phospholipase A and fatty acid oxidase activities were elevated with PUFA feeding, while succinate dehydrogenase activity was decreased (Elson and Shrago, 1975). Presently, the overall relations of high polyunsaturated fat intakes to increased susceptibility to skin, eye and respiratory infections, increased mortalities, tissue fragilities, membrane alterations are not well understood. To explore the cause of cardiac fragility observed in this study, two subsequent experiments (Experiment 2 and 3) were designed to study functional and gross morphological changes related to high levels and prolonged feeding of safflower oil.

SECTION II. CARDIAC STRESS-STRAIN RESPONSE AND MORPHOLOGY

Review of Literature

Definitions

The literature on cardiac mechanics contains several inconsistencies in terminologies employed by investigators. It is therefore appropriate to define the terms which will be related directly or indirectly to this study.

A stress-strain (SS) curve measures the extension of a material in the presence of a force. As the material stretches, the length and volume become deformed.

Stress is defined by Vidik (1973) as the force per unit cross-sectional area of the material. It measures basically the intensity of forces. The units commonly employed are g/mm^2, g/cm^2, $dynes/cm^2$, etc.

Strain is a dimensionless quantity and is produced by the application of a stress. It indicates the change from the original or unstressed to the final stressed dimension. Mirsky and Parmley (1973) defined Lagrangian strain as $(l-l_o)/l_o$ where l_o is the length at zero stress and l is the instantaneous length. Since zero-state lengths are technically difficult to assess, l_o is often replaced by initial length or the end diastolic length. Natural strain is defined as $\log_e (l/l_o)$ and is a term more applicable to strain for biological materials. In contrast to the Lagrangian strain, this definition does not assume that the cross-sectional dimensions remain unaltered and that strains are uniform along the length of the fiber.

Distensibility is normally employed in pressure-volume change studies of the cardiac chambers. It is the change in volume V relative to an alteration in pressure P and is expressed as dV/dP. The term is used interchangeably with compliance.

Specific compliance is the change in volume per unit volume relative to a pressure change, i.e., dV/VdP. The inverse, VdP/dV, is referred to as volume elasticity and has the dimensions of stress.

Young's modulus describes the mechanical characteristic of an elastic material which is compressed or stretched. A material that obeys Hooke's law has a stress-strain relationship that is linear. Young's modulus, E, is thus expressed as $\sigma = E\varepsilon$, where σ and ε are the stress and strain, respectively. Most biological materials deviate from this linear relationship. They generally follow a curvilinear path which is usually exponential in form. In this instance, Young's modulus is replaced by the term tangent modulus. Young's modulus is usually expressed as dynes/cm^2.

Tangent modulus or elastic stiffness according to Vidik (1973) and Mirsky and Parmley (1973) is the instantaneous slope at any point of the SS curve. If the SS relationship is exponential, the elastic stiffness ($d\sigma/d\varepsilon$) vs. stress relationship is linear (Figure 9). The slope k of this line is termed the elastic stiffness constant with C as the intercept of the y-axis. If the material obeys Hooke's law, the elastic stiffness is constant and is independent of the stress level.

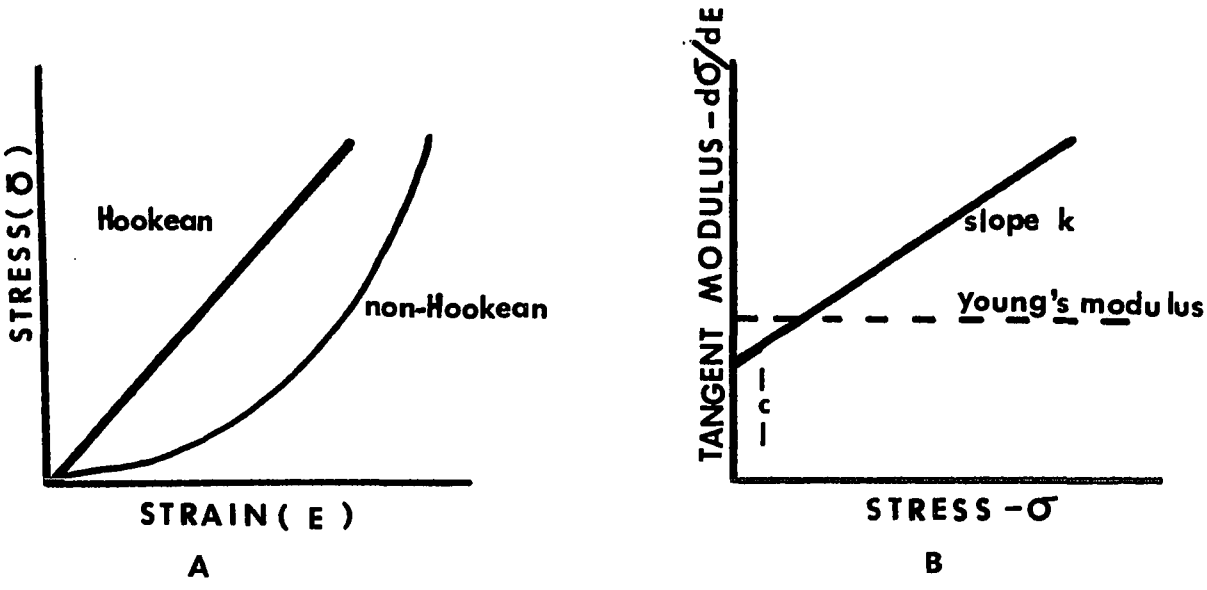


Figure 9. Stress-strain and elastic stiffness (tangent modulus) vs. stress relationships: A = $\sigma - \varepsilon$ relationship for a Hookean and non-Hookean (biological tissues) material; B = $\frac{d\sigma}{d\varepsilon}$ vs. σ relationship; for non-Hookean material this relationship is linear with k as the slope and C the y intercept; for Hookean material the relationship is a constant described by the Young's modulus, E (Mirsky and Parmley, 1973).

Stress-strain equation

A mathematical description of the stress-strain history law of tissues is usually required before theoretical mechanisms can be understood. Unfortunately at the present time, there is no satisfactory constitutive equation derived for any biological material (Fung, 1967). Extensive two- and three-dimensional testing program is not feasible on a viable specimen without damaging its integrity. Hence most studies are limited to a one-dimensional stress field at a time. This is true of experiments with papillary muscle, intact and perfused heart. A quasi-linear stress-strain-history relationship for soft tissues was developed by Fung (1967) for rabbit mysentery. In biological systems, several contributing

factors to the SS relationship have to be considered, for example viscosity, plasticity, numbers of elements (i.e., fiber types), orientation and arrangement of these fiber elements (Gratz, 1931; Vidik, 1973). The fiber elements referred to here involve collagen, elastin and the gel-like matrix consisting primarily of muccopolysaccharides. Haut and Little (1972) attempted to formulate a constitutive equation for collagen fibers. The equation predicted strain rate and hysteresis effects only. It did not work for sinusoidal phenomenon.

The approach of using an analogy consisting of idealized elements was used on biological materials by Alexander (1962) for the body wall of the sea anemone and by Snedlin (1965) for human bone. On the basis of the curves generated, Vidik (1969) suggested a mechanical analogy which was subsequently analyzed mathematically and verified experimentally by Frisen et al. (1969). It would seem from these studies that most biological tissues contain two types of element arrangement models. The Kelvin model combines the elastic and viscous elements in parallel, while the Maxwell model uses in-series coupling.

Components in SS curves

Burton (1954) proposed a structural-functional relationship of elastin, collagen and smooth muscle in the arterial wall. From elastic diagrams, he calculated the dynamic tangential modulus to be 6×10^4 dynes/cm^2 for smooth muscle, 3×10^6 dynes/cm^2 for elastin and 10^9 dynes/cm^2 for collagen. Based on the shape of the curves generated, he hypothesized that the collagen fibers had to be arranged parallel to the elastic fibers. This meant that the elastic fiber would be stretched

first when the vessel was distended. The collagen fibers, being stiffer, would be stretched at increased pressures. The stretching of the elastic fiber functioned to provide maintenance tension against normal blood pressure and pressure fluctuations. The smooth muscle provided active tension and contributed little to maintenance tension. In this conceptual model the most vital role of elastin fibers was to provide graded contractions together with the smooth muscle.

Burton's hypothesis was substantiated in 1957 (Roach and Burton). From distensibility curves, the functional interrelations of the various components in the arterial wall of human iliac arteries were elucidated. Elastic tension diagrams were obtained with fresh, formic acid-treated (to remove collagen) and trypsin-treated (to remove elastin) vessel segments. Curves recorded from fresh and formic acid-treated segments showed little change in shape at low pressures, though formic acid-treated segments were less distensible than fresh segments. At higher pressures (100 mm Hg) the trypsin-treated segments were more distensible. These findings confirmed earlier predictions that the elastic fibers functioned at low pressures, and played an insignificant role at pressures over 100 mm Hg. Conversely, collagen fibers functioned to maintain tension at increased pressures.

Bergel (1961) demonstrated increased elastic modulus during dilation of the arterial wall. Mean values for static elastic modulus at 100 mm Hg pressures were in dynes/cm^2, 4.3 x 10^6 for thoracic aorta, 8.7 x 10^6 for abdominal aorta, 6.9 x 10^6 for femoral artery, 6.4 x 10^6 for carotid artery and 10^6 dynes/cm^2 for smooth muscle. These values exceeded those obtained for smooth muscle by Burton by a factor of 15.

Burton (1954) and Elden (1970) proposed that the modulus for the lower SS curve for ligament and skin in uniaxial tensile testing was mainly due to elastin fibers and the higher modulus on the steep part of the curve to be due to collagen fibers. This concept was partly supported by Daly (1969), who showed that skin treated with elastase has a steeper SS toe portion. Later work by Stevens et al. (1974) confirmed this finding. These workers used highly purified elastin fibers of adult bovine ligamentum isolated in three ways (enzymatic digest, alkaline and formic acid pretreatments). The purity of these elastin fibers were controlled by amino acid analyses. Mechanical stretching and electron microscopy were performed simultaneously. Mechanical testing of the intact ligamentum nucleae showed two distinct regions during extension; the first was a reflection of elastin fibers while the second reflected collagen fibers. By subjecting each of these stretched muscle strips to digestion with either elastase or collagenase, specific slopes contributed by either elastin or collagen were generated. It should be emphasized that the elastin or collagen slope does not indicate the amount or degree of cross-linkages of elastin or collagen in the tissue under examination. Biochemical quantifications have to be performed to determine the concentration of these proteins.

Very little is known about the elements which contribute to the ground substance of the tissue. Consequently the middle portion of the SS curve which reflects these elements is difficult to interpret. Kinedi et al. (1965) emphasized the significance of the geometrical orientation of the ground substance to the elastin fibers in SS relationships. Vidik

(1969) formulated that viscosity of the tissue is partly influenced by some fraction of this component. Jackson (1969) indicated that glycoaminoglycans contributed to the cohesion of the fibrillar structure. Not all the glycoaminoglycans involved are known. Biochemical analyses of the interfibrillar substances have yielded conflicting results with respect to SS relationships (Elden, 1970).

Factors influencing the components of the SS curve - lipids

Elastin The eventual loss of elastic tissue function appeared to be the resultof a gradual uptake of lipid by the elastic lamellae followed by enzymatic fragmentation and solubilization (Robert et al., 1972; Szigeti et al., 1972). Lipid deposition near or around the elastic fiber of the aorta was shown using labeled cholesterol in human arteriosclerotic patients (Kramsch et al., 1971). Similar findings in skin and aorta were reported with rats fed normal and high cholesterol diets (Szigeti et al., 1972). Increased lipid content in skin elastic fiber was demonstrated in aged and arteriosclerotic patients (Bouissou et al., 1973). In rat aorta and sponge tissue, the total amount of radioactive cholesterol incorporated into the insoluble fibrous fraction, containing structural glycoproteins and elastin, depended on the cholesterol content of the diet (Jacotot et al., 1973). Kramsch et al. (1974) interpreted the accumulation of lipid at fragmented internal elastica as early manifestations of atherosclerosis. The extent of fragmentation cannot be quantitated at present.

Elastin is capable of binding large quantities of lipid (Kramsch et al., 1971; Kramsch and Hollander, 1973). Normal arterial and lung elastin, even after alkali-extraction, contained 1-3% lipid. Elastin from plaques

from atherosclerotic patients contained as high as 37-40% lipid (Kramsch et al., 1971; Kramsch and Hollander, 1973). The increase in lipid content was attributed to large increases in cholesterol (80 fold increase), especially cholesterol esters, with minor changes in phospholipid and tryglyceride levels. The phospholipid-cholesterol (P/C) ratio declined with increasing severity of atheroma (Kramsch et al., 1971). This P/C ratio in aortic plaques of younger subjects was similar to that of normal aorta in older subjects. An abrupt fall in P/C ratio, due to an elevation of cholesterol, occurred in the plaques of older patients (Adams and TuQan, 1961). It had been argued that the binding of lipid to elastin in plaques was in fact due to an altered form of elastin. Kramsch et al. (1974) demonstrated the presence of lipopeptides in plaque elastin. These peptides usually appeared as products when alkali-insoluble elastin was treated with elastase. However, the origin of these peptides, that is, whether or not they originated from glycoprotein, collagen or elastin, remained unclear.

Several mechanisms have been proposed for the association of elastin and lipid. Beta-lipoproteins appeared to be the main vehicles responsible for lipid and cholesterol entry into the arterial wall (Sandberg et al., 1969; Gero et al., 1961; Jacotot et al., 1971). Their passage through the endothelium was demonstrated biochemically and radioautographically (Stein and Stein, 1971; 1972). The fragile ULDL and LDL lipoproteins were capable of squeezing through elastic laminae, which possessed changeable pore sizes depending on pH and other conditions (Robert et al., 1972). During their passage, the β-lipoproteins were irreversibly deformed and denatured, leading to separation of the lipid and peptide components by

separation. The presence of such peptides was demonstrated using immunofluorescent techniques (Walton and Williamson, 1968; Stein and Stein, 1972). The lipids liberated could react with elastin by intercalating in the hydrophobic peptide folds via hydrophobic stacking (Robert et al., 1972) or with the intercellular matrix.

The role of other macromolecules in lipid deposition has also been considered. Bihari-Varga et al. (1968) and Gero et al. (1961) reported that proteoglycans could interact with lipoproteins through their acidic polysaccharide chains. Consequently lipoproteins were denatured, and deposited in situ. As proteoglycans of the heparin-sulphate and dermatan-sulphate type occurred in the arterial wall (Richard, 1962), the first and final phases of lipid deposition were facilitated. Structural glycoproteins from pig aortas had 20-40% of the total dry weight as lipid, even after rigorous delipidation (Moczar and Robert, 1970). These lipids might arise, at least partially, from cell membranes.

A mechanism has been proposed for the deposition of lipid into elastic fiber, built on the hydrophobic property of elastin. A schematic diagram of the process is shown in Figure 10. Lipids settle in the hydrophobic folds (hydrophobic stacking) of elastin. The fiber becomes distended and deformed, thus exposing susceptible peptide bonds for proteolytic cleavage. Elastolytic enzymes were shown to exist in leukocytes (Janoff, 1972) and blood platelets (Robert et al., 1970; 1971). Cathepsins were shown to be affiliated with lipoproteins (Robert et al., 1972). Other tissue elastases produced in situ might also be present. These enzymes could be responsible for the slow degradation of the elastic fiber.

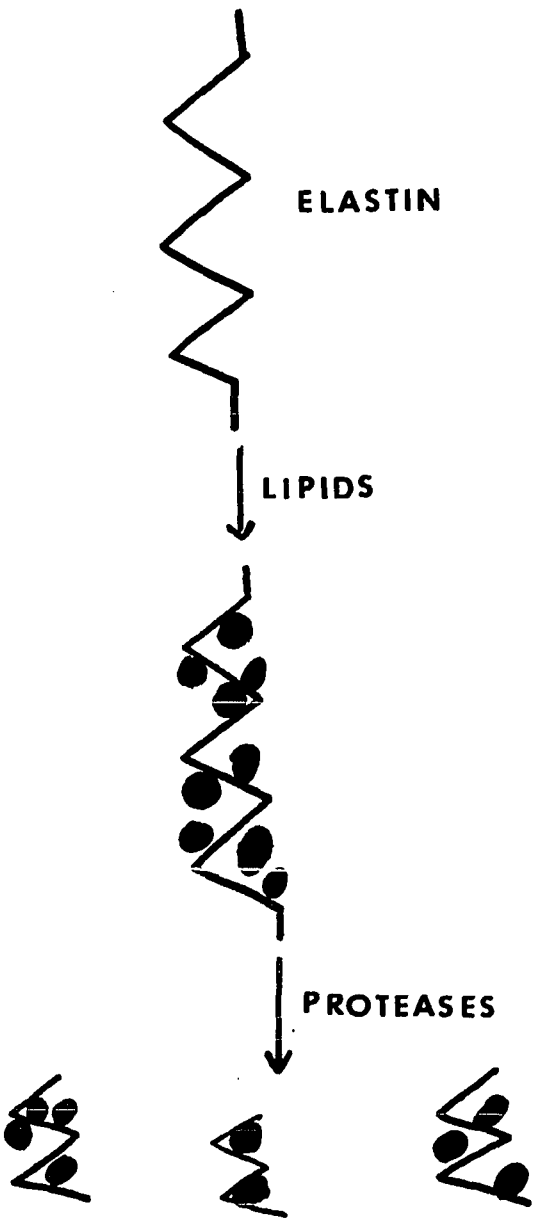


Figure 10. Elastic fiber was represented as a peptide spring which became deformed and denatured by the insertion of lipid molecules within the hydrophobic peptide folds. Consequently the molecules became increasingly exposed to proteolytic digestion (adapted from Jacotot et al., 1971).

With a loss in the integrity of the hydrophobic interaction of the peptide chains of elastin, a gradual loss in elasticity resulted from this lipid accumulation (Robert and Poullain, 1966). An increase in the tangential dynamic of Young's modulus occurred in rabbit aorta when high levels of cholesterol were given (Band et al., 1973). Similar alterations occurred in cholesterol-fed chicks (Newman et al., 1971).

Collagen Collagen, like elastin, possesses similar affinity to bind lipids, especially cholesterol. The accumulation of lipid into collagenous fiber might proceed at a slower rate than into elastin, though the mechanism of lipid infiltration was similar to that of elastic tissues (Nikkari and Heikkinen, 1968).

The alterations in connective tissue as a result of elevated cholesterol intake, seemed to be mediated by cholesterol itself. Diets high in fat, particularly in saturated fats, which may induce elevated levels of circulating and tissue cholesterol and other lipids, could affect connective tissue via a mechanism similar to that of a cholesterol-rich diet. On the other hand, the influence of polyunsaturated fatty acids (PUFA) on elastin and collagen seemed to be more subtle. Their action has been associated with that of prostaglandins (PG) (Struyk et al., 1966; Vergroesen, 1972). Coronary flow was stimulated by PGF_1 and PGF_2, while with certain cation ratios (K-Ca) $PGF_{1\alpha}$ and $PGF_{2\alpha}$ could stimulate the contractile force in isolated frog and rat heart (Vergroesen and De Boer, 1968). The influence of dietary linoleic acid on PG biosynthesis was studied by Hoor and deDeckere (1976). Preliminary data (Hoor et al., 1973) indicated a 50% ($P<0.01$) decrease of maximum contraction force in isolated papillary muscles from rats fed EFA-deficient diets compared with

that of rats fed 5% of calories as sunflower seed oil (5SSO). Rats on a 2 week, fat-free dietary regimen revealed a 60% decline in papillary muscle contractile force compared to controls fed 10% of calories as SSO (10SSO). Coronary flow in isolated perfused hearts of animals on 50% of calories as sunflower seed oil, soybean oil (SO), olive oil (OO), hydrogenated soybean oil (HSO) or lard for 6 days showed a dependency on the type of dietary fat. Coronary flow rates with the different diets were in decreasing order SSO > SO = HSO = OO > lard (DeDeckere and Hoor, 1975). Left ventricular function of rats consuming 50% of calories as SSO was greater than those on mixed fat diets of either 45% of calories as HSO + 5% of calories as SSO or 45% of calories as HCO + 5% of calories as SSO, respectively (Neely et al., 1967; Hoor and deDeckere, 1976). These effects were achieved regardless of length of feeding period (3 days vs. 21 days). These results indicated that elevating the amount of dietary linoleic acid led to enhanced coronary flow and left-ventricular function, suggesting a function of linoleic acids involving PG synthesis. The underlying mechanism awaits elucidation.

Age

Changes of connective tissues with age showed several common features, namely: cell death (Holliday and Tarrant, 1972), increased catabolic activity (Hjertquist and Wasteson, 1972), increase in cross-linking (Bailey, 1965), lipid infiltration (Beaumont et al., 1963; Kramsch et al., 1971; Szigeti et al., 1972), calcification (Ouzilou et al., 1974) and changes in immune response (Walford, 1969).

Elastin In a 50-week study (Berry et al., 1972; Looker and Berry, 1972), chemical and microscopic determinations indicated increases in

total elastin content with increasing age in male rat aorta. Previous work by Kao et al. (1961) had indicated negligible elastin turnover in rat aorta. Incorporation of ^{14}C-lysine into polymeric elastin of rabbit aorta suggested continued synthesis with little exchange or turnover. Cross-linkages in elastin also increased with aging. In aging skin, Kenedi et al. (1965) found the "toe" part of the SS curve to diminish, while Jansen and Rottier (1957) failed to observe change. In aging humans, arterial elastin degenerated and became replaced by collagen fibers (Leoroy and Taylor, 1966). In contrast, Miller and Perkins (1927) reported an increase in elastic tissue with age in human cardiac ventricles. Fahr (1906) found no variation in elastic fiber quantity in human atria. Lev and McMillan (1961) found no significant increase in elastin and collagen in the ventricles.

Collagen The literature had yielded inconsistent reports on ventricular collagen change with age. Findings of decrease during the first 10-30 years of life (Wegelius and Knorring, 1964), increase (Clausen, 1962) and no variation (Blumgart et al., 1940; Lenkiewicz et al., 1972) in human hearts had been reported. Biochemical quantitation showed that collagen amounted to about 1% of wet weight of heart tissue (Blumgart et al., 1940; Clausen, 1962).

With increasing age, soluble collagen and glycoaminoglycans decreased, whereas insoluble collagen and the collagen/glycoaminoglycan ratio increased (Vogel, 1974). In rat skin, a correlation of tensile strength with either the amount of insoluble collagen or total hydroxyproline at various age intervals was reported.

Methods and Materials

Design of experiment

Two experiments, 2 and 3, were designed. In Experiment 2 the effects of dietary variations (fat level, fat saturation, frequency of feeding and length of feeding) on cardiac function were studied. Except for the omission of the 60 day feeding period, the design was identical to that used in Experiment 1, Section I. The design of Experiment 2 is shown in Figure 11. Stress-strain response was used as an index of cardiac function.

In order to correlate cardiac function to morphology, Experiment 3 was designed. The histopathology of the myocardial cell in the right ventricle and ventricular septum was examined by electron microscopy. The design is like Experiment 1 with only one time period and one frequency of feeding. Design of Experiment 3 is shown in Figure 12.

Animal treatment Animal treatment was as described in Experiment 1. In Experiment 2, two feeding periods were used. Rats were put on dietary regimens for either 30 or 90 days. In Experiment 3, littermates in sets of three were assigned to three diets. Only one feeding frequency (MF) and feeding period (90 days) was used. In Experiment 2 rats were killed after 30 and 90 days on the diet. They were lightly etherized then paralyzed by cervical dislocation. The heart was removed, washed, dried on filter paper, weighed and stored immediately in sealed plastic pouches at -20°. Cardiac stress-strain curves were run a week later.

In Experiment 3 animals were guillotined on day 31 between 8-9 AM. Heart was excised by severing the major vessels. When contraction ceased, sections of the right ventricle and ventricular septum were taken.

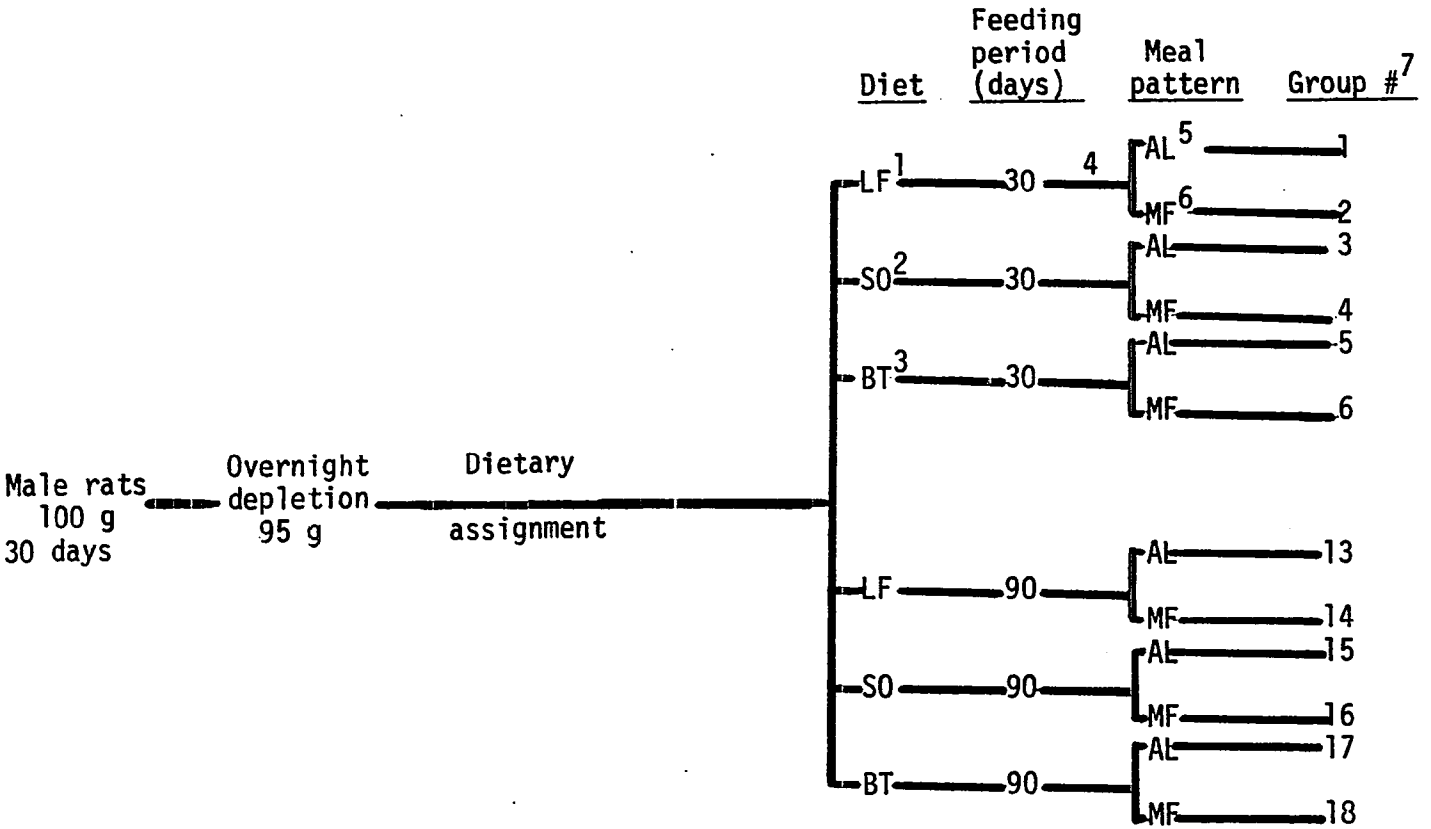


Figure 11. Schematic design of experiment 2: 1 = 2% safflower oil as calories; 2 = 38% safflower oil as calories; 3 = 38% beef tallow as calories; 4 = littermate; 5 = ad libitum (24 hr feeding out of a 24 hr period); 6 = meal fed (3 hr feeding out of a 24 hr period); 7 = 10 rats per group.

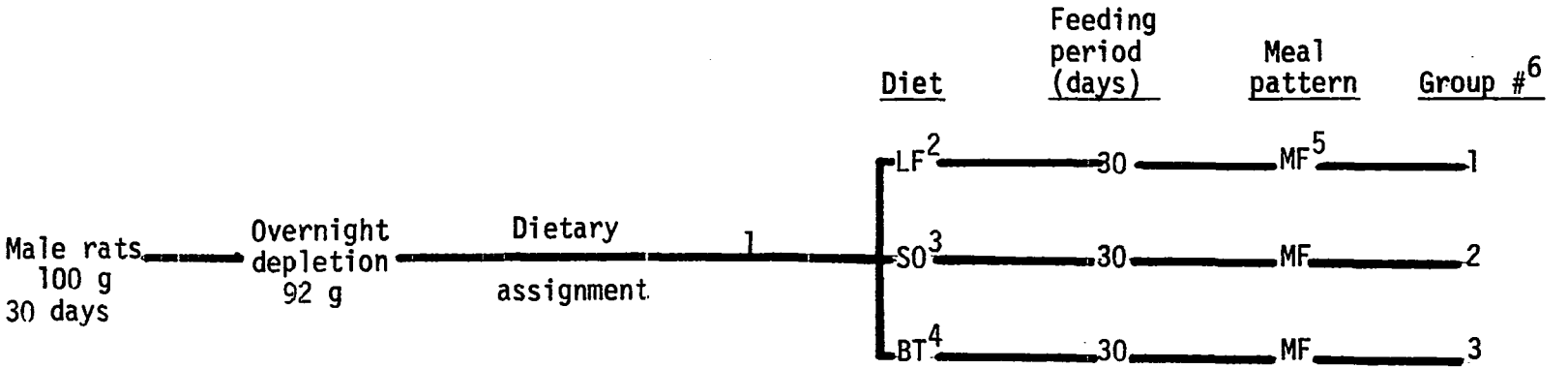


Figure 12. Schematic design of experiment 3: 1 = littermates in sets of three, each assigned to a diet; 2 = 2% safflower oil as calories; 3 = 38% safflower oil as calories; 4 = 38% beef tallow as calories; 5 = meal fed (3 hr feeding out of a 24 hr period); 6 = 5 rats per group

Procedures

Stress-strain measurement

Tissue preparation Hearts were thawed at 4°, then brought to room temperature in rat Kreb-Ringers solution (Table 18). This solution was prepared fresh weekly and stored at 4°. The tissue was mounted on a soft cork board for sectioning. A representative longitudinal base to apex rectangular strip was removed from the ventricular septum. Removal procedures were as follows:

(1) remove atria

(2) remove right ventricular wall with a wide U cut along the sides thus exposing the septum

(3) cut left ventricle into longitudinal halves, then pinch down the flaps

(4) section a thin rectangular strip of septum with a double-bladed knife. Blade separation was set at 0.5 cm. While sectioning, equal pressure to the knife was applied by pressing both blades simultaneously. This prevented tearing along the muscle edges.

(5) record initial length, width and thickness of the unstretched septum with a ruler.

Uniform sectioning was extremely critical in order to reduce muscle orientation difference which would affect stretching behavior.

Stress-strain (SS) curves The instrument used to obtain stress-strain curves consisted of a microdirect-drive manipulator[1] controlled by

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[1] D. Koft Instrument, Model 1207B, Chicago, Illinois.

Table 18. Kreb-Ringers Solution

	%	gm	volume(l)	Solution
NaCl	0.90	18	2	1
KCl	1.15	23	2	2
$CaCl_2$	1.22	12.2	1	3
KH_2PO_4	2.11	21.1	1	4
$MgSO_4.7H_2O$	1.85	38.2	1	5
$NaHCO_3$	1.30	13.0	1	6

Kreb-Ringers stock concentrate

500 mls solution 1

20 mls solution 2

15 mls solution 3

5 mls solution 4

5 mls solution 5

Store frozen for 1 month.

Kreb-Ringers dilute solution

200 mls stock concentrate

32 mls solution 6

928 mls distilled water

Store $0\text{-}4^{0}$ for 1 week

a master unit.[1] This provided the mechanical force for stretching the muscle. The latter was mounted in series with a glass force-displacement transducer coupled with a recorder.[2] The muscle was immersed completely in Krebs-Ringers solution in a 3" x 4" x 3" Plexiglas chamber. Chamber temperature was maintained at 38° by a water bath[3] equipped with a circulating pump and a heat exchanger. Temperature in the chamber was checked periodically with an electrical thermistor. The muscle ends were attached between a lower stationary clamp and a movable upper clamp set in series with the transducer. The transducer was mounted on a moving platform driven by the manipulator. A signal generator was used to produce precise increments of stretch in microns. The transducer-recorder system was calibrated before each stretching period with a 30 g weight. This gave a pen deflection of 6 divisions on the chart paper. The baseline of the SS curve was taken at the point of zero strain. This implied that the muscle was under negligible stress. Three successive curves were generated for each muscle. At a paper speed of 50, each curve took 8-10 minutes to complete. The first curve was discarded. The second and third curves were superimposed on a light box. Superimposibility was taken as the criterion for uniform experimental conditions, e.g., clamp tightness, paper speed, muscle integrity, bath temperature, etc. The second curve was used to provide data for muscle elongation under given tensions. Each curve was considered complete when either the pen deflection exceeded

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[1] D. Koft Instrument, Model 1207S, Chicago, Illinois.

[2] Bausch and Lomb, Rochester, New York.

[3] Haake, F. J., Chicago, Illinois.

maximum chart space or when the tissue was torn. A calibration constant in μ/cm was taken for each curve. At the end of each experiment an average of all the calibration constants was taken. This value was used to compare instrumental variability from day to day.

Final length, width and weight of the stretched muscle were measured. Final length was taken as the inner distance between the clamp marks. Volume of the septum was determined by liquid displacement using a picnometer with a thermometer affixed to the stopper.

Corrected SS curves Each curve traced out the length elongation pattern (strain) of the muscle under given loads (stress). This was a SS curve unadjusted for cross-sectional area and length difference. To obtain cross-sectional area, the volume of the sample was divided by its length. To adjust for length, an elongation % was used. The actual strain became:

$$\frac{\text{elongated length (l)}}{\text{original length (lo)}} \times 100$$

Thus a new plot of stress (g/cross-section area = cm^2) against strain (l/lo x 100) was made for each muscle.

From each SS curve the following parameters were evaluated:

(1) initial slope representing elastin slope (ES)

(2) final slope representing collagen slope (CS)

(3) area under the arc, representing matrix (M)

A composite SS curve (CSS) for each treatment group was also plotted. This curve represented an average of all the SS curves in a treatment group. Since there were 12 dietary treatments, 12 CSS curves were obtained. By using similar scales for the X and Y ordinants for each CSS a

fast way of comparing cardiac distensibility between treatments was developed. A muscle became more or less distensible if its CSS was shifted right or left with respect to another CSS from another group.

Collagen quantitation

Collagen in most tissues is the only major source of hydroxyproline. Quantitation of hydroxyproline in experiment 2 yielded an indirect assessment of collagen content in the heart. The method of Klein (1970) using ion-exchange chromatography was adopted. This method was based on the chromatographic separation of amino acids on sulfonated polystyrene cationic exchange resins (Moore and Stein, 1969). Hamilton and Anderson (1969) had demonstrated that hydroxyproline was the first unmodified amino acid to be eluted when citrate buffer (pH 2.9) was used. The separation from other amino acids was clean, fast and specific, thus permitting the use of any colorimetric procedure for hydroxyproline.

Tissue hydrolysates

Septa in each dietary treatment were pooled. They were minced on a watch glass and about 0.8-1 g of tissue was weighed on glassine paper. The tissue and 10 ml of 6 N HCl in a 20-ml screw-capped Kimex culture tube were placed in boiling water for 1 hr or until the tissue was dissolved. The mixture was autoclaved for 3 hr at 15 lb pressure and 120°. The acid hydrolysate was evaporated over a rapid steam bath under a well-ventilated hood. A piece of moist pH paper was used to check acidity. Presence of HCl in the hydrolysate could also be detected easily by its characteristic pungent odor. When the hydrolysate was relatively free of detectable acid, it was dried and reconstituted with 4 ml of 0.1 M citric buffer (pH 2.90±.02). Black precipitate in the hydrolysate was removed by

filtration over a slow-flow filter paper (Whatman #2) or by centrifugation at 2,000 rpm for 5 minutes. Supernatant was remade to 4 ml with citric buffer. A 1 ml aliquot was layered on the column. Remaining supernatant, when well-covered, could be stored indefinitely at 4° without loss of hydroxyproline.

Columns

Packing Twelve glass columns in the specifications described by Klein were set up. Dowex-50 cation exchange resin[1], 200-400 mesh, 8% cross-linked was washed twice with water and 0.25 N NaOH in alternation. The moist slurry was poured with stirring into the columns. Each column was filled to designated heights in less than two additions. This prevented later cracking of the resin. The packed columns were washed with 1 volume 0.25 N NaOH. Before each use the columns were regenerated by adding 0.2 M citric buffer (pH 2.90) until the eluant pHydrion paper[2] turned the same color as the citric buffer. Columns could be left indefinitely in 0.25 N NaOH, or for 18 hrs in 0.2 M citric buffer, pH 2.90.

Calibration All twelve columns were calibrated simultaneously. Before the addition of the standards, top resin was flattened with the back of a transfer pipet. This provided a uniform surface for sample to sink through at equal rate. One ml calibrating hydroxyproline stock solution (100 µg/ml) was layered with a pipet, which was rinsed twice with buffer. When all the standard had been taken up by the resin the column was filled to the top with 0.2 M citric acid (pH 2.90). The first 20 ml

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[1]Sigma Chemical Co., St. Louis, Missouri.

[2]Micro Essential Laboratory, New York.

of eluant was discarded. Ten 5 ml fractions were collected in 12 ml centrifuge tubes. A 10 ml fraction was collected thereafter. Hydroxyproline was measured colormetrically by the method described by Klein.

Colorimetric measurement

To 2 mls of sample or standard in a 12 ml centrifuge tube was added 1 ml of freshly prepared 0.015 M $CuSO_4$-15% NaOH mixture. Contents were mixed. Subsequently, 0.5 ml of 8.4% $H_2O_2$was added and the mixture thoroughly mixed for 5 minutes to complete hydroxyproline oxidation. This was followed by incubation in a water bath at 70° for 10 minutes with constant agitation to destroy excess H_2O_2. Two and half ml Ehrlich's reagent was added and the tubes returned to the bath for 25 minutes. Tubes were cooled and optical densities were read within 1 hour at 540 nm on a spectrophotometer[1] with a digital readout system.[2] Standards containing 2, 4, 8, 12 or 16 mcg hydroxyproline per 2 ml solution were assayed simultaneously.

Hydroxyproline was eluted in the fractions collected between 50 and 100 ml. Overall % recovery was 94.1. Elution time was about 20 ml/45 minutes.

Samples One ml of sample was layered as described earlier. The first 50 ml were discarded, and the following 50 ml were collected in a 50 ml graduate cylinder. An additional 10 ml was collected in a 25 ml Erlenmeyer flask. This fraction was reserved in the event that hydroxy-

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[1]Beckman D.U. spectrophotometer, model 2400, Beckman Instruments, Fullerton, California.

[2]Update Inc., Madison, Wisconsin.

proline elution was delayed. This happened when one or more of the following had occurred:

(1) the resin was left dry in the column for too long

(2) the resin was used repeatedly without consistent and thorough washing with 0.25 N NaOH

(3) the tissue hydrolysate was too acidic, i.e., HCl evaporation incomplete

(4) pH of the column or citric buffer was below 2.90±0.02

Elastin quantitation

Elastin was quantitated by the method of Newman and Logan (1950). Pooled septa from each dietary treatment was used. Results for collagen and elastin amount were expressed as % hydroxyproline in each fraction.

Electron microscopy

Muscle sections from the right ventricle and ventricular septum were fixed for 3 hrs in Karnowski para-formaldehyde glutaldehyde mixture. They were stored overnight in isotonic Millinigs phosphate buffer (pH 7.2-7.4), later post fixed with osmium tetroxide. Sections were embedded in epon-araldite mixture and dehydrated by a graded series of acetone (25, 50, 70, 95 and 100%). After resins had polymerized, sections were cut and stained with uranyl-acetate in methanol. Post-staining with lead citrate followed. Photographs were obtained using an electron microscope (RCA EMU-4).

Statistical Analysis

Statistical analysis was done as for experiment 1, using an unweighted analysis of variance for overall group comparisons, and a t-test between dietary treatments.

Results

Two experiments, 2 and 3, were conducted. Experiment 2 assessed cardiac functional alterations. Experiment 3 focused primarily on morphological cellular changes of the ventricular myocardium utilizing electron microscopy. Variables for each of the experiments were given in the procedural section.

Body weight, weight gain, food intake and food efficiency

Data for body weight, weight gain, food intake and food efficiency for Experiment 2 only are given in the Appendix in Table 23. Results from these parameters confirmed the findings of Experiment 1 with similar diets and meal patterns as variables. General trends for all parameters were consistent in corresponding treatment groups of the two experiments. Data pertaining to cardiac function obtained in Experiment 2 are therefore applicable to corresponding groups in Experiment 1.

Cardiac weight (Table 19)

Cardiac weights were expressed in g and g/100 g body weight (CW/BW). Cardiac weights expressed in either absolute or relative terms were unaffected by dietary fat concentration. However LF-fed groups tended to have lower cardiac weights than those on high-fat diets. Values were 1.25 vs. 1.40 g. Such trend was not observed in the CW/BW ratio (39 vs. 39) between the two fat levels.

Cardiac weights for SO groups were lower than those of BT groups ($p<.05$). This was seen in both the 30- and 90-day treatments. The CW/BW values for both groups were not different.

Table 19. Cardiac weights

	30 days		90 days	
	g	g/100gBW x10^{-2}	g	g/100gBW x10^{-2}
LF-AL	1.16±.04[a]	38±1	1.58±.08	30±1
LF-MF	0.87±.04	40±2	1.40±.05	32±2
SO-AL	1.25±.03	40±1	1.58±.05	32±1
SO-MF	1.10±.03	39±1	1.46±.08	31±2
BT-AL	1.29±.03	38±1	1.74±.07	33±1
BT-MF	1.14±.03	40±1	1.63±.06	34±1

ANOVA

	g	g/100gBW
Fat level	NS	NS
Fat saturation	p<.05	NS
Age	p<.01	p<.05
Meal pattern	NS	NS

[a]Mean ± SEM.

With age, there was a concomitant increase in cardiac weights ($p<.01$) and a decrease in CW/BW values ($p<.05$) since rates of body weight increase exceeded those of cardiac weight. Between the two time periods, cardiac weight increased by approximately 38%, while body weight increased by 69%. This implies that cardiac weight gain might be proportional to gain in lean body mass.

Meal-fed animals, because of smaller body weights, had lower cardiac weights compared to ad libitum-fed controls. Consequently CW/BW values for both patterns were similar.

Ventricular thickness (Table 20)

A variable mean of 0.21 cm was obtained regardless of amount of fat consumed. Consequently fat level did not affect ventricular thickness. A similar effect was seen with varying fat saturations. Mean values were 0.21 cm and 0.22 cm for SO and BT groups, respectively. However, there was an increase in ventricular thickness with age for all treatments ($p<.05$). Meal-fed rats had thinner ventricular walls than ad libitum-fed controls ($p<.01$).

Stress-strain curves

A model stress-strain response curve is shown in Figure 13. The stress-strain (SS) curve for each septum was normalized for length and cross-sectional area. For each group a composite stress-strain (CSS) curve, representing the group average, was constructed. Since 12 treatment groups were involved, 12 CSS curves were generated (Figures 14-19).

Table 20. Ventricular septal cross-sectional area, thickness, length and weight

	30 Days				90 days			
	Cross-sect. cm^2	Thick-ness cm	Length cm	Weight g	Cross-sect. cm^2	Thick-ness cm	Length cm	Weight g
LF-AL	0.095	0.21	0.99	0.120	0.086	0.25	0.97	0.101
LF-MF	0.089	0.17	0.99	0.092	0.091	0.19	0.98	0.110
SO-AL	0.110	0.20	0.97	0.105	0.100	0.28	0.99	0.090
SO-MF	0.098	0.17	0.92	0.099	0.103	0.19	0.99	0.101
BT-AL	0.126	0.20	0.99	0.120	0.116	0.29	1.01	0.096
BT-MF	0.102	0.18	1.01	0.102	0.120	0.19	0.99	0.091

ANOVA

Thickness

Fat level	N.S.
Fat saturation	N.S.
Aging	$P<.05$
Meal pattern	$P<.01$

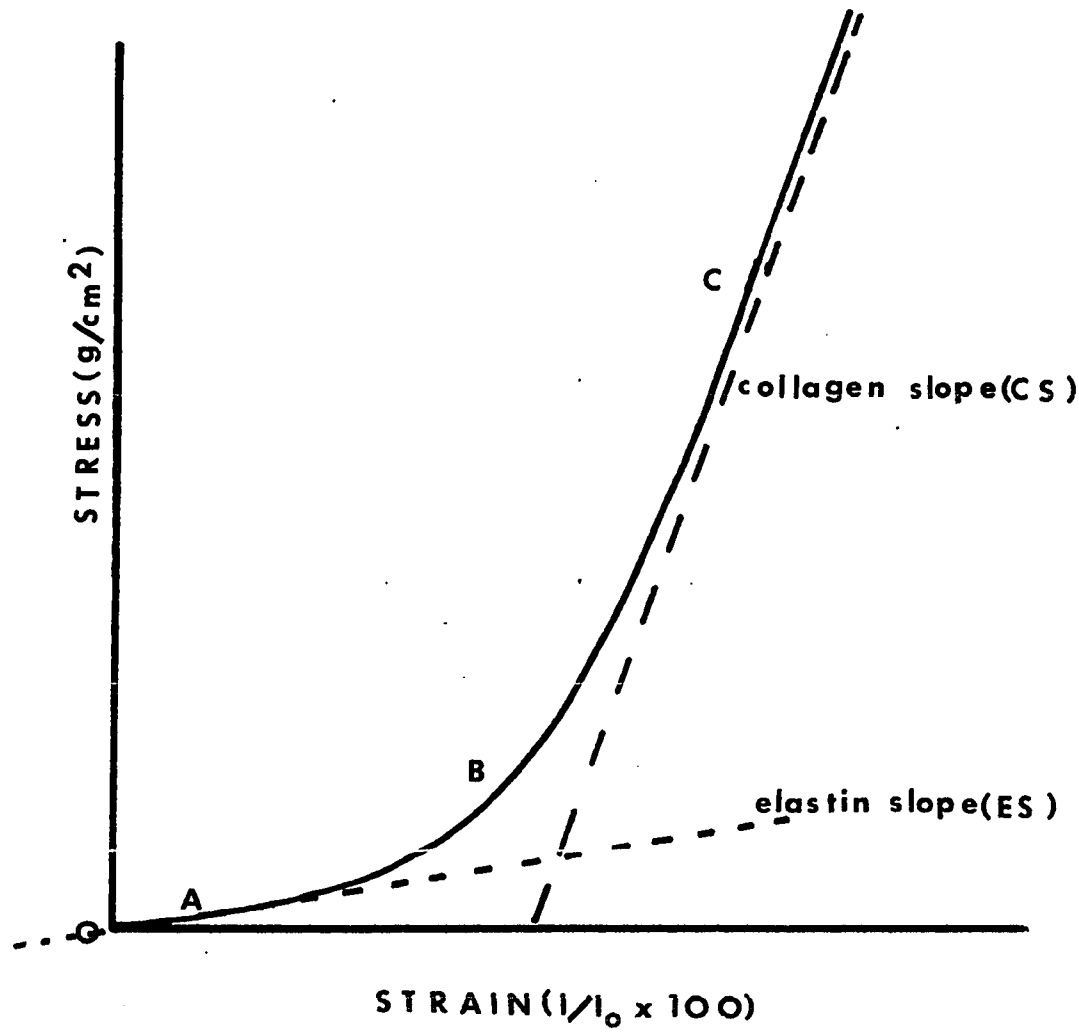


Figure 13. A model stress-strain response curve. Stress is expressed as g/cm^2, strain as $\frac{\text{elongated length(l0}}{\text{original length (lo)}}$ x 100. Portion A of the curve is influenced predominantly by elastin, portion B by the ground substance (matrix) and portion C by collagen.

The following basic rules were employed in analyzing CSS curves. A shift in a curve to the left with respect to a designated control was considered to be less distensible or stiffer. Conversely, a shift to the right with respect to the control was considered to be more distensible or less stiff.

Each curve was divided into three regions, namely initial (elastin), middle (matrix) and final (collagen). The slopes at the initial and final regions were measured (Figure 13). A displacement of a stress-strain curve to the right of a control curve (i.e., the curve was more distensible than the control curve) could be the result of one or several of the following: a decrease in elastin slope, an increase in the length of the elastin region, a decrease in collagen slope, and finally a decrease in matrix slope. Likewise, when the curve displacement was to the left of control, the opposite events occurred.

Data for the matrix portion are not presented because of theoretical uncertainties related to curve analysis.

Composite stress-strain curves (Figures 14-19)

The influence of dietary fat level on the position of composite stress-strain (CSS) curves appeared to depend on the fat source. When dietary fat level was elevated, the CSS curve of the high fat (SO + BT) groups was shifted to the right of the LF CSS curve (Figure 14). However, this displacement was influenced primarily by group SO and not BT. The CSS curves from group BT appeared to be similar to those of group LF based on very small shift differences. The pattern of shift between these groups, however, was not consistent, e.g., CSS curves from groups

Figure 14. Composite stress-strain curves with fat level as a variable. Y axis represents stress (force/cross-sectional area = g/cm^2) and X axis represents strain (elongation %). Animals were fed for 3 (MF) or 24 hours (AL) per day, diets containing as % calories either 2% safflower oil (LF), 38% safflower oil (SO) or 38% beef tallow (BT) for 30 and 90 days.

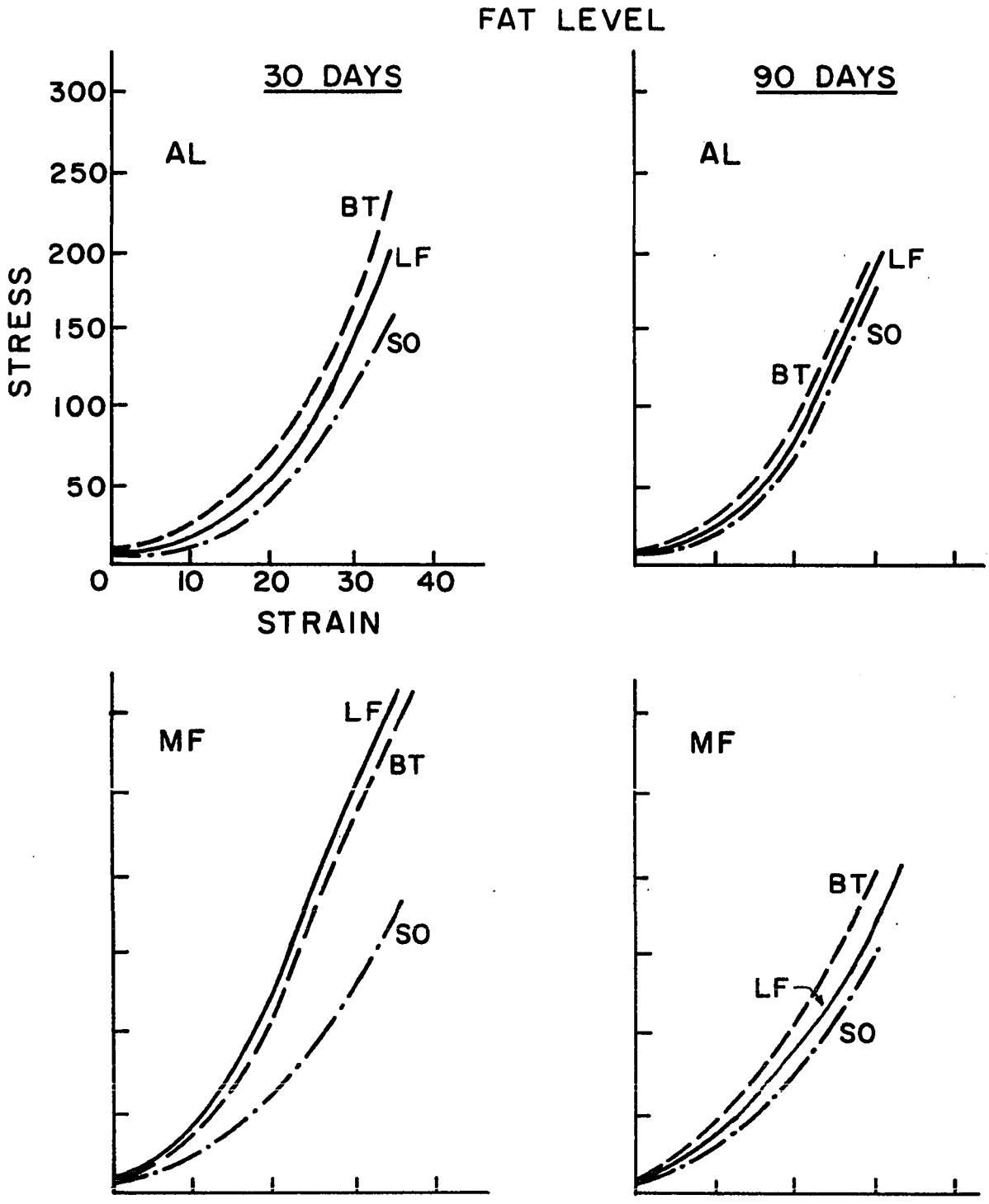

FAT LEVEL
30 DAYS
90 DAYS
AL
AL
MF
MF
BT
LF
SO
STRESS
STRAIN
300
250
200
150
100
50
0
10
20
30
40

Figure 15. Composite stress-strain curves with fat saturation as a variable. Y axis represents stress (force/cross-sectional area = g/cm^2) and X axis represents strain (elongation %). Animals were fed for 3 (MF) or 24 hours (AL) per day, diets containing as % calories either 2% safflower oil (LF), 38% safflower oil (SO) or 38% beef tallow (BT) for 30 and 90 days.

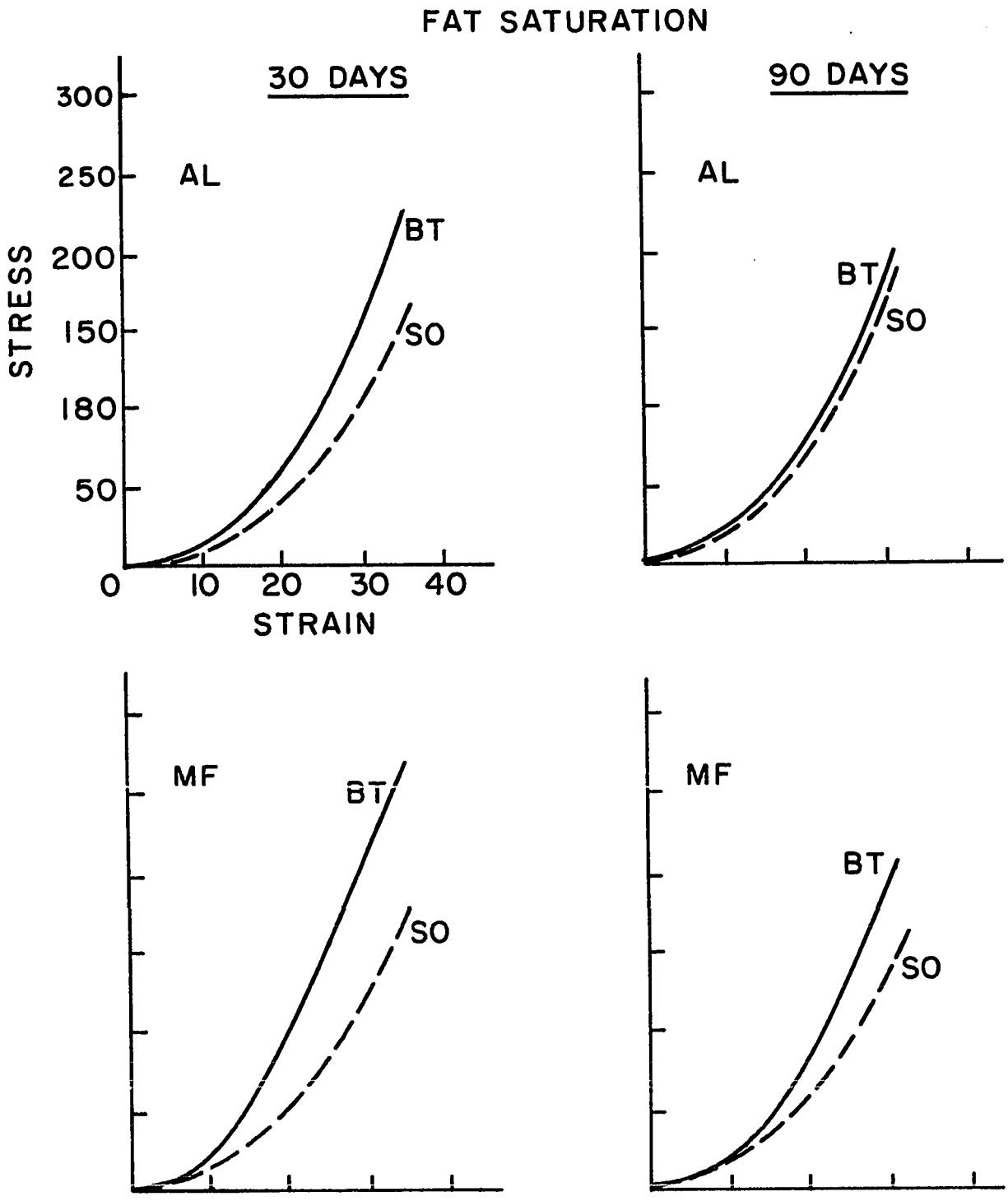
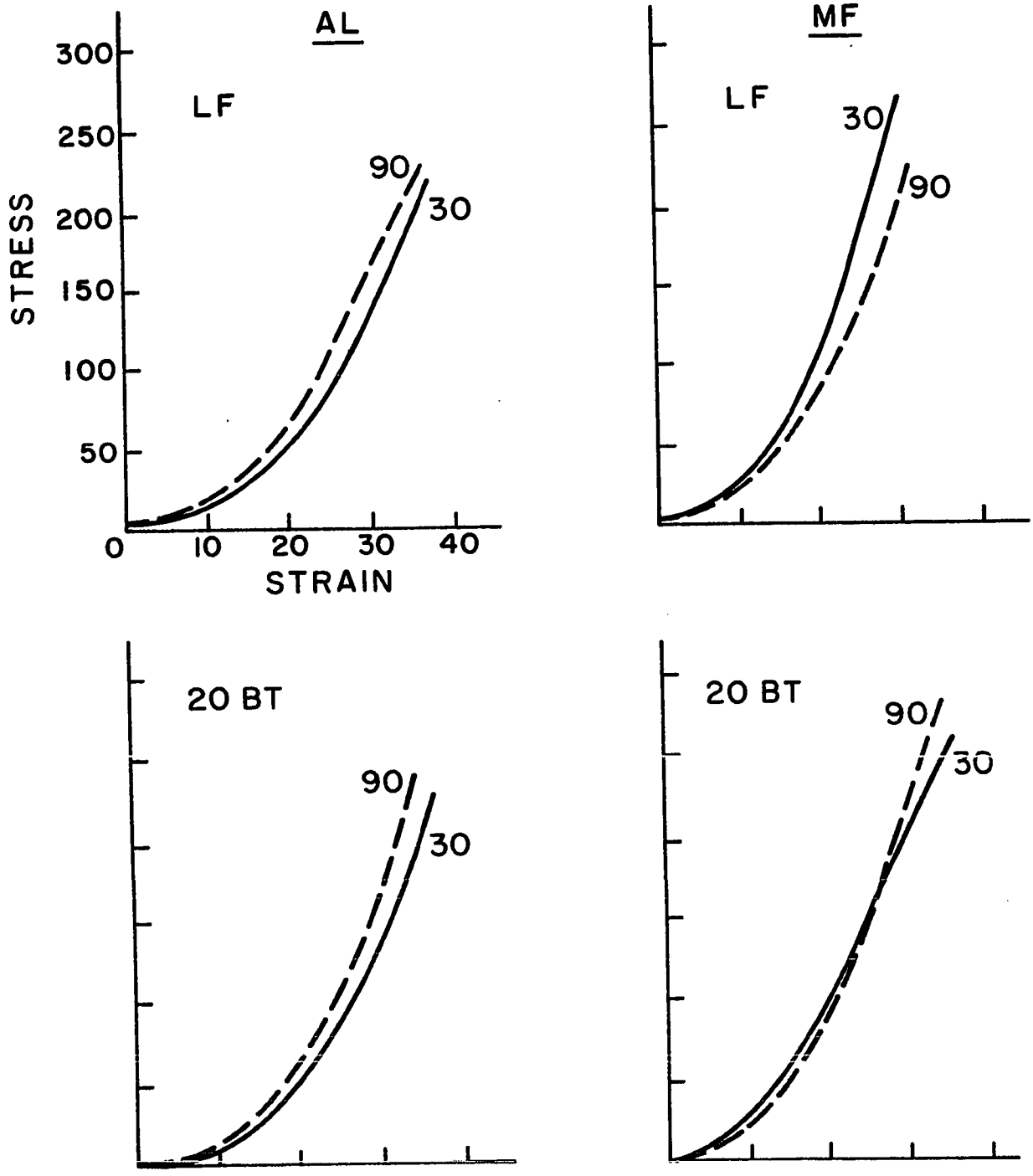

FAT SATURATION
30 DAYS
90 DAYS
AL
AL
MF
MF
BT
SO
BT
SO
BT
SO
BT
SO
STRESS
300
250
200
150
180
50
0
10
20
30
40
STRAIN

Figure 16. Composite stress-strain curves with aging as a variable. Y axis represents stress (force/cross-sectional area = g/cm^2) and X axis represents strain (elongation %). Animals were fed for 3 (MF) or 24 hours (AL) per day, diets containing as % calories either 2% safflower oil (LF), 38% safflower oil (SO) or 38% beef tallow (BT) for 30 and 90 days.


AL
LF
90
30
STRESS
300
250
200
150
100
50
0
10
20
30
40
STRAIN
MF
LF
30
90
20 BT
90
30
20 BT
90
30

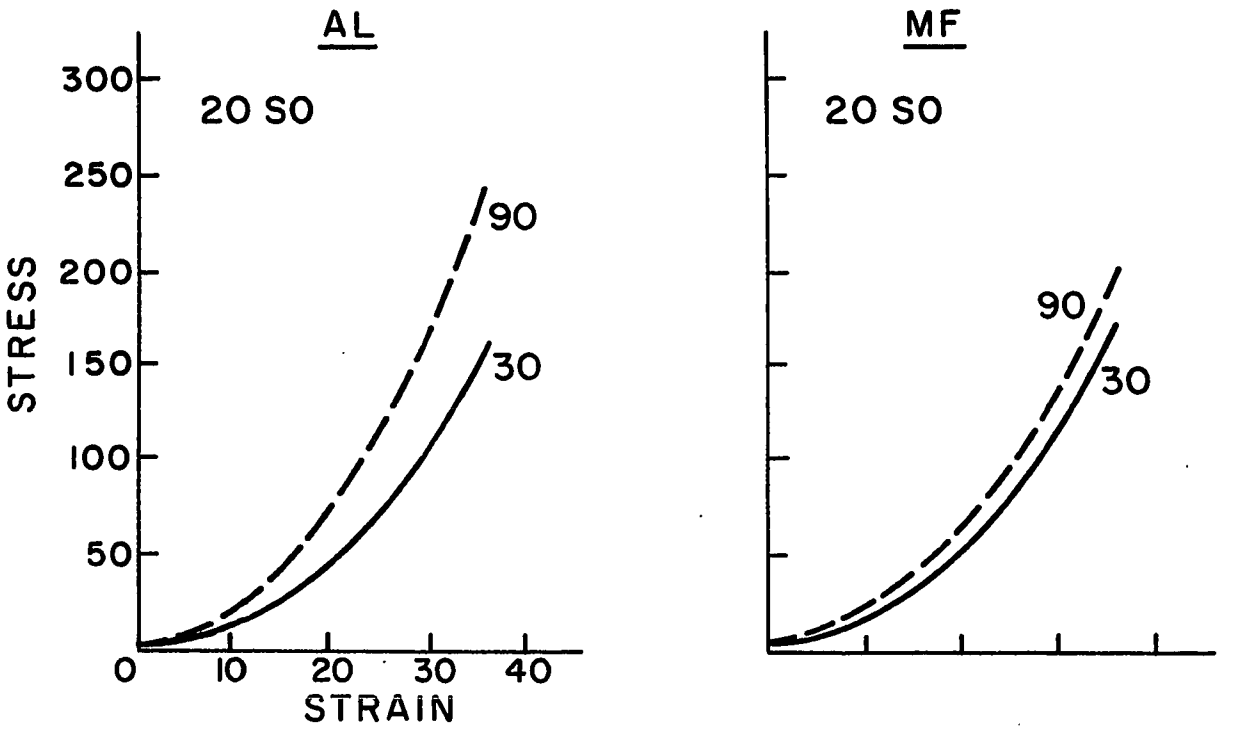
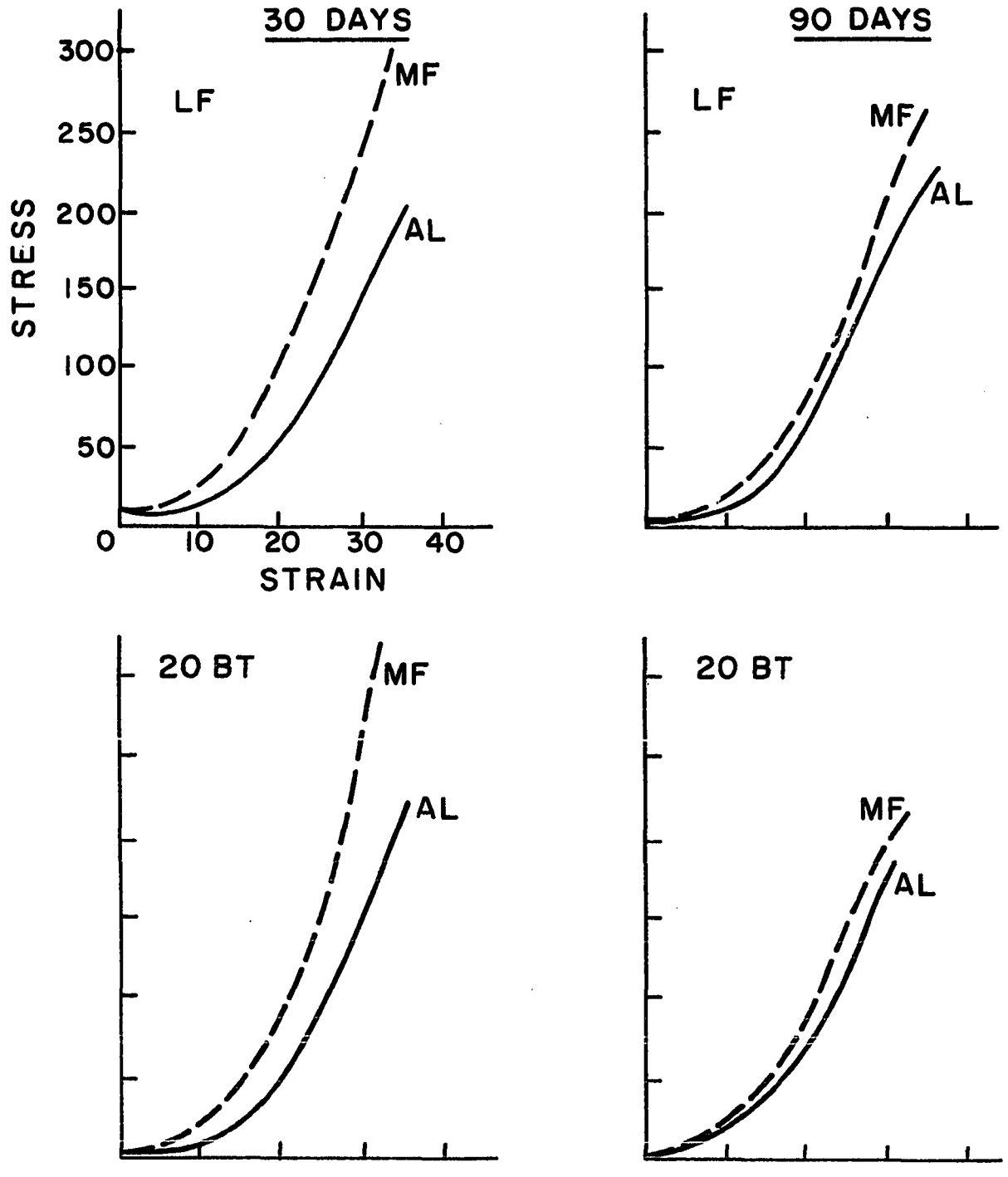


Figure 17. Composite stress-strain curves with aging as a variable. Y axis represents stress (force/cross-sectional area = g/cm^2) and X axis represents strain (elongation %). Animals were fed for 3 (MF) or 24 hours (AL) per day, diets containing as % calories either 2% safflower oil (LF), 38% safflower oil (SO) or 38% beef tallow (BT) for 30 and 90 days.

Figure 18. Composite stress-strain curves with meal pattern as a variable. Y axis represents stress (force/cross-sectional area = g/cm^2) and X axis represents strain (elongation %). Animals were fed for 3 (MF) or 24 hours (AL) per day, diets containing as % calories either 2% safflower oil (LF), 38% safflower oil (SO) or 38% beef tallow (BT) for 30 and 90 days.

MEAL PATTERN



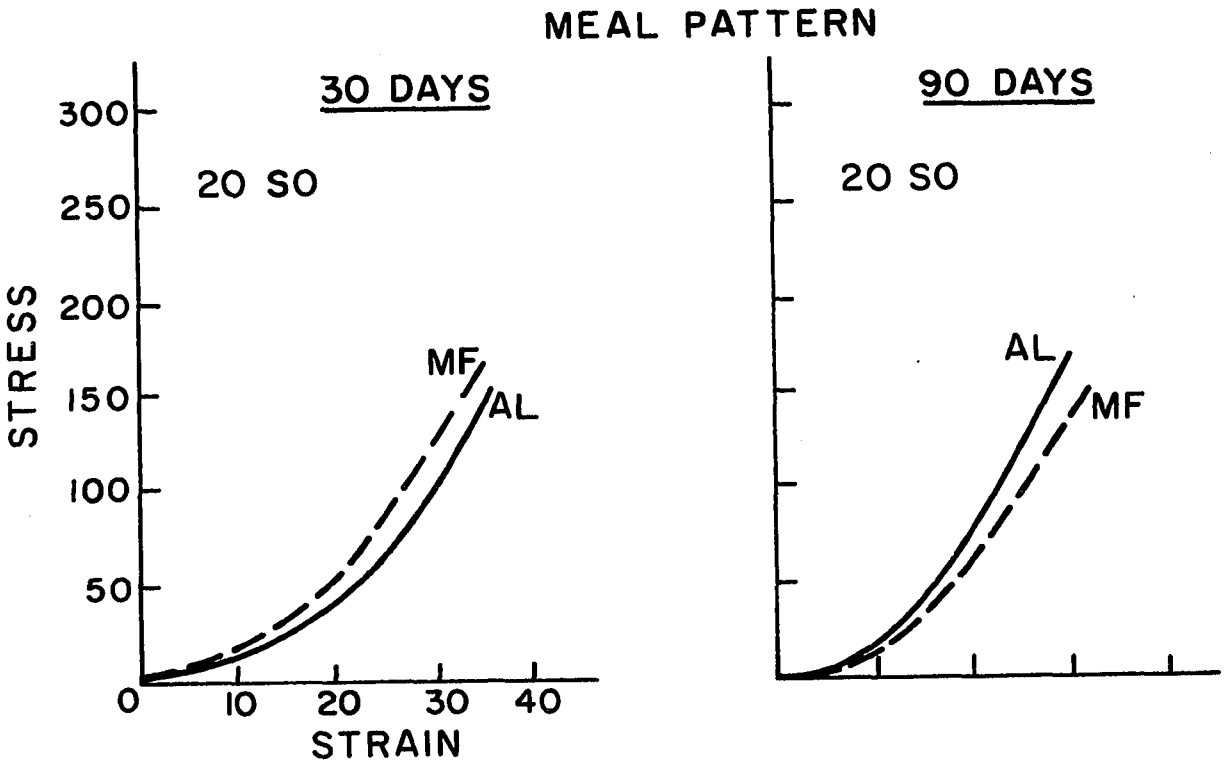


Figure 19. Composite stress-strain curves with meal pattern as a variable. Y axis represents stress (force/cross-sectional area = g/cm^2) and X axis represents strain (elongation %). Animals were fed for 3 (MF) or 24 hours (AL) per day, diets containing as % calories either 2% safflower oil (LF),38% safflower oil (SO) or 38% beef tallow (BT) for 30 and 90 days.

30-BT-AL, 90-BT-AL and 90-BT-MF were shifted slightly left of the corresponding LF groups. Conversely, the CSS curve from group 30-BT-MF was displaced slightly to the right of that of 30-LF-MF (Figure 14).
The shift of the CSS curves from SO groups was predictable, being consistently to the right of CSS curves from either LF or BT groups. This displacement to the right of SO curves, with respect to either BT of LF curves, was seen in both feeding periods, being more apparent after the 30-day period and especially with meal feeding (Figures 18-19).

In most groups CSS curves tended to be shifted to the left with aging, CSS curves from most treatments tended to be shifted left (Figures 16-17).

The CSS curves of most groups, except for those of group 90-SO-MF, were shifted to the left with meal feeding. In general the meal feeding effect was more apparent at the 30- compared to the 90-day feeding period.

The shifts in CSS curves may be related to variations in cardiac distensibilities. In general, cardiac distensibility was increased with safflower oil feeding, and decreased with either beef tallow or low fat feeding. Furthermore beef tallow feeding generated a similar ventricular distensibility response as did low fat feeding. Increased ventricular distensibility occurred as early as day 30 with safflower oil feeding. Aging caused an apparent increased resistance to stretch in most groups. In addition, meal feeding appeared to reduce distensibility, regardless of dietary types.

Elastin slope (ES) Table 21

Elastin slopes were not influenced by variations in dietary fat level. There was no significant difference when the elastin slope of group LF was compared to that of either group SO or BT due in part to large individual variations in group 30-LF-MF. Moreover, the mean elastin

Table 21. Elastin and collagen slopes of CSS curves

	Age 2 months		Age 4 months	
	30 Days		90 Days	
	Elastin $x10^{-1}$ g/cm^2	Collagen g/cm^2	Elastin $x10^{-1}$ g/cm^2	Collagen g/cm^2
LF-AL	2.6±.5[a]	3.7±.6	2.4±.3	3.8±.4
LF-MF	0.5±.1	4.4±.4	2.4±.5	3.9±.3
SO-AL	1.6±.2	3.9±.5	2.9±.4	3.1±.2
SO-MF	1.2±.1	3.4±.4	3.7±.4	2.9±.3
BT-AL	1.6±.4	3.4±.3	2.9±.4	3.6±.3
BT-MF	2.4±.4	4.4±.5	3.8±.7	4.3±.4

ANOVA

Fat level	Elastin	Collagen
LF vs. (SO + BT)	NS	NS
LF vs. SO	NS	NS
LF vs. BT	NS	NS
Fat saturation		
SO vs. BT	$p<.025$	NS
Age		
30 vs. 90	NS	NS
LF 30 vs. 90	NS	NS
SO 30 vs. 90	$p<.05$	NS
BT 30 vs. 90	$p<.05$	NS
Meal pattern overall	NS	NS
30	NS	NS
90	NS	NS

[a]Mean ± SEM.

slope value for this group was unusually low for reasons which will be presented in the discussion. There was, however, a fat-saturation effect. This was apparent when group SO was compared to group BT ($p<.025$). The decrease in elastin slope with SO was most apparent at 30 days. At 90 days, the elastin slope value for group SO was similar to that of group BT. An overall age related increase in elastin slope was observed. This effect, however, was attributable to significant increases in slopes of groups SO ($p< .05$) and BT ($p<.05$). There was no age related increase in diet LF, due to large standard deviations.

There did not seem to be a consistent pattern of variation in elastin slope values with meal feeding. Consequently a significant meal pattern effect was not observed.

Collagen slope (Table 21)

In general, collagen slopes did not seem to be influenced by elevation in diet at any fat level. The overall fat level effect was not significant when group LF was compared to groups SO and BT. Collagen slope values of group SO were consistently lower than those of groups LF and BT at both feeding periods. The mean values for group SO, LF and BT were 331, 396 and 393 g/cm^2, respectively. The collagen slope response with beef tallow feeding was similar to that with low fat feeding.

When SO and BT groups were compared, the greatest differences in collagen slopes were obtained with the 90-day treatment ($p<.01$).

Neither age nor meal pattern had any significant effects on collagen slope values.

Elastin and collagen content (Table 22)

The % hydroxyproline in either elastin or collagen was used to estimate tissue level of these two proteins.

When group LF was used for comparison, a significant increase in % hydroxyproline in elastin was apparent when safflower oil was used ($p<.05$). However the % hydroxyproline in elastin varied little when beef tallow replaced safflower oil as source of fat. The variable mean values were 1.12%, 1.35% and 1.15% for diets LF, SO and BT, respectively. However, when the two high fat groups were combined and then compared with the LF group, as shown in the ANOVA table, the overall fat level effect was lost. The variable means values were 1.12% and 1.26% for LF and SO + BT, respectively.

On the other hand, the % hydroxyproline in the collagen fraction was not influenced significantly by either fat level or fat saturation. The range values for the three diets were small, falling between 1.5-1.8%.

In general, there was an apparent decrease in % hydroxyproline in elastin with aging. The average decrease for all treatment groups between the two time periods was approximately 38%. Meal feeding did not appear to affect % hydroxyproline in elastin to a significant level. The % hydroxyproline in collagen appeared not to be influenced significantly by either age or meal pattern.

Collagen to elastin ratio, C/E (Table 22)

The collagen to elastin ratio, C/E, was calculated by dividing % hydroxyproline in collagen by % hydroxyproline in elastin.

The influence of dietary fat level on the C/E ratio appeared to depend on the type of fat used. When beef tallow was used as fat source,

Table 22. Cardiac muscle: % elastin (E) or collagen (C) based on OH-proline, and C/E ratio

Treatment		Age (mth) 2			Age (mth) 4		
		30 Days			90 Days		
		Elastin	Collagen	C/E	Elastin	Collagen	C/E
LF	AL	1.11±.1[a]	1.5±.2	1.35±.05	1.07±.2	1.6±.05	1.49±.05
	MF	1.10±.1	1.6±.1	1.45±.04	1.20±.3	1.7±.1	1.41±.04
SO	AL	1.42±.1	1.5±.1	1.05±.02	1.35±.2	1.5±.1	1.11±.03
	MF	1.31±.05	1.6±.1	1.22±.02	1.30±.2	1.7±.1	1.30±.03
BT	AL	1.20±.1	1.7±.1	1.41±.03	1.17±.2	1.8±.05	1.54±.05
	MF	1.25±.1	1.8±.1	1.44±.06	1.08±.1	1.7±.05	1.57±.05

ANOVA

	Elastin	Collagen	C/E
Fat level	N.S.	N.S.	N.S.
Fat saturation	P<.05	N.S.	P<.05
Age	N.S.	N.S.	N.S.
Meal pattern	N.S.	N.S.	N.S.

[a]Mean ± SEM.

there was little difference in C/E ratios between BT and LF groups. The variable mean values for these groups were 1.49 and 1.43 for BT and LF, respectively. Conversely, when safflower oil was substituted as fat source, the C/E variable mean value for group SO was reduced to 1.17. This decrease was significantly lower ($p<.05$) than either that of group LF or group BT in both the 30- and 90-day feeding periods.

In general, C/E tended to increase with age. The percent increase from day 30 to day 90 with diets LF, SO and BT was 4, 6 and 9, respectively. Similarly, the C/E ratio appeared to increase with meal feeding.

Electron microscopy

Left ventricular and ventricular septal mycocardial cells were analyzed for fine structure modifications (Figures 20-25).
The following organelles were examined: (1) plasma membrane, (2) nuclear membrane, (3) mitochondrial membranes, (4) sarcoplasmic reticulum, (5) Golgi apparatus, (6) intercalated discs, (7) myofibrils, (8) collagen fibers, (9) nuclei and nucleoli, (10) abnormal lipid inclusions, (11) glycogen deposits, (12) vacuoles, (13) lysosomes.

No differences in ultrastructures were found which could be associated with any of the diets (LF, SO and BT) used. No structural or cytoplasmic abnormalities were observed; nor was any type of damage detected. Plasma, nuclear and mitochondrial membranes appeared normal; no increase in number of mitochondria was observed. Glycogen deposits were low in all instances, with no detectable group differences. Areas of myofibrillar disruption or degeneration did not occur. Ribosomes, nuclei and nucleoli were normal. Lysosomes were observed occasionally in all

Figure 20. A thin myocardial section from the right ventricle of a rat allowed access to food 3 hours per day (10480). Animal consumed a diet containing 2% safflower oil as calories for 90 days. Magnification x20,000. A. Mitochondria. B. Myofibril. C. Z line.

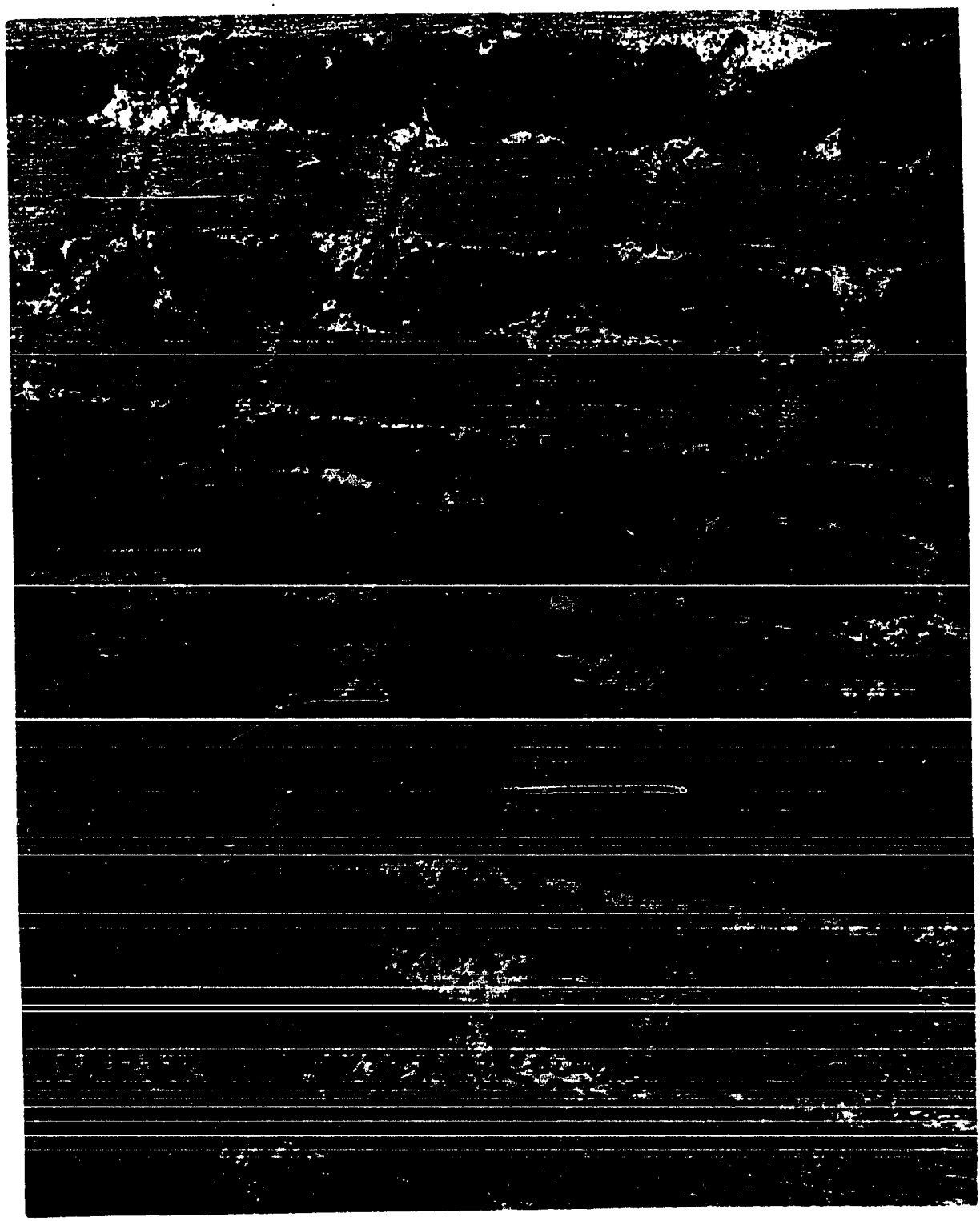


Figure 21. A thin myocardial section from the right ventricle of a rat allowed access to food 3 hours per day (10731). Animal consumed a diet containing 38% safflower oil as calories for 90 days. Magnification x17,000. A. Mitochondria. B. Sarcolemma. C. Myofibril. D. Vesiculated region of sarcolemma. E. Lipid droplet.

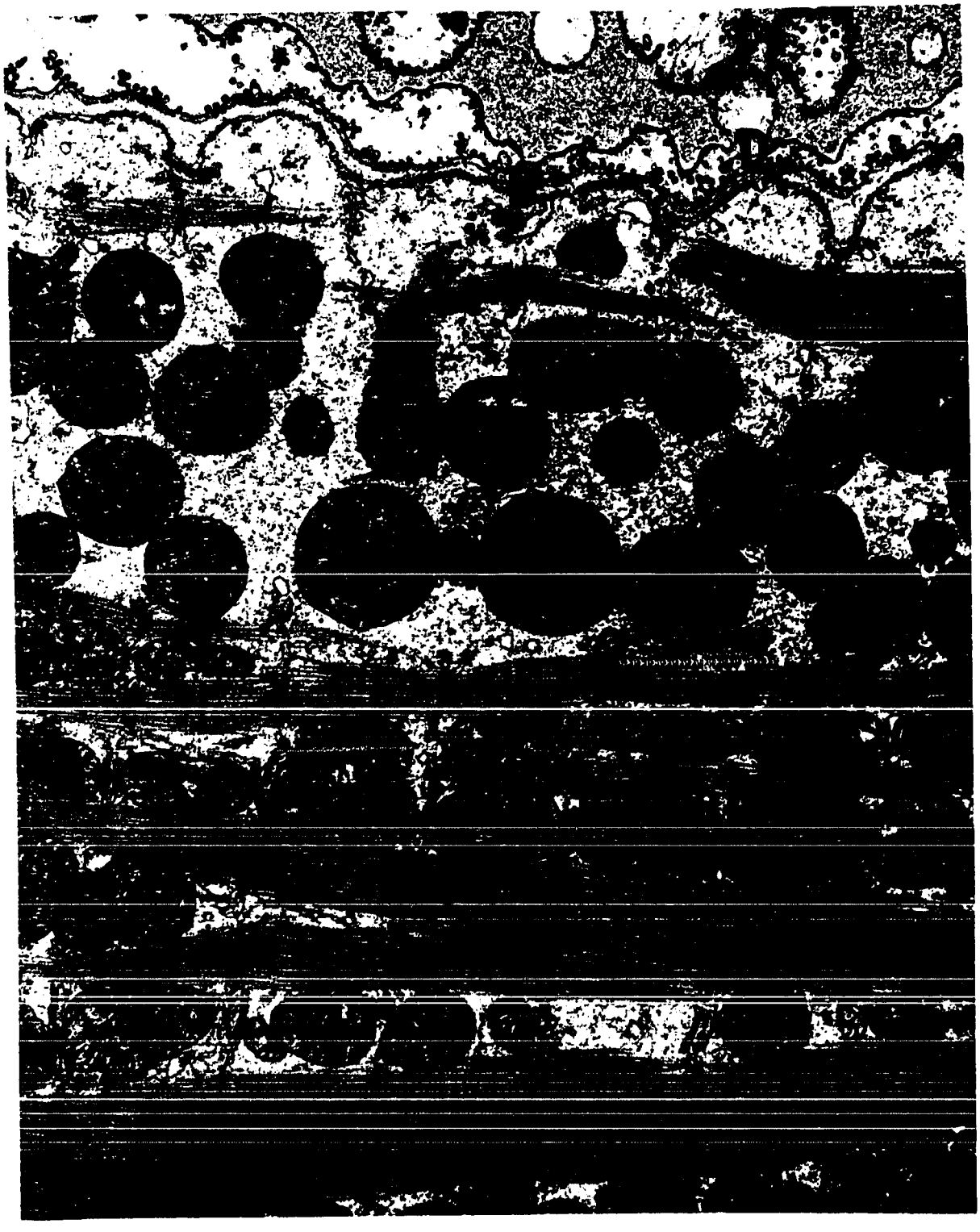


Figure 22. A thin myocardial section from the right ventricle of a rat allowed access to food 3 hours per day (10484). Animal consumed a diet containing 38% beef tallow as calories for 90 days. Magnification X18,000. A. Mitochondria. B. Sarcolemma. C. Intercalated disc.

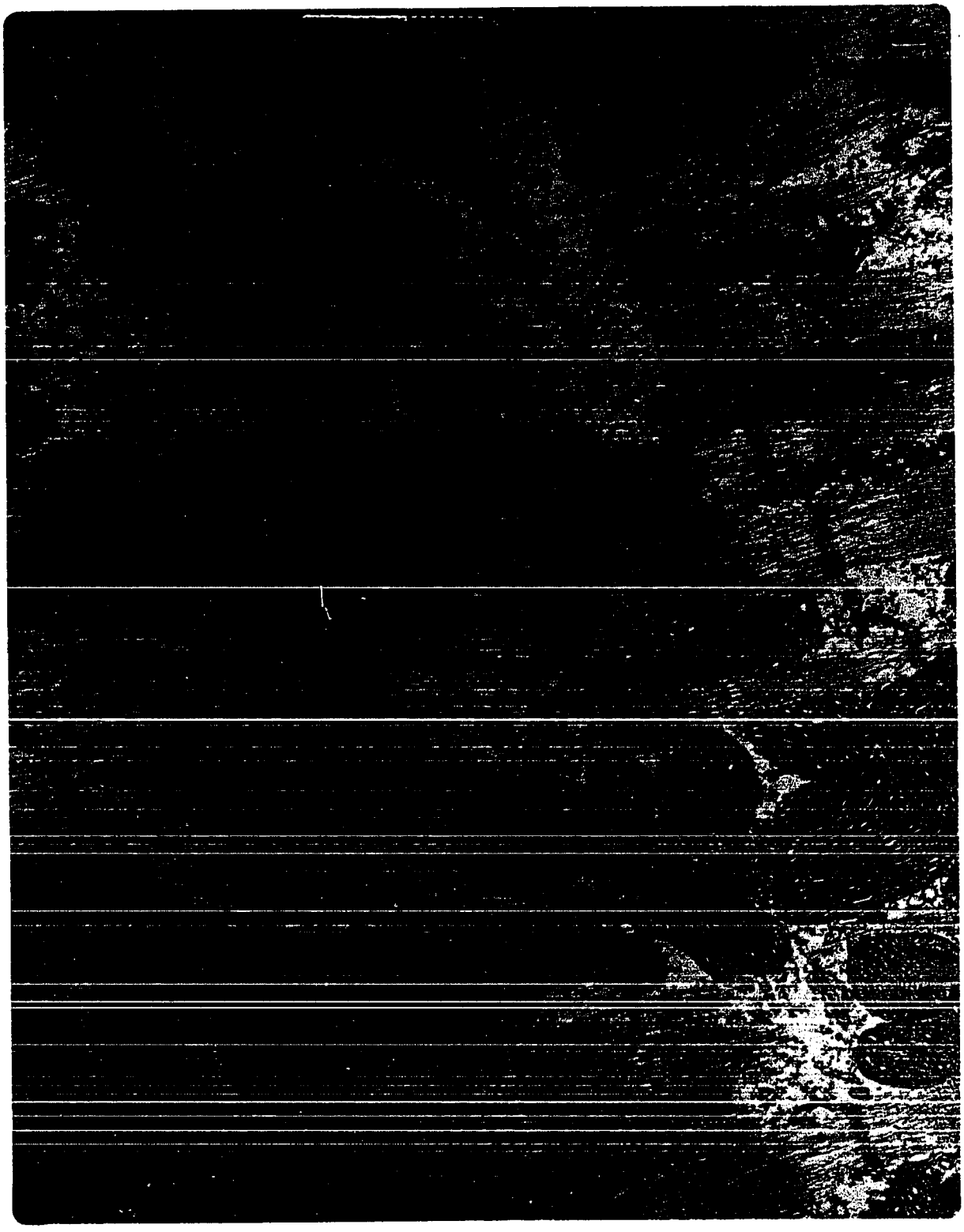


Figure 23. A thin myocardial section from the ventricular septum of a rat allowed access to food 3 hours per day (10710). Animal consumed a diet containing 2% safflower oil as calories for 90 days. Magnification x27,400. A. Sarcolemma. B. Mitochondria. C. May be nucleus. D. Z line. E. Cross-sections of collagen fibers (white spots). F. May be membrane whirls.

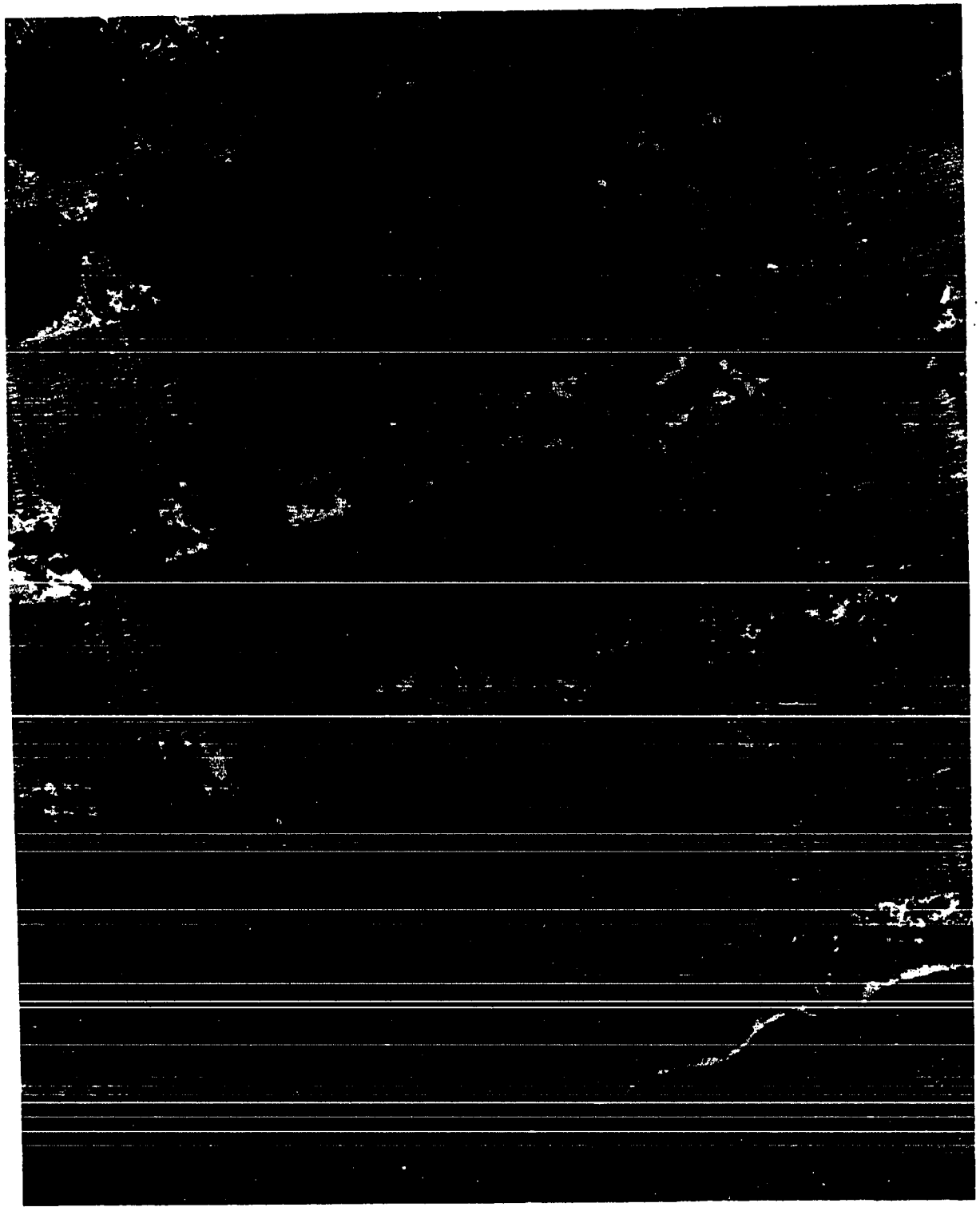


Figure 24. A thin myocardial section from the ventricular section of a rat allowed access to food 3 hours per day (10786). Animal consumed a diet containing 38% safflower oil as calories for 90 days. Magnification x20,000. A. Mitochondria. B. Z line. C. Lipid droplet.

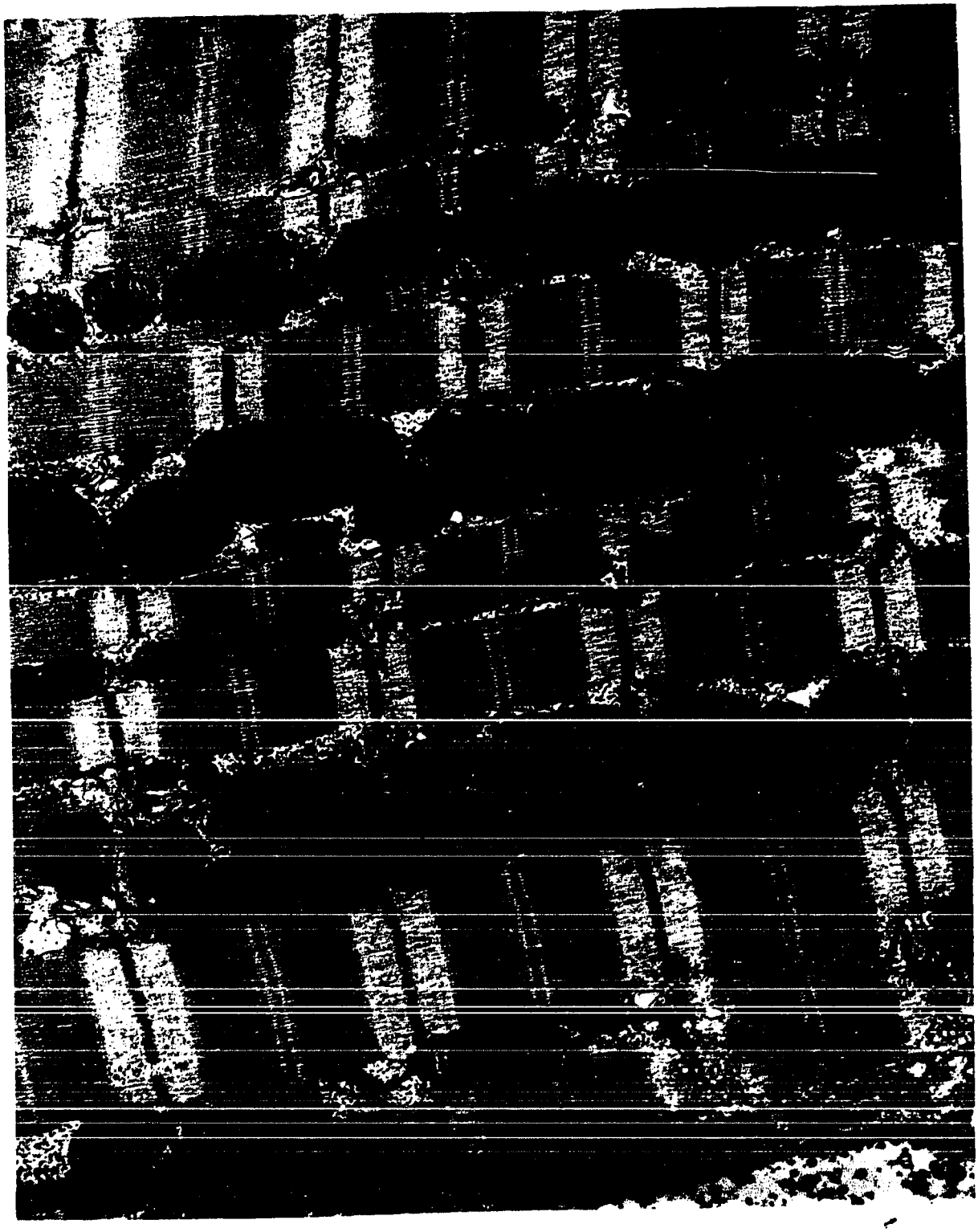
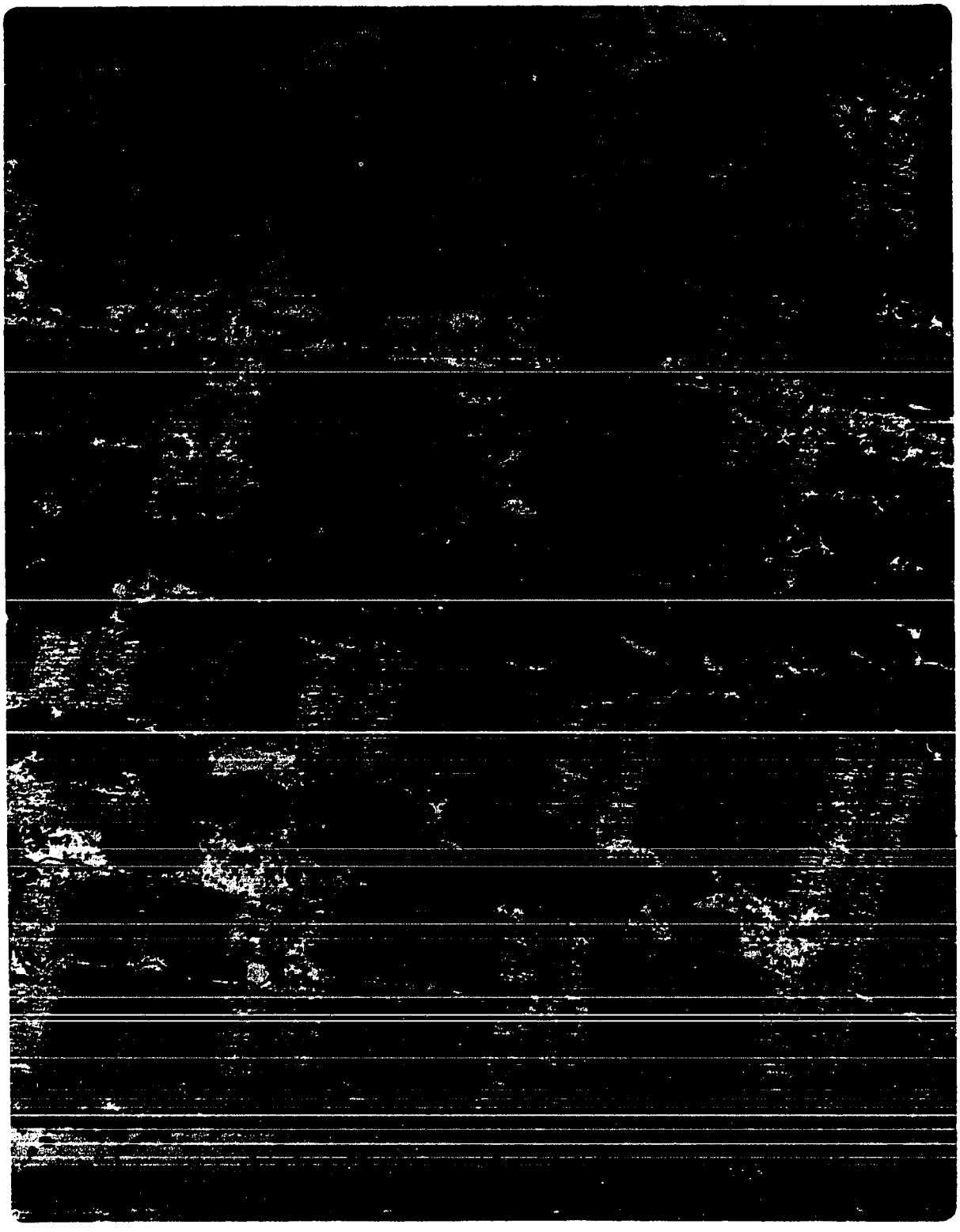


Figure 25. A thin myocardial section from the ventricular septum of a rat allowed access to food 3 hours per day (10495). Animal consumed a diet containing 38% beef tallow as calories for 90 days. Magnification x17,600. A. Mitochondria. B. Z line. C. Myofibrils.



groups. Collagen fiber width was approximately 600 Å, which is within normal range. A multiple intercalated disc was seen in one case (BT-septum), though intercalated junctions were normal in all instances. No unusual lipid droplet proliferation was seen in any of the groups.

Discussion

The functional-structural significance of the stress-strain curve has been determined experimentally and mathematically (Burton, 1954; Roach and Burton, 1957). Each curve is influenced simultaneously by three factors, namely elastin, interfibrillar substances (matrix) and collagen. The initial linear portion is attributed to the influence of elastin, the middle curve portion to the matrix, and the final linear portion to collagen. Due to the heterogeneity of the stress-strain curve, the use of linear regression during statistical analysis has been avoided since a regressed line may discount contributions of each individual factor. Consequently each curve was analyzed by measuring initial and final slopes, and area under the curve in the middle portion (Figure 13). This method, however, had its drawbacks. It was difficult to measure initial slopes for curves with short initial portions. In particular, curves from group 30-LF*MF had unusually abrupt initial portions, making assessment of the initial slopes by angle measurements difficult. As a result, elastin slopes might have been underestimated in this group. This problem arose when the muscle strip was too short to fit completely between the clamps during mounting. Consequently, the muscle was stressed at the beginning of the experiment. The baseline taken did not register true zero tension, and the instrument was not sensitive enough to adjust

for this. In addition, a short muscle left little end spaces for a tight grip of the clamps. Subsequently, slippage occurred occasionally. When this happened the stress-strain curve was distorted and pushed out of alignment. Muscle strips from group 30-LF-MF were shorter because heart sizes were smaller, compared to other groups. Similar problems with improper baselines and false starting points marring interpretations had been reported by other investigators (Hembrough and Riedesal, 1971).

The influence of the matrix on either elastin or collagen slope was not assessed. To obtain meaningful data, digestion of the muscle with either elastase or collagenase, following stretching, would be required. The digested muscle would have to be stretched under conditions (temperature, load calibration, etc.) identical to those used previously for the initial stretch experiment. The resulting curves would represent essentially pure elastin or collagen curves. This procedure, however, lacks reliability, since enzyme digestion does not always yield pure products. In addition, the digested muscle has to be free of tear before restretching.

In addition to the technical difficulties just described, the functional-structural significance of the matrix curve portion is only poorly understood. Some acid mucopolysaccharides in the matrix, e.g., hyluronic acid, chrondriotin, chrondriotin sulphates, denuratan sulphates, heparin, etc., are reported to affiliate in the lipids and to vary in concentrations with high cholesterol intakes, the presence of atherosclerotic lesions, and aging. Unfortunately, the impact of these changes on viscoelastic properties is not known. Consequently, stress-strain data from the matrix cannot be related to structural alterations.

Measurements of stress-strain response of the ventricles upon mechanical stretching indicated increased distensibility in groups receiving 38% safflower oil as calories in contrast to groups receiving either 38% beef tallow as calories or 2% safflower oil as calories. It appeared that increased ventricular distensibility was caused, in part, by changes in the elastin component. These changes occurred as early as 30 days on the diet, becoming less apparent with prolonged feeding to 90 days. Elastin slopes and collagen to elastin ratios decreased with high level of polyunsaturated fat feeding. These declines were associated with increased elastin in tissues of groups receiving 38% safflower oil. Conversely, collagen slopes and tissue collagen levels were not varied significantly by any of the experimental variables. These observations seem to imply that alterations in collagen (structural and tissue level) either do not occur with any of these variables, or occur at a slower rate than those related to elastin, and that the duration of our experiment was not long enough to cause major changes. Though fasting had been shown to decrease collagen synthesis in guinea pig skin (Chvapil, 1958), it could be that the 21 hours fast imposed here on meal fed rats were not severe enough to reduce significantly collagen synthesis. Work by Nikkani and Heikkinen (1968) indicated that in arterial vessels lipid infiltration into the intercarlated spaces of collagen fibers occurred via the same mechanism as in elastin fibers, though at a slower rate.

Increased ventricular distensibility correlated well with reduced C/E ratios. In blood vessels, a similar correlation was also observed (Burton, 1954; Roach and Burton, 1957). Conversely, in blood vessels, a

high C/E ratio was reported when collagen fibers increased leading to reduced distensibility and increased inclination to atherosclerosis. The findings of Wong et al. (1975) indicated reduced C/E ratios in aortas of cholesterol fed cockerels subjected to physical exercise compared to increased C/E ratios in cholesterol fed, unexercised birds. In addition, cockerels on plain mash demonstrated little changes in C/E ratios. The authors concluded that the reduced C/E ratios in the exercised group indicated reversal of the severity of atherosclerosis.

In the present study, collagen level was not influenced by any of the dietary variables used. Elastin level, on the contrary, was increased with safflower oil feeding, and not with beef tallow- or low-fat feeding. The study of Wong et al. (1975) with birds showed decreases in both collagen and elastin levels with cholesterol feeding and no exercise. The apparent increase in elastin in the present study presents an interesting question as to the effects of polyunsaturated fatty acids on elastin metabolism.

Increased elastin in the tissue of SO-fed rats in our experiment could be due to a) increased synthesis or decreased degradation of elastin as induced by safflower oil feeding, or b) increased catabolism stimulated by beef tallow and low fat feedings. The effects of high level linoleic acid (about 79% in safflower oil) on elastin metabolism are not known, though diets rich in linoleic acid (e.g., 50% sunflower seed oil) are reported to stimulate coronary flow and left ventricular functions. These increases are associated with increases in prostaglandin (PG) synthesis of which linoleic acid is known as a precursor (Vergroesen, 1976). Hwang et al. (1975) demonstrated that the serum concentration of PGE_1 and $PGF_{2\alpha}$ were higher in rats fed a linoleate-rich diet than in rats fed a diet

supplying equivalent levels of fat, but a low amount of linoleate. Prostaglandins PGE_1 and PGE_2 are reported to stimulate coronary flow of several animal species, including rats, while $PGF_{1\alpha}$ and $PGF_{2\alpha}$ under certain conditions of Kt/Ca^{+2} can stimulate contractile forces in isolated rat hearts (Vergroesen, 1972). At concentrations of $10^{-11}M$, PGE_1 elevates cAMP levels in myocardial preparations via stimulation of adenyl cyclase activity (Klein and Levey, 1971). In addition, PGE_2 and PGA_1 produce similar effects at $10^{-4}M$, whereas $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are not active. The F type apparently does not interact with adenyl cyclase, however cGMP may be a mediator pf PGF_{α} action in blood vessels (Dunlam et al., 1973). The effects of PG and cAMP on smooth muscle activity and elastin synthesis are less certain. Though safflower oil feeding indicates higher elastin content, we are unable to say conclusively from our experiment whether this increase is a genuine increase over normal due to the absence of a proper control group consuming a nonpurified diet. Consequently, a mechanism for increased distensibility with SO feeding cannot be deduced without further experiments.

Animals on either beef tallow- or low-fat diets indicated decreased ventricular distensibility compared to ventricular distensibility of animals on safflower oil diet. Stress-strain curves from beef tallow groups behaved relatively similar to those from low-fat groups. Similarity in distensibility between beef tallow- and low-fat groups is not fully understood. It may be that the rates of lipid infiltration into cardiac elastin are similar in these animals. The finding of Dupont et al. (1972) reported a tendency of beef tallow- and low-fat rats to accumulate

saturated and monounsaturated fatty acids in their tissues. In general, decreased distensibility may be related to one or several of the following: a decrease in elastin, an increase in collagen, an increase in either collagen or elastin cross-linkages, and finally an increase in matrix viscosity attributable to alteration in matrix composition. in elastin, an increase in collagen, an increase in either collagen or elastin cross-linkages, and finally an increase in matrix viscosity attributable to alteration in matrix composition.

Decreased distensibility in blood vessels correlates with increased tissue and serum lipids (Jacotot et al., 1973; Newman et al., 1971; Band et al., 1973). Focal deposition of cholesterol, primary cholesterol esters, phospholipids and tryglycerides are causes of elastin fragmentation as well as early signs of atherosclerosis (Kramsch and Hollander, 1973; Adams and TuQan, 1961). These lipids are derived from serum LDL and ULD fractions (Robert et al., 1972). In our experiment cardiac total lipid content was not assessed. However, the finding of Deere (1973) indicated little variation in cardiac total lipid content when adult rats were fed diets either free of fat or containing 40% of calories as safflower oil or beef tallow. Similar results were obtained when young rats were fed diets containing either corn oil or beef tallow for 15 weeks (Egwin and Kummerow, 1972). In both studies, the cardiac phospholipid fraction increased with feeding safflower oil.

Band et al. (1973) reported a negative correlation of serum cholesterol concentration to thoracic aorta distensibility in rabbits. In contrast, serum cholesterol concentration and aortic distensibility were not correlated in rats. Similar findings were reported by Hembrough and

Riedesal (1971) who examined abdominal aorta of rats. In Experiment 1 of the present study, plasma cholesterol did not correlate with cardiac distensibility, and was not influenced significantly by diet. Total plasma cholesterol concentration may not be a good measure of cholesterol entrapment into connective tissue. Rather it is the compartmentation of plasma cholesterol that may be critical, i.e., cholesterol distribution in the lipoproteins, specifically in LDL and VLDL, since cholesterol transports across membrane and into elastin fibers is facilitated by low density lipoproteins.

In a preliminary study with selected groups, we found little variation in cardiac cholesterol concentrations in rats on the 90-day regimen, regardless of diet. In every group, free cholesterol far exceeded that of esterified cholesterol, e.g., 96% in group LF, 99% in group SO and 98% in group BT. Heere (1975) had shown a similar predominance of free over esterified cholesterol in cardiac tissues of adult rats fed diets similar to those used in this present study. The mean values were 72%, 99% and 92% with LF, SO and BT diets, respectively. Furthermore total cardiac cholesterol concentration was not influenced by any of the diets used. Carlson (1975) reported that though cardiac cholesterol concentrations were not influenced by diets (low fat, safflower oil and beef tallow) in the adult rats, the incorporation of H-^{14}C-cholesterol into cardiac tissue of safflower oil fed rats was higher than that of either low fat or beef tallow fed animals.

Evidence is lacking at the present time for an association of cardiac lipids (content and composition) to cardiac distensibility. Cardiac lipids

are not as responsive to dietary modifications, particularly dietary fat alterations, as arterial lipids.

The effects of aging and meal feeding on cardiac tissue were similar. Both variables reduced cardiac distensibility. Other laboratories (Robert et al., 1972; Szigeti et al., 1972; Bouissou et al., 1973) have reported increased elastolysis in blood vessels and dermal layer of the skin with aging, due in part to lipid deposition in elastin fibers. Aging may increase elastin-cross linkages, resulting in stiffer, less elastic fibers. Our results indicated a tendency toward decreased elastin content with age. This decrease, however, was not significant due to large standard deviations within groups. The work of Kane et al. (1976) with Syrian golden hamsters reported decreased ventricular distensibility with aging. The authors suggested that the aging heart does not normally undergo substantial alterations in passive properties that affect the muscle cells and fibers, but rather that observed changes in stress-strain relationships are attributable to alteration in ventricular size only.

The effect of meal feeding on cardiac distensibility is not well documented. However, studies related to fasting have been reported. Fasting and subsequent refeeding are known to cause hypertension in rats, dogs and man (Bernardis and Brownie, 1965; Brozek et al., 1948; Wilhelmj et al., 1951). Hembrough and Riedesal (1971) demonstrated decreased distensibility in the rat abdominal aorta with long term fasting and subsequent refeeding. Decreased distensibility, however, was not related to diet, i.e., control versus dextrose. In this present experiment, decreased distensibility with meal feeding cannot be explained by either changes in

elastin slopes, which do not show a consistent pattern of variation, or changes in elastin content. We speculate therefore that decreased distensibility with meal feeding may be influenced by the matrix. Unfortunately there is little information on the effect of meal feeding on the components of the matrix.

Ultrastructural examination failed to bring out differences in myocardial cells, regardless of dietary variables tested. Reeves (1971) who used rat hearts perfused with either glucose or palmitate, also found little differences in microscopic examinations of cardiac tissues, in spite of differences in contractibilities. Likewise Sulkin and Sulkin (1967) reported no differences in cardiac mitochondria, sarcoplasmic reticulum or Golgi apparatus between young and old rats.

SUMMARY AND CONCLUSION

Three experiments were designed to study changes in cholesterol metabolism, cardiac stress-strain response and cardiac morphological alterations associated with variations in dietary fat level and saturation, meal pattern and age. A model using male rats, undergoing rapid development, was used. In experiment 1, 30 days old rats weighing approximately 100 g, were assigned to one of three diets containing as % calories: 2% safflower oil (LF), 38% safflower oil (SO) or 38% beef tallow (BT). Animals consumed these diets for either 30, 60, or 90 days, corresponding to chronological ages of 2, 3 or 4 months. Animals ate their food ad libitum (AL) or in a 3 hour meal (MF). Lighting schedule was reversed with dark hours coinciding with feeding hours.

The same experimental design was used in Experiment 2 with the omission of the intermediate (3 months) age group. In Experiment 3, only the longest feeding period (90 days, 4 months old) and only the meal-feeding pattern were used.

In Experiment 1, acute changes in cholesterol metabolism, specifically in the rapidly equilibrating pools of serum, liver, and small intestine were assessed. Low-level radiotracers were administered to estimate relative rates of synthesis, degradation, excretion and distribution of endogenous cholesterol. Each animal was injected 2.5 μC/0.5 ml saline 4-^{14}C-cholesterol, 6 days prior to, and 50 μC/0.5 ml saline ^{3}H-acetate, 2 1/2 hours prior to termination. Cholesterol was assessed as digitonin precipitable steroids (DPS). Recovery of ^{14}C-counts in plasma, hepatic and intestinal DPS was interpreted as indicative of relative rates of

cholesterol distribution of retention in these tissues. Recovery of ^{3}H-counts in plasma, hepatic and intestinal DPS was used to estimate relative rates of cholesterol synthesis in liver and small intestine from ^{3}H-acetate, 2 1/2 hours after administration. Recovery of ^{14}C-counts in acid steroid fractions from liver, small intestine and feces (including large intestinal contents) was interpreted to represent cholesterol degradation and excretion.

In vivo data related to body weight changes, food intakes and food efficiencies were similar to previous findings in our laboratory with adult rats using relatively similar diets (Reeves and Arnrich, 1974; Derer, 1974). Animals on BT diet consumed more food and gained more weight than animals on LF diet. Food efficiencies were not different for the three diets used. Food efficiencies decreased markedly with aging (2-3X). Tissue weights of liver, epididymal fat pads, kidneys, stomach and heart were all sensitive to dietary manipulations and aging. However, spleen weight reached maximal weight by age 2 months, and was not influenced by further variations in either fat level, fat type or meal pattern.

Plasma cholesterol concentrations responded little to variations in the fat component of these diets or to meal frequency. However, plasma cholesterol concentrations tended to increase as rats matured.

This age-related overall mean increase in plasma cholesterol concentration approximated 23% for all dietary regimens for the age span between 2 to 4 months. Failure of plasma cholesterol concentration in young rats to respond to variation in dietary fat could be due to low plasma choles-

terol levels characteristic of young animals, and to elevated rates of cholesterol exchanges between plasma and the slowly equilibrating pool such as muscle, skin and other body systems.

Plasma triglyceride concentrations like those of cholesterol were also not influenced by either dietary fat or by meal pattern. Other investigators have indicated that circulating triglyceride levels influence plasma cholesterol levels indirectly.

Plasma levels of two metabolites of thyroxine were measured to assess thyroid status in young rats. Dietary fat and meal pattern had no influence on plasma thyroxine levels measured either as plasma T3, T4, T3/T4 or TI (T4 x 0.653) in any of the treatment groups. Data on plasma thyroxine and triglycerides correlated with the data obtained for plasma cholesterol concentrations with the variables used.

Meal feeding did not elevate plasma cholesterol concentrations in all the dietary groups examined. This finding contrasts to those reported for the adult rats from our laboratory. Failure to demonstrate differences in plasma cholesterol levels between ad libitum controls and meal fed animals could be due to adaptation to meal feeding after 30 days on the diet. Since preexperimental plasma cholesterol levels were not measured, the possibility exists that after 30 days on the diet, the animal had already adapted physiologically to the meal feeding pattern.

High fat diets (SO and BT) stimulated increased rates of ^{14}C-cholesterol synthesis from ^{3}H-acetate in liver and small intestine throughout the three age periods studied. It has been suggested that increased cholesterogenesis with high fat diets is due to decreased hepatic lipogenesis resulting in increased substrate availability for cholesterol

synthesis such as acetyl CoA. In addition, high fat diets, particularly with safflower oil as fat source, induced cholesterol accumulation in liver and small intestine. Cholesterol degradation to bile acids in the liver and excretion as bile acids in the small intestine and excreta were likewise elevated with SO feeding. In contrast, feeding beef tallow invoked in many instances similar rates of degradation and excretion as did feeding a low-fat diet.

The most striking finding of polyunsaturated fat feeding in maturing rats was the increased bile acid content, measured as ^{14}C-acid steroids, in liver, small intestine and feces plus large intestinal contents. This finding of increased bile acid formation and excretion with safflower oil feeding is supported by experiments of others, where bile acid half life was reduced with polyunsaturated fat feeding. The finding that hepatic synthesis was not stimulated with increased unsaturation of dietary fat was unexpected since it is generally accepted that polyunsaturated fat feeding stimulates hepatic holesterogenesis. Cholesterol content in liver, quantitated chemically, was elevated with safflower oil feeding. However, radiochemical data on ^{14}C-cholesterol content failed to demonstrate difference with polyunsaturated fat intake. Cholesterol synthesis in the small intestine, however, was suppressed with decreasing dietary fat saturation. Such decrease could be attributed to the increase in cholesterol accumulated in this tissue. This finding supports the report of decreased HMG-CoA reductase activity with polyunsaturated fat feeding in young rats (Shefer et al., 1973). Thus it appears from this study that, with polyunsaturated fat feeding, changes in cholesterol degradation to bile acids and excretion of bile acids precede changes in hepatic

cholesterol synthesis and plasma cholesterol concentrations. The mechanism by which polyunsaturated fat influences cholesterol degradation and excretion is open to further study.

An age effect on cholesterol metabolism has been demonstrated in this experiment. Young rats maturing from 2 months to 4 months had increased rates of cholesterogenesis in liver and small intestine. Similar increases with age were noted for acid steroid formation and excretion, cholesterol content in plasma, liver and small intestine. In most instances, age-related increases were associated with increases in organ weights.

Meal feeding in young animals had little effect on cholesterol metabolism on a unit tissue weight basis. Cholesterol metabolism for these animals was measured at a time of day when cholesterogenesis was not at peak rate. It seems possible, therefore, that in the meal fed groups, cholesterogenesis would have been higher than determined here if this time of animal sacrifice had been near peak cholesterogenesis for the meal fed model.

Animals fed 38% of calories as safflower oil showed a relatively enhanced incidence of respiratory, skin and eye infections.

Cardiac and intestinal tissues of safflower oil fed animals appeared fragile to physical manipulation. Cardiac fragility in these animals appeared to affect mechanical function measured by stress-strain response, even though examination of ultra-structures of the myocardial cells were normal, and did not differ from those of animals on low fat and beef tallow diets (Experiments 2 and 3). In general, ventricles of safflower oil fed rats showed increased cardiac distensibility upon mechanical

stretching. Increased distensibility was related to increased elastin content, which in turn may be related to increased tissue prostaglandin synthesis from high levels of linoleate present in safflower oil. Tendency to decrease cardiac distensibility with advancing age and with meal feeding may be associated with decreased elastin content. Collagen content was not influenced by any of the variables used. Ultra-structures of myocardial cells were normal. Examinations indicated little differences in fine structures with variations in dietary fat level and saturation.

In conclusion, the effects of varying dietary fat level and saturation, age and meal pattern in young rats appear to be:

1. Increased hepatic cholesterogenesis with increased dietary fat level regardless of fat source.
2. Increased bile acid formation and excretion with polyunsaturated fat feeding.
3. No difference in hepatic cholesterogenesis with polyunsaturated fat feeding, though small intestinal cholesterol synthesis was suppressed. Suppression of intestinal cholesterogenesis was attributed to the increase in cholesterol content in this tissue from both chemical and radiochemical data. Increased cholesterol content in liver was shown chemically, but not radiochemically.
4. Little difference in plasma cholesterol, triglyceride and thyroxine concentrations by variations of either dietary fat level or fat saturation in all the treatment groups. Mean plasma cholesterol concentration tended to increase with age.

5. Increased animal susceptibility to respiratory, skin and eye infections, as well as increased cardiac and intestinal fragility with polyunsaturated fat feeding. Cardiac fragility correlated to increased ventricular distensibility as measured by stress-strain response. Increased distensibility with safflower oil feeding was associated to increased elastin content which may be related to the in vivo increase of prostaglandin from linoleate. Ultra-structures of myocardial cells did not indicate alterations with variations in dietary fat level and saturation.
6. Alterations of cholesterol metabolism with aging and meal feeding appeared affected primarily through changes in tissue size. In addition, these variables tended to decrease cardiac distensibility, though they had no effect on fine structures of myocardial cells.

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APPENDIX

Table 23. Body weight, weight gain, food intakes and food efficiency (FE), Experiment 2

	2 months				4 months			
	Body weight g	Weight gain g	Food intake kcal	FE g-100 kcal	Body weight g	Weight gain g	Food intake kcal	FE g-100 kcal
LF-AL	302±8[a]	6.1±.5	72±5	10±2	514±17	0.7±.03	76±6	0.9±.1
LF-MF	216±7	6.5±.5	50±3	12±2	440±14	1.0±.1	64±4	1.6±.1
SO-AL	318±7	6.5±.5	68±4	10±1	492±10	1.2±.1	72±6	1.7±.2
SO-MF	286±5	6.6±.4	60±5	11±2	470±11	1.2±.1	70±5	1.7±.1
BT-AL	334±7	6.7±.6	77±6	9±1	526±14	0.7±.04	74±6	0.9±.1
BT-MF	285±5	6.8±.5	65±5	10±1	487±13	1.3±.1	73±6	1.6±.2

[a]Mean ± SEM.