Influence of meal pattern and dietary fat on cholesterol metabolism in adult rats recovering from undernutrition

by

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GLOSSARY

OF,OP - contains negligible quantities of fat and protein

OF - contains negligible quantities of fat

20SO - 20% by weight safflower oil

20BT - 20% by weight beef tallow

P - 4.3% of kcal as lactalbumin protein

4P - 17.2% of kcal as lactalbumin protein

AL - ad libitum; allowed continual access to food

MF - meal fed; fed for 8 hours out of 24

DPS - digitonin precipitable sterol; cholesterol

dpm - disintegrations per minute

FFS - fat free solids

LSD - least significant difference

SEM - standard error of the mean

INTRODUCTION

"There is abundant evidence that the risk of developing coronary heart disease is positively correlated with the level of cholesterol in the plasma."[1] A causative relationship, however, has not yet been indisputably established. Epidemiological, clinical and experimental investigations indicate that a number of factors affect serum cholesterol concentration. Variations in diet or activity which alter serum cholesterol concentration require further investigation to elucidate the mechanisms of their effects. Meal pattern or feeding frequency is one such variable. Other variables are the degree of saturation of dietary fat and the protein intake.

Observations made in the 1950s first indicated that serum cholesterol concentrations might be increased by decreasing feeding frequency. Many such studies, representing work with different species, sexes, ages, diets and meal patterns have been reported (Cohn et al., 1961; Gopalan et al., 1962; Wells et al., 1963; Cohn, 1964; Leveille and Hanson, 1965; Reeves and Arnrich, 1974).

In our laboratory a model, developed to study rapid lipogenesis in adult rats recovering from chronic undernutrition, has proven useful for studying the effects of feeding frequency and polyunsaturated fat upon cholesterol metabolism. With this model, serum cholesterol concentrations were found to be elevated during realimentation when rats consumed their

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[1] Diet and Coronary Heart Disease. A joint statement of 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.

daily allotment within an 8 hour period (MF) compared with controls consuming similar amounts during a 24 hour period (Reeves and Arnrich, 1974).

Polyunsaturated fat decreased serum cholesterol concentration (Avigan and Steinberg, 1958; Bloomfield, 1964; McGovern and Quackenbush, 1973b). Increased dietary protein concentration also decreased serum cholesterol concentration (Kenney and Fisher, 1973). Although there is controversy as to the mechanisms of these changes, available evidence points to an acceleration of cholesterol turnover as the result of high dietary intakes of both polyunsaturated fat and dietary protein.

Recommendations made by health professionals[1] indicate that individuals at risk from coronary heart disease should, in practice, increase their intakes of polyunsaturated fats to decrease serum cholesterol concentrations. There is growing evidence, however, that polyunsaturated fat itself may pose risks to health. For example, prolonged consumption of vegetable oils high in polyunsaturated fat has resulted in increased mortality rates in rats. Membrane structures may be altered functionally when high levels of linoleic acid replace other fatty acids.[2] Investigations in this laboratory have indicated increased in vitro tissue fragility and altered cardiac performance following safflower oil feeding which may indi-

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[1] Diet and Coronary Heart Disease. A joint statement of 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.

[2] A. A. Spindler, M. M. Mathias and J. Dupont, Colorado State University, unpublished communication, 1975.

cate decreased tissue collagen concentration.[1] These findings indicate a need for caution in making recommendations for dietary change solely for the purpose of altering serum cholesterol concentrations.

One aspect of overall metabolism which is not well understood is cholesterol metabolism following dietary alterations. In humans there is little opportunity to study simultaneously a number of factors involved in cholesterol metabolism. For this reason a study with rats was designed to investigate as many facets of cholesterol metabolism as possible with variations in feeding frequency, dietary fat and dietary protein. It was hoped that this study would help to explain fluctuations observed in serum cholesterol concentrations with these variables. A model which showed rapid changes in serum cholesterol concentration with variations in feeding frequency was used throughout the study. Variations in concentration and degree of saturation of dietary fat and in concentration of dietary protein were also incorporated into the design. A dietary fat concentration of 20% by weight or 40% of calories was chosen as an approximation of fat intake by the average Western individual.

Following a preliminary experiment, radioactive tracers were introduced to assess rates of cholesterol biosynthesis and catabolism. The acute turnover of cholesterol into bile acids and transport between serum, liver and intestines were followed with 4-^{14}C-cholesterol. Acetate labeled with ^{3}H was used to investigate rates of cholesterol and fatty acid biosynthesis.

It is well documented that variations in serum cholesterol concentra-

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[1]C-S. Heng, Iowa State University, personal communication, 1975.

tion result from alteration of one or more of the following aspects of cholesterol metabolism: 1) differences in absorption of cholesterol from the diet; 2) shifts in tissue levels of cholesterol; 3) alteration in the rate of cholesterol biosynthesis; 4) differences in the rate of cholesterol degradation; 5) differences in recycling of cholesterol; and 6) differences in excretion of cholesterol and its metabolites. It is hoped that this study will contribute to knowledge of serum cholesterol regulation after investigation of the metabolism of cholesterol following alterations in feeding frequency and dietary fat and protein concentrations.

LITERATURE REVIEW

A number of investigators have observed that dietary fat and frequency of food consumption affect serum cholesterol concentration. Few studies, however, have dealt with the influence of these variables on cholesterol metabolism. It is generally accepted that observed alterations in serum cholesterol concentration are associated with one or more of the following factors: differences in absorption of dietary cholesterol from the gut, shifts of tissue cholesterol, altered cholesterol biosynthesis and (or) altered degradation and excretion of cholesterol and its metabolites.

This review is divided into three sections. The major section is concerned with control of cholesterol metabolism. Another section deals with the influence of dietary variables, especially dietary fat, on cholesterol metabolism. Finally, the literature pertaining to the influence of periodicity of eating on cholesterol metabolism is summarized.

Investigations into control of cholesterol metabolism have been advanced with the recognition that rates of cholesterol biosynthesis and degradation peak at mid-night, unless the normal pattern of food consumption is reversed. Another important advance came with the purification of β-hydroxy-β-methylglutaryl coenzyme A reductase (HMG-CoA reductase, E.C. 1.1.1.34). Biochemical and immunological techniques may now be used in studying control of cholesterol metabolism.

Several recent reviews have covered aspects of control of cholesterol metabolism in greater depth than will be attempted here. They may be useful as a supplement to this review. Control of steroid biosynthesis has been reviewed by Bortz (1973) and Dempsey (1974). McIntyre and Isselbacher

(1973) have discussed the role of the small intestine in cholesterol metabolism.

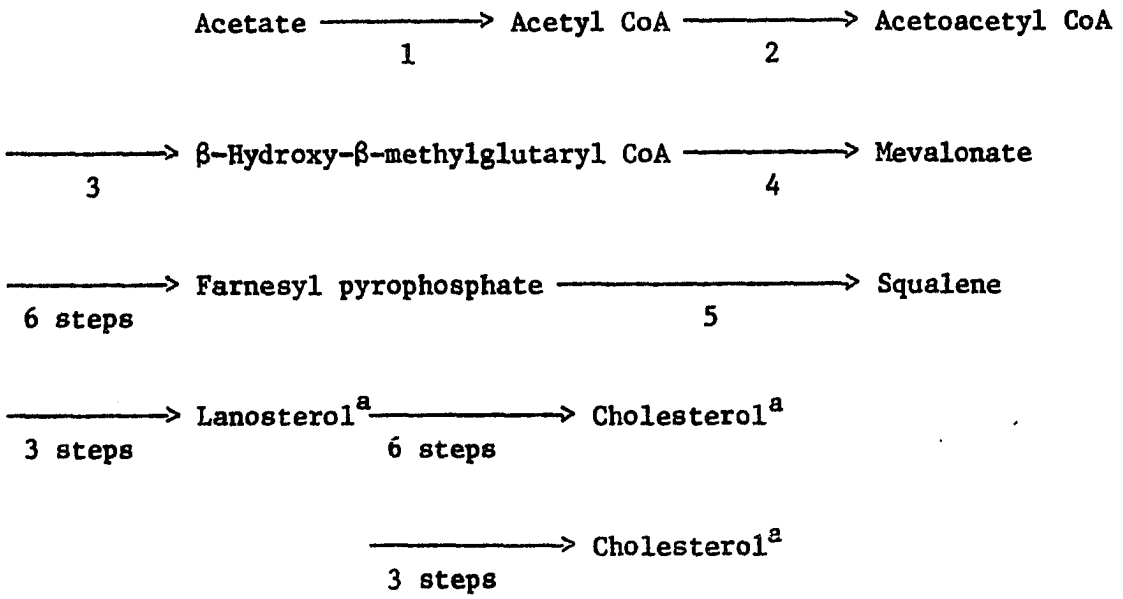
Factors Contributing to Homeostasis in Cholesterol Metabolism

Control of hepatic cholesterogenesis

Cholesterol metabolism was first reported to be under homeostatic control in 1933 (Schoenheimer and Breusch). Since that time, the negative feedback regulation of hepatic cholesterol synthesis has been well documented in a number of species, including man (Blattathiry and Siperstein, 1963; Siperstein and Fagan, 1966; Grundy et al., 1969).

When cholesterol is fed, thereby depressing hepatic cholesterogenesis, body cholesterol does accumulate through absorption and synthesis. The relative contributions of exogenous and endogenous cholesterol to the circulating pool are species dependent. Cholesterol is more readily absorbed in the dog and rat than in man. When labeled cholesterol is fed to these three species, 85-90% of serum cholesterol in dog and rat and 40% of serum cholesterol in man is of dietary origin (Wilson, 1968). Thus under conditions of depressed hepatic cholesterol synthesis, dog and rat synthesize about 10-15% of body cholesterol, while in man this figure is about 60%. Most of this synthesis is presumed to occur in the small intestine (Dietschy and Siperstein, 1967).

Regulation of hepatic cholesterol biosynthesis may occur at several enzymatic steps in the biosynthetic scheme. Most investigators have concluded, however, that the major site of cholesterol feedback is the enzymatic step converting β-hydroxy-β-methylglutarate to mevalonate. The enzyme involved is HMG-CoA reductase (Figure 1). Early data from Bucher et al.



1 Acetyl CoA synthetase.

2 Acetoacetyl CoA thiolase.

3 HMG-CoA synthase.

4 HMG-CoA reductase.

5 Squalene synthetase.

[a]Digitonin precipitable sterol.

Figure 1. Conversion of acetate to cholesterol (White, Handler and Smith, 1973)

(1959) indicated that the major control mechanism of biosynthesis occurred prior to mevalonate formation. Fasting and cholesterol feeding decreased conversion of acetate to cholesterol more than conversion of mevalonate to cholesterol. Siperstein and Fagan (1966) localized control at the level of HMG-CoA reductase. Cholesterol feeding decreased mevalonate synthesis but not synthesis of β-hydroxy-β-methylglutarate in their experiments.

Control of HMG-CoA reductase Liver HMG-CoA reductase activity has been shown to be influenced by a number of conditions including fasting, cholesterol feeding, diurnal cycling, hormone levels and bile acid concentrations. Effects of these variables on hepatic cholesterogenesis will be discussed independently, though they are probably interrelated to a large extent.

Feedback control by dietary cholesterol Negative feedback control by dietary cholesterol (Bucher et al., 1959; Siperstein and Fagan, 1966) was the earliest recognized control of HMG-CoA reductase. Until very recently, investigators believed that depression of sterol synthesis by dietary sterol was due entirely to decreased synthesis of HMG-CoA reductase. There is now direct evidence that cholesterol feeding has at least two effects on HMG-CoA reductase activity: an initial inhibition independent of protein synthesis, and a latent depression of new enzyme synthesis (Higgins and Rudney, 1973; Zavoral et al., 1973).

Diurnal control Hamprecht et al. (1969) first showed that HMG-CoA reductase activity varied diurnally, peaking in activity at midnight and having a low at noon. Shapiro and Rodwell (1969) confirmed their work and further demonstrated a requirement for protein synthesis in diurnal control. Cycloheximide injection completely prevented both the

rise in enzyme activity at night and the loss of activity during the day. Edwards et al. (1972) demonstrated circadian rhythmicity of hepatic cholesterogenesis in the rat in vivo. Hepatic microsomal HMG-CoA reductase activity and incorporation of ^{14}C-acetate and $^{3}H_2O$ into cholesterol were highest at about midnight and lowest at noon.

Studies in Edwards' laboratory led to the postulation that the circadian rise in enzyme activity was due to ingestion of food. In the first study, diurnal rhythmicity of HMG-CoA reductase was reversed in rats by reversing normal illumination (Edwards and Gould, 1972). Most food consumption occurred during the dark hours. In another experiment, with rats trained to eat from 9 A.M. to 1 P.M. under normal illumination, maximal hepatic cholesterogenesis occurred at 6 P.M. compared to 12 P.M. for ad libitum fed controls (Edwards et al., 1972).

Fat flow to the liver following alimentation may stimulate cholesterogenesis by inducing increased formation of HMG-CoA reductase (Bortz et al., 1973; Bortz and Steele, 1973). A small peak in plasma free fatty acid levels between 3 and 6 P.M. in fed rats, just prior to the rise in hepatic HMG-CoA reductase activity, has been observed. Arguments for elevation of hepatic cholesterogenesis by free fatty acids are weakened by observations that fasting elevated plasma free fatty acid but decreased cholesterogenesis compared to alimentation.

Hormonal control There is strong evidence for a physiological control of HMG-CoA reductase by insulin (Lakshmanan et al., 1973). Activity of HMG-CoA reductase was increased 2-7 times following subcutaneous administration of insulin to both normal and diabetic rats. Reductase activity began to increase after 1 hour, rose to a maximum in 2-3 hours, and

declined to control levels after 6 hours. The response was elicited at a time during the day when the normal diurnal variation in reductase activity approached a minimum. It was also elicited when animals did not have access to food. The known relationship between insulin and food consumption makes physiological induction of cholesterol biosynthesis by insulin an attractive hypothesis.

Lakshmanan and coworkers (1973) also injected adrenalin and thyroxine into rats. These hormones increased HMG-CoA reductase activity with activity maxima at 12 and 30 hours, respectively. It is unlikely that these hormones influence the diurnal rise in enzyme activity, but they may influence the enzyme in some physiological conditions.

Thyroxine affects cholesterol biosynthesis at a step prior to mevalonate formation (Fletcher and Myant, 1958). Rats made hyperthyroid synthesized cholesterol from acetate at an increased rate, but rate of mevalonate synthesis was unchanged. Hypothyroid rats incorporated less acetate and similar amounts of mevalonate into cholesterol compared with normal animals.

Feedback control by bile acid Large increases in cholesterol biosynthesis have been observed following biliary drainage (Dietschy and Siperstein, 1965, Dietschy and Gamel, 1971). The increase in biosynthesis probably resulted in part from a decreased ability to reabsorb cholesterol from the gut, thereby releasing negative feedback inhibition of hepatic cholesterogenesis.

Increased hepatic cholesterogenesis following biliary drainage could be due, however, to release of feedback inhibition from bile salts rather than from cholesterol. The reciprocal situation, that is depression of

liver HMG-CoA reductase activity and cholesterogenesis by bile salts, has been shown to occur (Hamprecht et al., 1971; Kandutsch and Chen, 1973). Cholic acid feeding prevented the diurnal rise in HMG-CoA reductase activity (Hamprecht et al., 1971). Evidence that bile salts mediate a change in enzyme content as opposed to simply decreasing activity comes from Barth and coworkers (1973). Cholesterogenesis in isolated rat livers was not decreased with portal infusion of taurocholate and cholate.

Research by Hamprecht and coworkers (1971) did not support the hypothesis that bile acids decreased HMG-CoA reductase activity indirectly by improving cholesterol absorption from the gut. Cholic acid fed to rats with lymph fistulas depressed peak HMG-CoA reductase activity compared with controls fed no cholic acid. The daily low reductase activity was not further depressed by cholic acid feeding.

Inhibition of cholesterol biosynthesis by cholesterol metabolites may be dependent upon the presence of a 7-hydroxy or 7 α-keto group on the molecule. In liver cell and L cell cultures, HMG-CoA reductase activities and rates of sterol synthesis from acetate were strongly inhibited by highly purified preparations of 7 β-hydroxycholesterol, 7 α-hydroxycholesterol and 7 α-ketocholesterol (Kandutsch and Chen, 1973). Other metabolites of cholesterol, including cholic acid and chenodeoxycholic acid, did not inhibit HMG-CoA reductase activity under conditions of the study.

Similarities in the diurnal patterns of hepatic cholesterol and bile acid synthesis (Danielsson, 1972; Danielsson, 1973; Bortz et al., 1973; Bortz and Steele, 1973) provide a physiological rationale for a control of HMG-CoA reductase activity by bile acid.

Increased rates of hepatic cholesterogenesis accompanied biliary obstruction (Ferris et al., 1972; Friedman and Byers, 1957; Byers et al., 1962) as well as biliary drainage. Since hepatic bile acid concentrations are increased with biliary obstruction and decreased with biliary drainage, there is not a simple inverse relationship between hepatic bile acid concentration and cholesterogenesis.

Rats subjected either to biliary drainage or to biliary obstruction have decreased bile acid concentrations in the small intestine. One could speculate that the effector of cholesterol biosynthesis in both situations is a blood factor produced by the small intestine under conditions of low bile acid concentration. Blood of biliary obstructed rats contained an agent which increased hepatic cholesterol biosynthesis. Ferris et al. (1972) postulated that this factor was of hepatic origin.

Control mechanisms Although it has been accepted that control of cholesterol biosynthesis occurs at the level of HMG-CoA reductase, little has been learned about the mechanism of control. Research in this area is ongoing in a number of laboratories.

Investigators have generally believed that sterol synthesis was controlled solely by the turnover of HMG-CoA reductase. Cycloheximide injection prevented the normal diurnal rise in HMG-CoA reductase activity (Shapiro and Rodwell, 1969). Therefore, synthesis of enzyme was required for the normal diurnal rise in cholesterol biosynthesis. Lakshmanan et al. (1973) found no evidence for activation of pre-existing enzyme by insulin in their studies. Furthermore, insulin-induced activity of HMG-CoA reductase required enzyme synthesis. The rise in activity of HMG-CoA

reductase, following removal of a serum factor which suppressed HMG-CoA reductase activity, required de novo protein synthesis (Brown et al., 1973).

Recent reports indicate that HMG-CoA reductase activity can be regulated more rapidly by mechanisms other than synthesis and degradation of the enzyme. Normal serum was infused into rats as a control. Normocholesterolemic serum was compared with hypercholesterolemic serum and (or) cycloheximide. Cycloheximide acted much more slowly in blocking synthesis of cholesterol than did injected cholesterol (Tanabe et al., 1972). In another study, depression of cholesterol biosynthesis occurred within one hour after infusion of hypercholesterolemic serum (Zavoral et al., 1973). An inhibitor of cholesterol biosynthesis detected in rat milk and bile also seemed to affect the activity of the enzyme directly (McNamara et al., 1972).

These results were consistent with direct inhibition of HMG-CoA reductase activity, but they did not preclude the possibility of enhanced degradation of enzyme. Purification of HMG-CoA reductase has recently allowed Higgins and Rudney (1973) to demonstrate conclusively that changes in the activity of the enzyme occur with cholesterol treatment. Using immunoprecipitation, the authors showed that feeding cholesterol did not alter the amount of HMG-CoA reductase within 4 hours, though the activity of the enzyme fell markedly within this period of time. Twenty-four hours later, the diurnal rise in activity was decreased in cholesterol fed animals. Decreased levels of enzyme, as shown by decreased quantities of precipitable protein, caused the change in activity. The authors concluded that cholesterol feeding has at least two effects on enzyme activity: a rapid inhibition of activity independent of protein synthesis, and subse-

quent depression of new enzyme synthesis.

A number of possible mechanisms for rapid control of the enzyme are under investigation. Phosphorylation-dephosphorylation has been studied as a possible control mechanism, but the evidence at this time is not conclusive. Cyclic AMP inhibited cholesterol biosynthesis in vitro (Goodwin and Margolis, 1973). The inhibition could involve protein kinase phosphorylation of a specific enzyme (Goodwin and Margolis, 1973). However, Raskin et al. (1974) believe that in vitro suppression of cholesterol and fatty acid synthesis with high concentrations of cyclic AMP are of questionable significance. In their studies, cyclic AMP levels in the intact rat were elevated 50 times with glucagon treatment. Neither lipid synthesis nor HMG-CoA reductase activity were decreased by these concentrations. Nevertheless, a control of this type is attractive, because it might account for known effects of hormones on in vivo cholesterogenesis.

Another possibility for control is suggested by recent findings of Knull et al. (1974). These investigators have evidence that rat brain hexokinase is controlled by microsomal membrane binding versus solubilization. A similar type of control might be postulated for HMG-CoA reductase activity since it also is a microsomally associated enzyme and since cholesterol is a constituent of the membrane.

It appears that HMG-CoA reductase has at least two binding sites for NADPH (Tormanen et al., 1975). Activity of the enzyme was increased markedly by preincubation with NADPH. Physiological conditions in which NADPH levels in the cell are elevated could maximally activate HMG-CoA reductase.

Control of other enzymes involved in cholesterogenesis A number of investigators have demonstrated that control of hepatic cholesterogenesis can occur other than at the level of HMG-CoA reductase. Though this enzyme still seems to be the major site of control, it is conceivable that in certain physiological or nutritional states control at one of the following levels may predominate.

Conversion of acetate to acetyl-CoA Studies of cholesterol biosynthesis have indicated that the conversion of acetate to acetyl-CoA (Figure 1) may be regulated (Bates and Rothblat, 1974; Haward et al., 1974). Evidence for regulation at this step, however, comes from cell culture studies only.

Conversion of acetyl-CoA to HMG-CoA Recently, both mitochondrial and cytoplasmic systems for production of HMG-CoA were shown to exist (Clinkenbeard et al., 1973). Therefore, the committed step in cholesterol biosynthesis may exist prior to that catalyzed by HMG-CoA reductase (Figure 1, Dempsey, 1974). Control of cholesterol biosynthesis may be at the levels of acetoacetyl-CoA thiolase and (or) HMG-CoA synthase.

Convincing evidence is lacking to support this hypothesis, but few comparisons of enzyme activities prior to mevalonate formation have been made. Most studies have compared synthesis of cholesterol from acetate and mevalonate. Of these, most have demonstrated that cholesterogenesis is controlled prior to mevalonate formation without delineating specific reactions. Multiple cytosolic forms of hepatic HMG-CoA synthase can occur, possibly regulating cholesterogenesis by this enzyme (Sugiyama et al., 1972).

Dugan et al. (1972) have evidence that the diurnal alteration in cholesterol biosynthesis is controlled by an enzyme prior to HMG-CoA reductase.

Conversion of mevalonate to cholesterol A number of investigators have found evidence of regulation between mevalonate and cholesterol. Bucher and coworkers (1959) concluded that at least two such regulatory steps existed. Carroll (1964) found two regulatory sites between mevalonate and squalene. Still other investigators found two sites of control, one between mevalonate and farnesyl pyrophosphate (Figure 1), and the other between squalene and digitonin precipitable sterol (Slakey et al., 1972).

With in vivo studies in rats, Shah (1975) found changes in both incorporation of mevalonate into squalene and squalene into digitonin precipitable steroid (DPS). The ratio of DPS to nonsaponifiable fat decreased between 7 and 34 days in rats, indicating a decrease in the conversion of squalene to cholesterol following weaning.

Control of small intestine cholesterogenesis

Dietschy and Siperstein (1967) showed in rats that hepatic and small intestine cholesterogenesis together accounted for approximately 90% of whole body synthesis. When cholesterol was fed, thereby depressing hepatic cholesterogenesis, small intestine cholesterol synthesis was little affected. Therefore, the highest rate of cholesterol synthesis in cholesterol fed rats appears to be in the gastrointestinal tract.

Chevallier and Lutton (1973) studied the quantitative relationship in excreta between cholesterol synthesized in the gut, cholesterol synthesized in other organs and dietary cholesterol. Synthesis in tissues other than

the small intestine provided a constant amount of approximately 5 mg cholesterol per day. Additional synthesis came from the gastrointestinal tract and was dependent upon the absorption coefficient. Absorption coefficients were between 35 and 95%. Total synthesis was between 10 and 25 mg per day. Therefore, gastrointestinal synthesis accounted for between 50 and 80 percent of total body synthesis in rats studied.

Investigators questioned if intestinally synthesized cholesterol could contribute to circulating pools. High rates of intestinal cholesterogenesis occurred with drastically curtailed cholesterol absorption in bile diverted animals (Dietschy and Siperstein, 1965). Lindsay and Wilson (1965) first showed that cholesterol synthesized in the intestinal wall entered into the circulating pool. In further experiments, these results were confirmed, and an attempt was made to quantify the contribution of the intestine to circulating pools of cholesterol (Wilson, 1968). Cholesterol was fed to squirrel monkeys to inhibit hepatic cholesterogenesis, and the intestinal lymph ducts were cannulated. Cholesterol synthesized from labeled acetate in the intestinal wall entered the lymph. From this, the authors calculated the contribution of intestinally synthesized cholesterol to the circulating cholesterol pool in intact monkeys.

Control by bile acids There is strong evidence that bile salts inhibit intestinal cholesterogenesis. In rat and man the intestine responds to removal of bile acid with increased cholesterogenesis. Diversion of bile from the small intestine of rats increased mucosal synthesis throughout the small bowel, while infusion of bile caused a striking inhibition of sterol production (Dietschy and Siperstein, 1965). Dietschy and Gamel

(1971) demonstrated greatly enhanced sterol synthesis in the distal ileum of man following interruption of enterohepatic circulation.

Bile salts were the constituents of bile which inhibited cholesterol synthesis in intact animals (Dietschy, 1968). Bile salts exerted their control in the biosynthetic sequence of cholesterol between acetyl-CoA and mevalonate. Bile acids appear to inhibit cholesterol biosynthesis by decreasing HMG-CoA incorporation into mevalonate (Hatanaka et al., 1972; Shefer et al., 1973). Biliary diversion or feeding of sitosterol enhanced the activity of HMG-CoA reductase in rat intestinal crypt cells, while administration of taurocholate and taurochenodeoxycholate reduced enzyme activity. Bile salts abolished the rise in intestinal HMG-CoA reductase activity in the next diurnal cycle (Shefer et al., 1973).

The structural specificity of bile acids required for inhibition of intestinal sterol synthesis was studied in cell free extracts of yeast by Hatanaka et al. (1972). The effects of 40 species of bile acids and related compounds on the incorporation of labeled acetate into sterol were compared. Inhibitory activity required the presence of a 3 or 7 α-hydroxy group and a carboxyl group at the terminal side chain. When an additional hydroxyl group was introduced at the 6 or 12 position, inhibition was abolished. Taurine conjugates of bile acids inhibited sterol synthesis as well as did free bile acids. Glycine conjugates of bile acids, however, inhibited sterol synthesis only about half as effectively as did free bile acids.

Feedback control by cholesterol Chevallier and Lutton (1973) concluded that biliary acids were not the primary regulatory agents of intestinal cholesterogenesis. Synthesis of cholesterol in the gastrointestinal tract was inversely correlated with absorption. Data from Cayen (1971) also support a direct role of cholesterol in the regulation of intestinal cholesterogenesis. Cholesterol biosynthesis increased in the intestine after binding of cholesterol by tomatine and without a change in bile acid production. Intestinal HMG-CoA reductase activity in rats was decreased with both cholesterol and bile acid feeding (Shefer et al., 1973), but the reduction in activity could not be ascribed to an increase in sterol concentration within the intestinal crypt cells.

Diurnal control Cholesterol biosynthesis in jejunum and distal ileum was diurnally regulated (Edwards et al., 1973). The amplitude of the diurnal rise was smaller than that observed in the liver. Maximal incorporation of labeled acetate into cholesterol occurred earlier in the intestine (between noon and 6 P.M.) than in the liver (6 P.M.). The authors suggested that the diurnal rise in HMG-CoA reductase activity in both tissues was related to ingestion of food.

Control of bile acid formation from cholesterol

The liver is the only organ capable of synthesizing bile acids from cholesterol. Its role in bile acid production was demonstrated by Harold et al. (1955). Cholic acid produced in the liver originated from cholesterol of both biosynthetic and exogenous origin (Rosenfeld and Hellman, 1959). Following administration of 4-^{14}C-cholesterol and ^{3}H-acetate to bile fistula subjects, time curves of ^{3}H:^{14}C were identical for cholic acid

and cholesterol ester.

Hepatic free cholesterol appears to be the precursor of bile acid synthesis. The specific activity of bile acids formed was slightly below that of hepatic free cholesterol but markedly higher than that of liver esterified cholesterol after labeled acetate injection (D'Hollander and Chevallier, 1972; Mathe et al., 1972). McGovern and Quackenbush (1973c) found greater conversion of ^{3}H-cholesterol than of labeled cholesteryl oleate and linoleate to bile acid. Six hours later half of the ^{14}C-cholesterol originally esterified was recovered in the liver as free cholesterol.

Recently investigators have postulated that newly synthesized cholesterol may be preferentially used for bile acid production (Mitroupolos et al., 1973; Balasubramanian et al., 1973). Cholesterol in rat liver microsomes was labeled by intravenous injection of ^{14}C-cholesterol. Cholesterol specific activity was about 3 times that of 7 α-hydroxycholesterol formed by the microsomes in vitro. Cholesterol reaching liver microsomes from the blood circulation mixed preferentially with the fraction of microsomal cholesterol not accessible to cholesterol 7 α-hydroxylase. These results raised the possibility that cholesterol synthesized in situ was the preferred substrate for bile acid production.

Feedback control by bile acid Hofmann (1965) proposed a theoretical model of enterohepatic circulation of bile acids. He emphasized that meaningful experiments to test for negative feedback control of bile acid on bile acid synthesis required knowledge of bile acid pool size and rate of enterohepatic circulation of the pool. Other investigators have used this model to study feedback control by bile acids on their own production

(Dietschy and Wilson, 1970; Shefer et al., 1969). Dietschy and Wilson termed the mass of bile acid transferred across the liver per unit time as "hepatic bile acid flux".

Negative feedback inhibition of bile acid synthesis by bile acid was demonstrated by Shefer et al. (1969), who calculated that the bile acid pool in the rat was approximately 14 mg/100 g body weight. A flux of 7 mg/100 g body weight/hour was required to maintain negative feedback inhibition. When taurocholate flux fell below 7 mg/100 g body weight/hour, bile acid synthesis was not inhibited. Thus 14 mg bile acid/100 g body weight had to circulate 12 times per day to maintain feedback inhibition of bile acid synthesis.

The first committed step in bile acid synthesis is catalyzed by cholesterol 7 α-hydroxylase. Feedback control by bile acid appears to occur at the level of this enzymatic step (Shefer et al., 1968; Shefer et al., 1970). Activity of cholesterol 7 α-hydroxylase was enhanced in livers of bile fistula or cholestyramine treated rats in vitro (Shefer et al., 1968). Later it was demonstrated in vivo that control of bile acid synthesis occurred at the level of cholesterol 7 α-hydroxylase (Shefer et al., 1970). Four labeled precursors of bile acids (acetate, mevalonate, cholesterol and cholest-5-ene-3(β)7(α)-diol) were injected into rats. Incorporation of acetate, mevalonate and cholesterol into bile acids was inhibited in the presence of the circulating bile acid pool. Injection of cholest-5-ene-3(β)7(α)-diol bypassed its normal catalytic formation by cholesterol 7 α-hydroxylase. This labeled metabolite was incorporated into bile acid equally well in the presence or absence of circulating bile acid.

The enzyme 12 α-hydroxylase was also stimulated by bile fistula and cholestyramine feeding but was quantitatively less important than stimulation of cholesterol 7 α-hydroxylase (Danielsson et al., 1967; Johansson, 1970). The enzyme may be most important in the qualitative control of bile acid synthesis. The production of cholate, but not chenodeoxycholate, requires 12 α-hydroxylase. Thyroxine is known to inhibit activity of this enzyme. Hyperthyroidism decreased the ratio of cholate to chenodeoxycholate from that of normothyroid animals (Mosbach, 1972).

Polyunsaturated fat may increase 12 α-hydroxylase activity. Feeding corn oil versus coconut oil (Lindstedt et al., 1965) or safflower oil versus beef tallow (McGovern and Quackenbush, 1973a) increased the ratio of cholate to chenodeoxycholate in rat bile.

There is evidence that cholic acid can influence production and turnover of chenodeoxycholate. Cholic acid feeding in man increased pool size of cholate 2-5 times while simultaneously decreasing pool size and turnover of chenodeoxycholate by about 50% in all subjects (Einarsson et al., 1973).

Induction by cholesterol Feeding cholesterol to rats can increase bile acid synthesis (Shefer et al., 1969). Investigators have reported increased excretion of bile acids following excessive absorption of cholesterol in rat and dog (Dietschy and Wilson, 1970).

Induction of bile acid synthesis by cholesterol appears to be at the level of cholesterol 7 α-hydroxylase. When cholesterol was added to the diet, cholesterol 7 α-hydroxylase activity was increased, but enzyme activity continued to vary diurnally (Mitropoulos et al., 1973).

Diurnal control Diurnal rhythmicity in bile acid production from cholesterol was first indicated in work by Chevallier and Lutton (1966). Following administration of 26-^{14}C-cholesterol, expired $^{14}CO_2$ was elevated at night. It was later demonstrated that cholesterol 7 α-hydroxylase was subject to diurnal variation with maximal activity at about 8 P.M. and minimal activity at noon (Danielsson, 1972).

Diurnal cycles of cholesterol and bile acid synthesis are similar (Bortz et al., 1973; Bortz and Steele, 1973; Mitroupolos et al., 1973). Presumably alterations in normal lighting and feeding would reverse rhythmicity of cholesterol 7 α-hydroxylase as it reverses rhythmicity of HMG-CoA reductase (Edwards et al., 1972; Edwards and Gould, 1972). Edwards and Gould concluded that the daily rise in HMG-CoA reductase activity was due to ingestion of food. The new cholesterol produced may cause an increase in cholesterol 7 α-hydroxylase activity.

Influence of Dietary Variables on Cholesterol Metabolism

Dietary fat

Cholesterol biosynthesis Early attempts to demonstrate an effect of fat feeding upon cholesterol biosynthesis were hampered by a lack of knowledge of the biochemistry of cholesterol biosynthesis and the diurnal alteration of synthesis. The studies of Brice and Okey (1956) and Russell et al. (1962) did not yield conclusive data on the effect of fat intake on incorporation of labeled acetate into liver lipid. Brice and Okey measured cholesterol biosynthesis during the diurnal low. Russell and coworkers used labeled mevalonate as a precursor of cholesterol biosynthesis. The

major site of control of cholesterogenesis has since been shown to occur prior to mevalonate.

Concentration of dietary fat Investigators have demonstrated increased incorporation of labeled acetate into cholesterol in liver slices following dietary treatment with fat compared to zero fat containing diets. However, these data are questionable because the zero fat diets may have led to an essential fatty acid deficiency (Mukherjee and Alfin-Slater, 1958). Acetate incorporation into cholesterol following dietary treatment with zero fat or 30% hydrogenated coconut oil was depressed by 90% compared to controls fed 15% cottonseed oil. The addition of only 100 mg linoleic acid daily to either the fat-free or the hydrogenated coconut oil diet increased cholesterol synthesis in these groups to almost the level of controls.

Linazasoro et al. (1958) and Hill et al. (1960) fed zero fat compared with fat containing diets. Diets were fed for only 3 days; thus the possibility of essential fatty acid deficiency in the zero fat group was eliminated. Fifteen percent corn oil, Wesson oil, Snowdrift or lard diets were compared with a zero fat diet. Fat feeding was accompanied by a decrease in hepatic lipogenesis. This decrease was followed 12 hours later by increased hepatic cholesterogenesis.

The relatively long period required for increased cholesterogenesis after feeding has been reported by other investigators under conditions of controlled feeding. Although fat feeding produced an early accumulation of fatty acyl-CoA derivatives and acetyl-CoA with a decrease in fatty acid synthesis, no alteration in cholesterol synthesis was seen until much later

(Bortz, 1967). Corn oil (20%) was a relatively weak inducer of HMG-CoA reductase, but it caused a prolonged elevation of enzyme activity. The elevation began late in the fasting part of the diurnal cycle (Goldfarb and Pitot, 1972).

Saturation of dietary fat At present, agreement is lacking as to the effect of degree of saturation of dietary fat on cholesterol biosynthesis. However, there is strong support for enhanced cholesterogenesis by polyunsaturated fat. For example, greater rates of hepatic cholesterogenesis were reported with linoleic acid versus coconut oil (Merrill, 1959), with rapeseed oil, corn oil, erucic acid or oleic acid versus coconut oil (Wood and Migicovsky, 1958) and with erucic acid versus oleic acid (Carroll, 1959).

Boyd (1962) showed accelerated biosynthesis of cholesterol with 10% linoleate or safflower oil as compared to 10% stearate. In another study, rat livers were perfused with labeled palmitate, oleate, linoleate or linolenate. Total radioactivity incorporated into free cholesterol was higher with linoleate and linolenate than with palmitate and oleate (Tria et al., 1971). Dupont et al. (1972) found greater rates of synthesis with corn oil than with beef tallow.

The data of Reiser et al. (1963) contradict available evidence that polyunsaturated fat increased cholesterol biosynthesis. The authors fed 30% fat diets for 2 weeks. Fat sources compared were tributyrin, tricaproin, tricaprylin, trilaurin, trimyristin, tripalmitin, triolein, trilinolein, lard, butter oil, safflower oil and a synthetic mixed triglyceride. Trilinolein and safflower oil depressed cholesterogenesis most. Tri-

palmitin gave the highest rate of cholesterol biosynthesis in their study.

Interpretation of this study is difficult because rats were injected with labeled acetate at 8 A.M. and sacrificed 1 hour later. At 8 A.M. animals should have been near their daily nadir for cholesterol biosynthesis. Furthermore, the poor digestibility of many of the fats fed make comparisons between fats difficult.

Matthias[1] recently indicated that he and his coworkers fail to find differences in synthesis of cholesterol between safflower oil and beef tallow fed rats. They have used alanine, glucose and acetate as labeled precursors of cholesterol.

Control of cholesterol biosynthesis by fat Work on the causative relationship between dietary fat and rates of cholesterogenesis has not yielded a unified theory, though a number of relationships have been reported. Bortz (1967) looked at the time relationship between decreased fatty acid synthesis and increased cholesterogenesis following fat feeding. He concluded that some mechanism other than surplus of substrate must explain increased cholesterol biosynthesis secondary to fat feeding.

Tormanen et al. (1975) recently demonstrated that HMG-CoA reductase has at least two binding sites for NADPH. Preincubation of enzyme with NADPH yielded a very high specific activity enzyme. Physiological conditions, such as obesity, in which levels of NADPH are elevated may result in maximally activated HMG-CoA reductase (Tormanen et al., 1975). Hypertriglyceridemic individuals synthesized about 3 times as much cholesterol as did normotriglyceridemic subjects (Sodhi and Kudchodkar, 1973). Plasma

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[1] M. Matthias, Colorado State University, personal communication, 1975.

free fatty acid concentrations and endogenous synthesis of cholesterol were highly correlated.

The normal cycle of cholesterogenesis following dietary fat consumption required 24 hours to establish (Hill et al., 1960). Synthesis of HMG-CoA reductase may be induced by fat flow to the liver following fat feeding (Bortz et al., 1973).

It is not known whether degree of saturation of dietary fat influences cholesterol biosynthesis; or if it does, if the influence is secondary to altered rates of intestinal absorption or transport of fat into the liver. If absorption played a role it would be necessary to postulate differences in rates of absorption between polyunsaturated and saturated fat sources. Some investigators have indicated that rates of fat absorption are dependent upon chain length and degree of fat saturation (Steenbock et al., 1936; Deuel and Hallman, 1940; Deuel et al., 1941). More recently, McGovern and Quackenbush (1973b) found no evidence that safflower oil and beef tallow were absorbed at different rates.

The effect of saturation of dietary fat on cholesterogenesis is complicated by the fact that vegetable fats, fed for their polyunsaturated fat content, contain plant sterols. The sterols have been shown to influence hepatic cholesterogenesis, and are discussed in a later subsection.

Excretion of cholesterol and bile acids Fecal losses of neutral and acid steroids account for most body steroid loss. Neutral steroids include cholesterol and metabolites of cholesterol formed by intestinal bacteria. Coprostanol is the predominant bacterial metabolite recovered in feces. Acid steroids in excreta originate from bile acids synthesized in

the liver which are not reabsorbed during enterohepatic circulation of bile acids.

Bile acid excretion Some animal species may respond to unsaturated fat by increasing degradation of cholesterol to bile acids. There is evidence that such a response occurs in rat and man. Substitution of safflower oil for butterfat (Moore et al., 1968) or sunflower seed oil for butterfat (Antonis and Bersohn, 1962) raised bile acid excretion significantly in humans. Connor et al. (1969) fed cholesterol free diets with either cocoa butter or corn oil to normolipemic men. Bile acid excretion was increased significantly by polyunsaturated fat feeding. Furthermore, the loss of acid and neutral fecal steroids after corn oil was twice as great as that calculated to leave the plasma.

The effect of polyunsaturated fat on bile acid excretion appears to be associated with a change in bile acid half life in both rat and human. Corn oil feeding decreased cholic acid half life in human subjects (Gordon et al., 1964; Lindstedt et al., 1965). Safflower oil versus beef tallow stimulated cholate formation and shortened its half life in rats (McGovern and Quackenbush, 1973a). Rat bile was collected directly using bile duct cannulation. In rats fed safflower oil versus beef tallow, output of bile acids from the liver and conversion of labeled cholesterol to bile acids were significantly increased (McGovern and Quackenbush, 1973c). Furthermore, conversion of cholesterol oleate to bile acid was 40% higher with safflower oil compared to beef tallow.

Wilson and Siperstein (1959) reported similar excretion of labeled bile acids after diets which were either fat-free or contained corn oil or

lard. This finding is in contrast to most reports in the literature.

Caution should be used in interpreting differences in bile acid excretion as the result of degree of fat saturation, since polyunsaturated fats are of vegetable origin and contain plant sterols. The influence of plant sterols on bile acid synthesis will be discussed later.

Qualitative changes in bile acids with polyunsaturated fat feeding have also been noted. Lindstedt et al. (1965) demonstrated an increased cholate to chenodeoxycholate ratio in excreta of humans fed corn oil versus butterfat. McGovern and Quackenbush (1973a) showed a similar increase in this ratio in bile of rats fed safflower oil compared with beef tallow.

Neutral steroid excretion Excretion of neutral steroid may be increased in response to polyunsaturated fat sources. But this response is usually less marked than the increase seen in bile acid excretion. In human subjects increased neutral steroid excretion has been found with corn oil versus cocoa butter (Connor et al., 1969) and with safflower oil compared to butterfat (Moore et al., 1968).

Neutral steroid excretion was increased with corn oil compared to butterfat, but primarily as a result of elevated elimination of plant sterols and their metabolites. Coprostanone and (or) cholesterol excretion increased with corn oil compared to butterfat, although coprostanol was the predominant neutral steroid found (Eneroth et al., 1964).

Control of steroid excretion by fat Bile acid synthesis may be proportional to liver cholesterol concentration. Feeding of cholesterol and polyunsaturated fats have been shown to increase both liver cholesterol concentration (McGovern and Quackenbush, 1973b) and bile acid synthesis

(Gordon et al., 1964; Lindstedt et al., 1965; McGovern and Quackenbush, 1973c). The cellular events of the relationship are not understood.

Increased bile acid production with unsaturated fat sources is not necessarily due to the degree of unsaturation alone. Plant sources of fat contain sterols which may increase bile acid excretion independently of fat saturation (Spritz et al., 1965; Antonis and Bersohn, 1962). In most studies, however, no attempt has been made to account independently for variation due to fat saturation and plant sterol.

Although excretion data on bile acids have been discussed here as if this parameter were a direct indicator of bile acid synthesis, rates of synthesis as well as secretion of bile acids could be influenced by the dietary fat source. Furthermore, reabsorption of bile acid could be modified by dietary fat. Substitution of safflower oil for beef tallow increased conversion of cholesterol to bile acids by 13.6% while secretion of acid label from the liver was increased by only 8.6% (McGovern and Quackenbush, 1973c). Thus it would appear that hepatic synthesis of bile acids was increased while enterohepatic circulation rates of bile acids were reduced with safflower oil compared to beef tallow.

Other dietary constituents

Several dietary constituents other than fat have been variables in the studies to be presented. Plant sterols, fibers and protein level appear to influence cholesterol metabolism. Some documentation of their influence on cholesterol metabolism will be presented here; where possible a brief statement as to underlying mechanism of affect will be given. The studies presented here are examples and do not constitute a comprehensive review

of the literature.

Plant sterols Interest in plant sterols in relation to cholesterol metabolism was stimulated by the finding that ingestion of β-sitosterol decreased serum cholesterol concentration (Beveridge et al., 1958; Betzien et al., 1961). It was postulated that the underlying mechanism was due to a competition of plant sterols with cholesterol for absorption from the gut. However, this idea has been questioned recently. Sylven and Borgstrom (1969) and Subbiah and Kuksis (1973) failed to produce evidence for a mutual interference of cholesterol and sitosterol absorption.

The lowering of plasma and tissue cholesterol associated with even modest intakes of sitosterol could be due to extrabsorptive effects of the plant sterols. Sitosterol can influence cholesterol metabolism. Intraperitoneal injection of 5 mg β-sitosterol daily for 25 days, to circumvent intestinal absorption, increased cholesterogenesis in rats (Gerson et al., 1964). Recently, Subbiah (1973) demonstrated that between 22 and 32% of plant sterols consumed by rats were absorbed. In another study, dietary soy sterols increased the incorporation of acetate into cholesterol by rat liver slices and prevented negative feedback by cholesterol on hepatic cholesterogenesis (Fishlir-Mates et al., 1973).

It appears that plant sterols may increase bile acid production. Synthetic fats with fatty acid patterns resembling corn or coconut oils depressed excretion of bile acids compared with excretion following consumption of natural, sterol-containing fat sources (Spritz et al., 1965).

Plant sterols may also modify neutral steroid excretion. Fecal neutral steroids were elevated with dietary fats containing plant sterols

compared with animal fat (Eneroth et al., 1964; Connor et al., 1969; Moore et al., 1968). Sitosterol compared to cholesterol feeding nearly doubled the quantity of neutral steroids excreted (Subbiah and Kuksis, 1973).

Fibers Fiber is another dietary constituent which has been implicated in cholesterol metabolism. The general class of fiber includes cellulose, hemicellulose, pentosan, lignin, pectin and certain fatty substances and gums. Cereal grains contain approximately 2 g of fiber/100 g. Legumes contain from 3-5 g of fiber/100 g (Trowell, 1972). Consumption of large quantities of natural fiber have been associated with a hypocholesterolemic effect. Saponin in chick diets (Griminger and Fisher, 1958) and chick peas in human diets (Mathur et al., 1968) decreased serum cholesterol concentrations.

Semipurified diets fed to laboratory rats usually contain cellulose as a bulking agent. There is evidence that the removal of natural fiber and replacement with the fiber cellulose may have a hypercholesterolemic effect. A number of laboratories have reported increased serum cholesterol concentrations with semisynthetic diets in a variety of species. Liquid diets fed to calves versus liquid diets to which some dry feed had been added resulted in elevated serum cholesterol concentrations (Jacobson et al., 1973). Elevated serum cholesterol levels were also reported in baboons fed semisynthetic diets compared to diets consisting of bread, fruit and vegetables (Kritchevsky et al., 1974). Similar results were found when rats were fed semisynthetic diets compared to stock diet (Balmer and Zilversmit, 1974). Serum cholesterol concentrations were increased in

humans and rats consuming baked products containing cellulose. Carcass cholesterol concentrations were also increased in rats. Cholesterol concentrations increased in proportion to the cellulose content of the products consumed (Ahrens et al., 1972).

The effects of some fibers on serum cholesterol concentration appear to be due to altered (increased or decreased) rates of cholesterol catabolism. Semisynthetic diets compared to normal or stock diets were accompanied by a decreased conversion of cholesterol to bile acid (Kritchevsky et al., 1974; Balmer and Zilversmit, 1974). Rats fed semipurified diets had slower rates of 7 α-hydroxylation of liver cholesterol than did rats fed stock diet (Johansson, 1970).

Certain dietary fat sources may overcome decreased bile acid excretion in response to low-fiber or fiber-free diets. In one study, fiber-free diets decreased bile acid excretion with butterfat but not with diets containing oil or hydrogenated oil (Antonis and Bersohn, 1962).

Other fibers may interfere in absorption of cholesterol. Saponins, which are widely distributed, particularly in leguminous forage plants such as alfalfa, form insoluble complexes with cholesterol in the gut.

Protein level Protein level is another dietary factor implicated in cholesterol metabolism. Plasma and liver cholesterol concentrations were higher on low protein than on high protein diets, but similar rates of cholesterol absorption were found at both levels of protein intake (Kenney and Fisher, 1973). Yeh and Leveille (1973) concluded that the hypocholesterolemic effect of high levels of protein in chicks was mediated through rapid removal of cholesterol from the blood and increased excretion

in the feces as cholesterol and bile acids.

Influence of Periodicity of Eating on Cholesterol Metabolism

Serum cholesterol concentrations

Our interest in meal pattern as a variable in the study of cholesterol metabolism stems largely from observations that consumption of the daily food allotment in a limited time period results in increased serum cholesterol concentration.

Although Okey et al. (1960) failed to observe differences in mean serum cholesterol levels between rats fed ad libitum and those fed for 3 hours per day, others have consistently observed an increase in serum cholesterol in a variety of species including rats. Thus chicks allowed access to food for 2 1-hour periods per day had higher serum cholesterol concentrations than did chicks fed ad libitum (Cohn et al., 1961). Monkeys fed for 2 or 3 1-hour periods per day had elevated serum cholesterol compared to animals allowed to consume their food ad libitum (Gopalan et al., 1962).

The effect of limited access to food on serum cholesterol levels may be transitory. Leveille and Hanson (1965) fed chicks for 2 1-hour periods per day. After 3-6 weeks, plasma cholesterol concentrations were elevated, but differences disappeared after the birds had been on the regimen for 10 weeks. However, Gopalan et al. (1962) found a larger difference between serum cholesterol concentrations of ad libitum fed monkeys at 8 than at 4 weeks. Reeves and Arnrich (1974), in this laboratory, found that the difference between serum cholesterol concentrations of ad libitum and meal

fed rats was more significant at 30 than at 10 days after feeding.

Data on humans are contradictory. Six individuals consuming 6 instead of 3 meals per day had decreased serum cholesterol levels (Cohn, 1964). In a recent study, Wadhwa et al. (1973) could not find an effect of meal pattern on serum cholesterol levels in man.

Synthesis of cholesterol

Few attempts have been made to assess the influence of meal feeding on cholesterol metabolism. Our literature search yielded five studies in which feeding frequency was limited. These studies were published between 1959 and 1966, before the diurnal control of cholesterol biosynthesis had been recognized.

Increased cholesterol biosynthesis followed increased utilization of fat, either from diet or from body stores (Dupont and Lewis, 1963). In another study, caloric allowances were restricted to 80, 60, 40 and 20% of the amount of food consumed ad libitum (Dupont, 1965). Caloric deficit and cholesterol biosynthesis were positively correlated.

In contrast, Cockburn and Van Bruggan (1959) showed that a 45 minute feeding period decreased incorporation of labeled acetate into cholesterol in gut, liver, carcass and skin in vivo. In the same laboratory, in vitro cholesterogenesis was maximal in the 2 hour fasted rat compared to ad libitum fed controls.

Although the bulk of the evidence supports the hypothesis that decreased feeding frequency increases cholesterogenesis, further study is needed.

Excretion

Data available on the catabolism of cholesterol to bile acids, and the excretion of these metabolites in response to caloric restriction, come exclusively from one laboratory. Bobek et al. (1973b) compared ad libitum fed rats to rats fed for 2 hours per day. A number of parameters measured during the 4 days following injection of 4-^{14}C-cholesterol were identical with both feeding patterns. They were specific activities of cholesterol in serum, liver, adrenal gland, small intestine and carcass; biological decay of serum cholesterol radioactivity; total excretion of radioactivity; distribution of bile acids and neutral steroids in excreta.

In another study, meal feeding shortened plasma cholesterol half life and raised fractional cholesterol turnover rate following injection of 26-^{14}C-cholesterol (Bobek et al., 1973a).

The experimental model used by Bobek and coworkers to study cholesterol metabolism with decreased feeding frequency may be criticized since serum and liver cholesterol concentrations are not altered by meal pattern (Bobek et al., 1972; Bobek et al., 1973a).

It is difficult to get a comprehensive picture of the influence of meal pattern on cholesterol metabolism. The problem is open to further investigation.

METHODS AND PROCEDURES

Selection and Treatment of Animals

Experimental design

Three studies were designed to evaluate the effects of periodicity of eating, variation in dietary fat and level of dietary protein on food efficiency, lipogenesis, tissue cholesterol levels and specific parameters related to cholesterol metabolism.

Pre-experimental Male adult Wistar rats from the stock colony of this department were used in all three studies. During the pre-experimental period, from weaning until they reached 500 to 525 g body weight at 4 to 5 months of age, rats were fed a modified Steenbock XVII ration (Table 1).

At this weight animals were transferred to a room where a reversed lighting schedule was maintained for the convenience of the researchers. Dark hours were maintained between 9 A.M. and 9 P.M.; light hours between 9 P.M. and 9 A.M. Each animal was housed individually in a one-half inch mesh wire cage. All laboratories were maintained at 24±1° with a relative humidity of approximately 40%.

Dietary restriction After a 16 hour fast, 500 g animals were given a depletion diet at 9 A.M. the following morning. The depletion diet contained essentially 100% of calories from corn starch. Daily supplements of α-tocopherol in corn oil and cod liver oil were given to maintain essential fatty acid and fat soluble vitamin sufficiency in the experimental animals. Animals received daily 0.75 mg DL-α-tocopherol made to 50 mg with

Table 1. Stock ration for male rats: Modified Steenbock XVII

Dietary component	Percent
Corn meal[a]	48.3
Linseed meal[b]	13.8
Skim milk[c]	10.3
Wheat germ[d]	8.6
Yeast, brewers[a]	8.6
Casein, high protein[a]	4.3
Alfalfa meal[e]	1.7
NaCl[f]	0.4
$CaCO_3$ plus trace elements[g]	0.4
Corn oil[h]	3.5
Corn oil plus Vitamin D_3[i]	0.1

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

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

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

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

[e]National Alfalfa, Lexington, Nebraska.

[f]Local grocer.

[g]Matheson Coleman and Bell, Division of Matheson Company, Inc., Norwood, Ohio.

[h]Mazola, Best Foods Division Commercial Products Company, New York, New York.

[i]Crystalline Vitamin D_3 diluted with corn oil to give 50 µg Vitamin D_3/kg diet.

corn oil and 50 mg cod liver oil. A water soluble vitamin mix was also given daily. Composition of the mix and size of dose are given in Table 2. Composition of the OF-OP (depletion) diet is given in Table 3.

When rats reached 400 or 380 g body weight (Experiments 1 and 3, and Experiment 2, respectively) calories were restricted severely. Two 2.5 g portions of OF-OP diet were given, one at 9 A.M. and one at 5 P.M. This feeding schedule was followed until the rats reached 300 g body weight. At this point, animals were either sacrificed (depleted controls) or refed for 10 days. Level and source of dietary fat, meal pattern and level of protein were treatment variables.

Realimentation Animals were assigned to treatment groups so that days required to deplete the animals from 500 to 300 g body weight were equalized for each treatment within the study. The dietary and meal pattern variables comprising treatments for each study are shown in Table 4. These variables will be discussed in this section under experimental diets.

Radiotracers In the last two studies 2.5 or 5 μC 4-^{14}C-cholesterol and 50 μC ^{3}H-acetate were injected intraperitoneally to measure acute turnover of cholesterol and cholesterol biosynthesis respectively. The labeled cholesterol was injected on the morning of the 5th day of refeeding. On the 10th day of refeeding, all animals in Experiment 2 were fasted from 5 P.M. to 9 A.M. In the 3rd study, ad libitum (AL) fed animals were allowed access to food from 5 P.M. to 9 A.M. as was their usual pattern. In both experiments, animals were allowed food from 9 A.M. to 10 A.M. They were then injected with labeled acetate and killed 2.5 hour later according to Dupont et al. (1972).

Table 2. Composition of water soluble vitamin mixture

Vitamin	Composition per rat per day	Composition per 1000 doses
Thiamine HCl[a]	40 µg	40 mg
Riboflavin	60 µg	60 mg
Pyridoxine HCl	40 µg	40 mg
Ca-pantothenate	100 µg	100 mg
Nicotinic acid	500 µg	500 mg
Folic acid	8 µg	8 mg
Biotin	1 µg	100 mg Biotin-dextrine mixture[b]
Vitamin B_{12}	.75 µg	750 mg B_{12}-mannitol mixture[c]
Ascorbic acid	1 mg	1 g
Choline chloride	5 mg	5 g
Inositol	10 mg	10 g
p-Amino Benzoate	10 mg	10 g
Dextrin to make 500 g		

[a]All vitamins were obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[b]Biotin was mixed with dextrin so that 100 mg of the mixture yielded 1 mg biotin.

[c]Commercially available Vitamin B_{12} in mannitol furnished 0.1 mg B_{12} per 100 g of mixture.

Table 3. Composition of experimental diets

Dietary component	Depletion diet	Diets refed ad libitum or for 8 of 24 hours for 10 days					
	OF-OP	OF-P	OF-4P	20SO-P	20SO-4P	20BT-P	20BT-4P
				% weight			
Corn starch[a]	93.5	88.0	72.7	66.0	46.8	66.0	46.8
Safflower oil[b]	0	0	0	20.0	20.0	0	0
Beef tallow[c]	0	0	0	0	0	20.0	20.0
Lactalbumin[d]	0	5.1	20.4	6.4	25.6	6.4	25.6
William-Briggs salt	3.5	3.5	3.5	4.4	4.4	4.4	4.4
Non-nutritive fiber[e]	2.8	2.8	2.8	3.0	3.0	3.0	3.0
NaCl, iodized	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Cholesterol	-	0.02	0.02	0.02	0.02	-	-
				% kcal			
Corn starch	100	94.5	78.1	56.2	39.9	56.2	39.2
Fat	0	0	0	38.3	38.3	38.3	38.3
Lactalbumin protein	0	4.3	17.1	4.2	17.0	4.2	17.0

[a]Argo, Best Foods Division Commercial Products Company, New York, New York.

[b]PVO International Corporation, Richmond, Cal. + 0.1% α-tocopherol.

[c]Tocopherol added, Oscar Meyer, Madison, Wisconsin.

[d]78% protein, Nutritional Biochemical Corporation, Cleveland, Ohio.

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

Table 4. Experimental protocol

Treatment			Experiment		
Diet		Meal pattern	1	2	3
			Pre-experimental		
Stock	-	AL	X	X	X
			Depletion		
OF-OP	-	MF	X	X	-
			Refeeding		
OF-P	-	AL	X	X	-
		MF	X	X	-
OF-4P	-	AL	X	-	-
		MF	X	-	-
20SO-P	-	AL	X	X	X
		MF	X	X	X
20SO-4P	-	AL	X	-	X
		MF	X	-	X
20BT-P	-	AL	-	X	X
		MF	-	X	X
20BT-4P	-	AL	-	-	X
		MF	-	-	X
Stock	-	AL	-	X	-

Diets

Stock diet The composition of the stock diet, a modified Steenbock XVII, is listed in Table 1. Animals consumed this diet ad libitum from weaning until they weighed between 500 and 525 g (the pre-experimental period). The stock diet was supplemented weekly with 15 g of lean ground beef, 20 g of carrot, 10 g of cabbage, 165 µg retinyl acetate and 1.25 mg Vitamin D_3. The two fat soluble vitamins were given directly by mouth in 50 mg corn oil.

Experimental diets The compositions of experimental diets fed to depleted rats are given in Table 3. Percentage contributions of corn starch, fat and lactalbumin protein to caloric intakes are also listed. Protein, salt mix and non-nutritive fiber were equalized on a caloric rather than a weight basis.

Fat and protein level were varied in these studies. Dietary fat provided 0 (OF) or 38.3 (20SO or BT) % of calories. Fat calories were provided by safflower oil (SO) or beef tallow (BT). Lactalbumin was the source of dietary protein for all refed diets, except the stock diet used for refeeding (Experiment 2). Two levels of dietary protein, 4.2 (P) and 17.0 (4P) % of calories, were fed. In 4P diets, calories from lactalbumin protein were increased compared to P diets at the expense of calories from cornstarch.

In addition to the experimental diets, water soluble and fat soluble vitamin supplements were given daily. The water soluble vitamins and doses are given in Table 2. Vitamins A and D were furnished by 50 mg cod liver

oil[1]. Vitamin E was provided as DL-α-tocopherol acetate diluted with corn oil[2] so that 50 mg daily provided 0.75 mg DL-α-tocopherol acetate. Fat soluble vitamins were measured using a dropper calibrated so that 2 drops closely approximated 50 mg.

Meal patterns

Diets were fed on two meal schedules. Rats were either allowed access to a given diet for 24 hours per day (AL) or for an 8-hour period (from 9 A.M. to 5 P.M.) out of 24 hours (MF). Food was given to MF animals daily at the beginning of the dark cycle. The majority of animal handling was done between 8 and 9 A.M. in the last hour of the light cycle.

Autopsy

All rats were sacrificed following injection with sodium pentobarbitol[3]. In Experiment 2 all animals were injected with 50 mg while in Experiments 1 and 3, 25 mg per 100 g body weight was injected. When the animals were unconscious, blood was removed by heart puncture. One drop of DL-α-tocopherol solution (1.6 mg/ml ethanol) was added per 2 ml blood to retard lipid oxidation. After clotting, blood samples were centrifuged in a clinical centrifuge at 4° for 20 minutes. Serum was carefully lifted out with a Pasteur pipette and stored in tightly covered vials at -20° for serum analyses.

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[1]Squibb Cod Liver Oil, USP, manufacturer guarantees 1700 IU of Vitamin A and 170 IU Vitamin D per g of oil.

[2]Mazola, Best Foods Division Commercial Products Company, New York, New York.

[3]Nembutol sodium, Abbott Laboratories, Chicago, Illinois.

Liver was excised immediately after removal of blood, weighed and quick frozen in liquid nitrogen. Heart, kidneys, epididymal fat and small and large intestines were also removed, weighed and quick frozen in liquid nitrogen. All tissues and the remaining eviscerated carcasses were heat sealed in Kapak pouches[1] and stored at -20° for later analysis. Feces excreted during the six days following injection of labeled cholesterol were also stored at -20° for later analysis.

Tissue Analysis

Serum

Lipid extraction In Experiment 1, serum lipid was extracted according to the method of Sperry and Brand (1955). After extraction, samples were evaporated to dryness under nitrogen at 35-40° and transferred to vials with approximately 5 ml chloroform. Lipid samples were stored under nitrogen in tightly sealed vials at -20° until used for column separation of lipid classes.

Separation of lipid classes Serum lipid extracts were removed from storage, evaporated to less than 200 µl under nitrogen, and fractionated according to the method of Lis et al. (1961). The cholesterol ester fraction and triglyceride fractions were checked for purity using thin layer chromatography.

Esterification Cholesterol ester fatty acids were methylated by a modification of Stoffel et al. (1959). Two % sulfuric acid in methanol was used to esterify cholesterol ester fatty acids. Hexane was used to extract the methyl esters of fatty acids.

[1]Kapak Industries, Inc., Bloomington, Minnesota.

Gas-liquid chromatography A Beckman GC-72-5 gas chromatograph equipped with dual flame ionization detectors and connected to Infotronics CRS 208 electronic integrator was used for analysis of fatty acid methyl esters. Instrument conditions for analysis were: stainless steel columns, 10 feet by 1/8 inch, packed with 3% ethylene glycol succinate on Gas-Chrom G 100/120 mesh HP; column temperature, 180°; injector temperature, 220°; detector temperature, 220°; detector line temperature, 250°; carrier gas flow, 30 ml per minute.

Cholesterol analysis Cholesterol was analyzed colorimetrically following digitonin precipitation by the method of Sperry and Webb (1950). Cholesterol was also analyzed radiochemically in Experiments 2 and 3 following digitonin precipitation and dissolution of the precipitate in methanol. Serum aliquots of 1 ml were extracted into acetone-ethanol (1:1). In Experiment 1, 2 6-ml and 2 3-ml aliquots of the extract were used for colorimetric analysis of serum free and total cholesterol (mg/dl), respectively. In Experiments 2 and 3, 6 3-ml aliquots were used, 2 for free and 4 for total cholesterol determination. The 3-ml aliquots used for free cholesterol were kept proportional with Experiment 1 by the addition of 1.5 ml digitonin solution to 3 ml extract (instead of 3 ml digitonin solution to 6 ml extract). After the acetone-ether (1:2) wash, 2 of the aliquots, prepared as for total cholesterol determination, were transferred quantitatively with methanol to scintillation vials. Methanol in excess of 1/2-1 ml was evaporated off.

Scintillation counting Scintillation cocktail (either 15 ml Spectrafluor PPO-POPOP[1] or Spectrafluor Butyl PBD[2] in toluene) was added to each sample. These samples were counted in duplicate on a Packard Tri Carb Liquid Scintillation Spectrophotometer (Model 3320) equipped with an external standard. External standardization and/or internal standardization with ^{3}H and ^{14}C-toluene[3] were used to correct for decreased efficiency due to quenching. Channels and gains were set for simultaneous counting of ^{3}H and ^{14}C.

Liver

Total lipid extraction The method of Folch et al. (1957), as modified by Stadler (1969) was used to extract total hepatic lipid. The extract was used for gravimetric determinations of lipid, separation of lipid classes (Experiment 1), ^{3}H-acetate incorporation into fatty acid and cholesterol, and colorimetric and radiochemical analyses of cholesterol (see scheme Figure 2).

Separation of lipid classes A modification of the method of Hirsch and Ahrens (1958) was used to separate cholesterol ester from total hepatic lipid. Silicic acid[4] was activated by Stadler's (1969) modification of Borgström's (1952) procedure. Each column used 6 g silicic acid. Between

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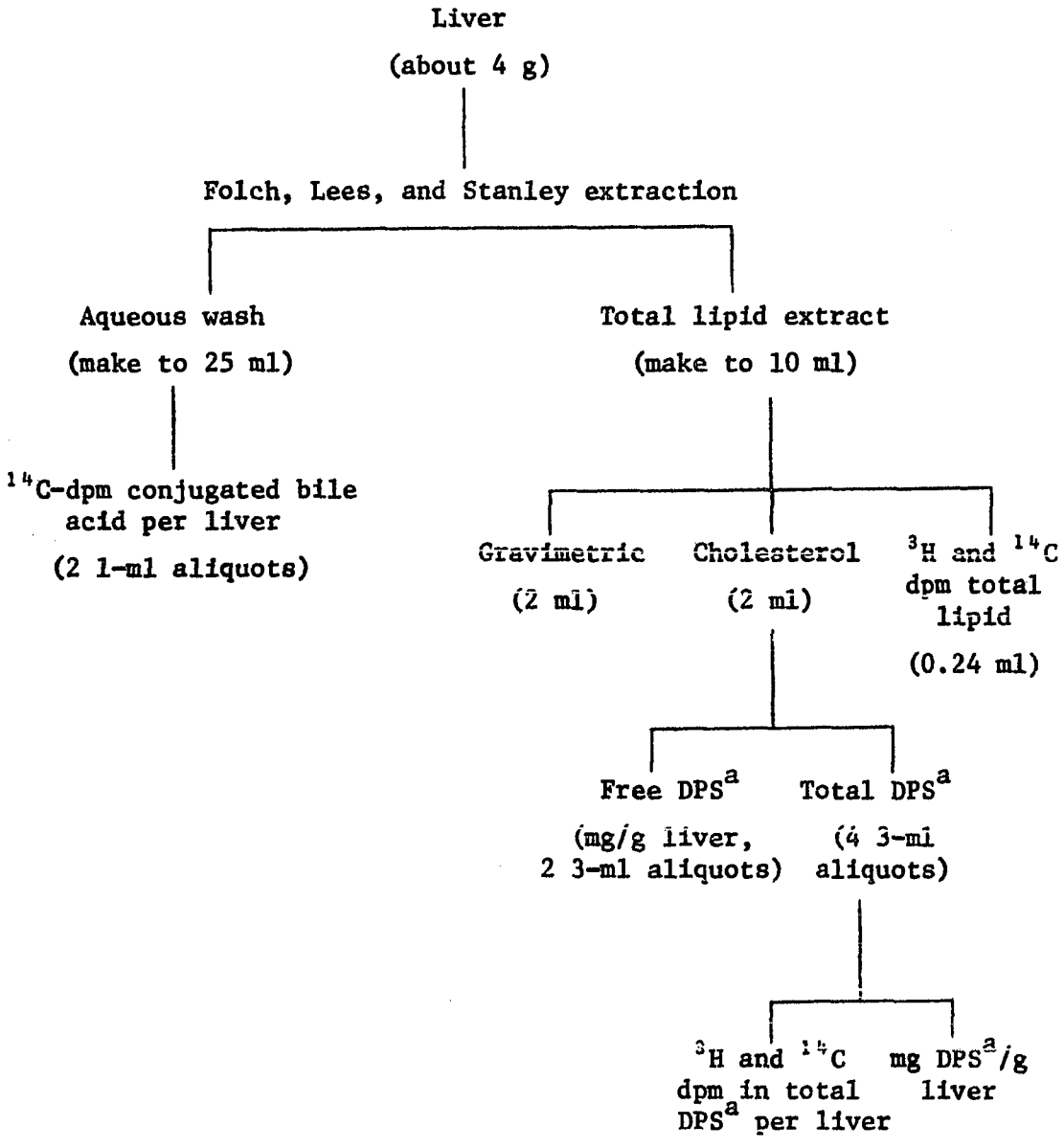
[1] Amersham/Searle Corporation, Arlington Heights, Illinois. Concentration of cocktail was made 1 1/2 times standard.

[2] Amersham/Searle Corporation, Arlington Heights, Illinois. Concentration of cocktail was made as recommended.

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

[4] Analytical grade, Mallenchrodt, 100 mesh, Mallenchrodt Chemical Works, St. Louis, Missouri.



[a]Digitonin precipitable steroid.

Figure 2. Schematic of liver analyses

90 and 120 mg of total hepatic lipid were used in each separation. Cholesterol ester was eluted with 120 ml of 1% diethyl ether in petroleum ether (b.p. 60-70°, redistilled).

Esterification The procedure used for preparation of methyl esters of cholesterol ester fatty acid was identical with that used for serum cholesterol fatty acid.

Gas-liquid chromatography Gas-liquid chromatographic analysis was identical with that used for serum fatty acid analysis.

Cholesterol A 2 ml aliquot, representing 1/5 of total liver lipid extract from approximately 4 g liver, was pipetted into a 25 ml volumetric flask. The choloroform was evaporated to dryness under nitrogen and acetone-ethanol (1:1) was added to the mark. Six 3-ml aliquots of the acetone-ethanol mixture were used, 2 for free and 4 for total cholesterol determination according to the procedure of Sperry and Webb (1950). Two of the total cholesterol samples were transferred with methanol to scintillation vials following an acetone-ether (1:2) wash. Samples were prepared and counted by the procedure described for serum cholesterol. The remaining samples were assayed colorimetrically according to the procedure of Sperry and Webb (1950).

Hepatic conjugated acid steroid-^{14}C The aqueous wash (upper phase) was quantitatively transferred to a 25 ml volumetric flask and made to volume with methanol. Two 1-ml aliquots were removed to scintillation vials and 15 ml scintillation cocktail was added to each vial. Samples were counted with channel and gain settings appropriate for double label counting of ^{3}H and ^{14}C. An external standard curve of a quenched set of ^{14}C

standards was used to correct for loss of efficiency. Data were expressed as ^{14}C-dpm acid steroid per liver.

Total hepatic lipid ^{3}H and ^{14}C A small aliquot of total hepatic lipid was counted to determine total liver lipid ^{3}H and ^{14}C. The aliquot was pipetted into a scintillation vial, the choloroform was evaporated and scintillation fluid was added. Samples were solubilized in scintillation fluid without the addition of methanol. Counting and efficiency corrections were carried out as previously described for serum and liver cholesterol digitonide.

Small intestine

Homogenization and extraction Small intestine with contents was minced slightly and homogenized in chloroform-methanol (2:1) for 5 minutes. A Lourdes[1] multi-mix homogenizer with a 250 ml stainless steel cup was used for the homogenization. Original homogenization was done with approximately 100 ml of solvent. The homogenate was made to 200 ml with chloroform-methanol giving a solvent to tissue ratio of 20:1. The homogenate was shaken well. A 40 ml aliquot was removed rapidly and placed in a stainless steel centrifuge tube. Samples were layered with nitrogen and capped. They were centrifuged[2] at -4° for 20 minutes according to Stadler (1969) to remove the small amount of fragmented tissue.

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[1]Brooklyn, New York.

[2]International High Speed Refrigerated Centrifuge, Model HR 1, International Equipment Company, Boston, Massachusetts.

Conjugated acid steroid-^{14}C The supernatant was poured into a 50 ml glass stoppered centrifuge tube. Eight ml of distilled water were added to the chloroform-methanol extract. About 2 ml of chloroform-methanol (2:1) was used to rinse the precipitated tissue and the centrifuge tube. This rinse was added to the glass stoppered centrifuge tube. The entire mixture was shaken vigorously for 2 minutes. The tubes were centrifuged for 15 minutes at very low speed in an International Model EXD centrifuge. The upper phase was quantitatively removed into two scintillation vials for ease of evaporation. An additional 5 ml water wash followed removal of the first wash. Samples were shaken for 1 minute and centrifuged as before. The upper phase was transferred to the same two scintillation vials. The upper phases were evaporated to dryness under nitrogen in an N-Evap[1] at 75-80°. Contents of both scintillation vials were recombined. They were transferred with methanol to the same 25 ml volumetric flask. Two 1-ml aliquots were removed to clean scintillation vials. Scintillation cocktail was added to each vial. Counting and correction for efficiencies were carried out as described for serum.

Cholesterol Two methods were used for digitonin precipitable steroid preparation. The less complicated method was a modification of the procedure used for preparation of liver digitonin precipitable steroid. This method is described below. It was used for analysis in both Experiments 2 and 3. The washed chloroform-methanol extract was made to 25 ml and 6 3-ml aliquots were removed for each animal. These aliquots were evaporated to dryness under nitrogen at between 30 and 35°. To each sample was

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[1]Organomation Association, Model 10, Worcester, Massachusetts.

added 3 ml acetone-ethanol (1:1). These aliquots were prepared for cholesterol analysis according to Sperry and Webb's (1950) procedure for serum lipid extract. Two samples were used for free and 2 for total chemical analysis of cholesterol. The remaining 2 samples were prepared as for total cholesterol determination, rinsed and transferred with methanol to scintillation vials. Most of the methanol was evaporated off, cocktail was added and counting and quench correction done as for serum cholesterol digitonide.

Another procedure used to extract digitonin precipitable steroid was a modification of the method of Grundy et al. (1965) for extraction of fecal neutral steroids. This method was used only in Experiment 2. It compared favorably with that described by McGovern and Quackenbush (1973b) for extraction of neutral steroid from washed intestines. It also compared well with modification of the method of liver neutral steroid extraction outlined in detail above.

A 40 ml aliquot of total small intestine homogenate was evaporated to dryness under nitrogen and saponified at 45° for 3 hours in 20 ml 1 N NaOH in 90% ethanol. After saponification, 10 ml water and 50 ml petroleum ether (Fisher Scientific, b.p. 37.7-48.9°) were added. Four 4-ml aliquots were removed, and evaporated to dryness under nitrogen. Acetone-ethanol (1:1) and digitonin solution were added as for serum and liver cholesterol analysis. Digitonin precipitation yielded four total cholesterol samples. Two were used for colorimetric analysis and 2 were dissolved in methanol and counted as described previously for serum cholesterol digitonide.

Large intestinal contents and feces

Homogenization Feces and large intestinal contents were washed into a 250 ml stainless steel cup attachment of a Lourdes multi-mix homogenizer. Feces were diluted 4-5 times with water. The mixture was homogenized for 5 minutes, or until a homogenous mixture was obtained. The homogenate was made to 200 ml (100 ml for depleted controls, 500 ml for stock refed) in water with methanol added as needed to break foams formed.

Aliquot preparation Two 5-ml aliquots were removed to small aluminum weighing dishes and lyophilized[1] overnight. The dry material was crushed and carefully removed to a 250 ml centrifuge bottle. Transfer and rinsing were accomplished with 20 ml 1 N NaOH in 90% ethanol.

Neutral steroid-^{14}C Samples were stoppered and heated at 45° for 1 hour in a water bath. Ten ml distilled water and 50 ml petroleum ether were added. The bottle was stoppered and shaken vigorously for 1 minute. The bottle was centrifuged at 1000 x g in an International Model EXD centrifuge. The upper phase was removed and the aqueous phase was reextracted two more times with 50 ml petroleum ether. Each extraction was followed by a 5 minute centrifugation at 1000 x g. The petroleum ether extracts were combined and backwashed with 10 ml of 1 N NaOH in 50% ethanol to remove any traces of acid steroid-^{14}C. The washed extract was evaporated to dryness on a steam bath, transferred quantitatively to a scintillation vial and evaporated to dryness again. Scintillation cocktail was added as described earlier and samples counted in duplicate. Single label counting of fecal neutral-^{14}C was done with channels set from 50-1000. Gains were

[1]Virtes Lyophilizer, Gardiner, New York.

set at 10 and 20 for channels 1 and 2, respectively, so that simultaneous counting at two gains occurred. Quench curves at these two gains were used. The optimum curve for each sample was used to correct for color quenching in fecal samples.

Acid steroid-^{14}C The aqueous phase remaining after petroleum ether extractions was acidified to pH 2 with concentrated HCl and extracted with 75 ml of chloroform-methanol (2:1) according to Grundy et al. (1965). Their rigorous saponification preceding chloroform-methanol extraction was omitted. The bottle was stoppered and shaken vigorously for 5 minutes at 1000 x g. The lower phase was removed and evaporated on a steam bath. The aqueous phase was extracted two more times with 50 ml chloroform. All three extractions were combined and evaporated to dryness. The viscous brown residue remaining was transferred to a 25 ml volumetric flask and made to volume with methanol. Two 1-ml aliquots were removed to scintillation vials and 15 ml scintillation cocktail was added to each aliquot. Samples were counted as for fecal neutral steroid-^{14}C.

Epididymal fat

Epididymal fat was extracted according to a modification of Stadler (1969) of the method of Folch et al. (1957). Total fat was determined by gravimetric analysis. In Experiment 2, an aliquot of total fat was evaporated to dryness, scintillation cocktail added and samples counted for H-acetate incorporation into fatty acid. Channel and gain settings appropriate for double label counting were used to correct for neutral steroid-^{14}C in total epididymal lipid.

Carcass

Homogenization A modification of the method of Judge[1] was used for homogeneous preparation of carcass (minus liver, epididymal fat and 10 ml blood). Carcasses were autoclaved in a quart jar at 15 pounds pressure for 1 hour. The warm carcass was transferred carefully to a large preweighed 4 quart Waring blender. An amount of water approximately equal to the weight of the rat was used to rinse the jar. Finally the jar was rinsed with about 10 ml ethanol.

The rat carcass was blended on lowest speed for 10 minutes. Blender sides were scraped down with a spatula. The blender was turned to low, then to medium speed. Blending was continued for 15 minutes longer. During the last 15 minutes, the blender motor was stopped two or three times to wash down lid and sides of the blender. The homogenate was cooled to 25-35°. Blender and homogenate were weighed and weight recorded. The mixture was blended for about 1 minute longer on low speed until well mixed. Samples were taken for moisture and fat analysis with blending between sampling to keep the mixture homogeneous.

Moisture Preweighed cups were used to dry samples of homogenate at 68° for 48 hours under vacuum. Moisture (%) was calculated as below:

$$\text{Moisture}(\%) = 100 - \frac{(\text{weight dry matter in aliquot} \times 100)}{\text{weight rat in aliquot}}$$

$$\text{where weight rat in aliquot} = \frac{\text{fresh carcass weight}}{\text{weight of homogenate}} \times \text{weight of aliquot.}$$

[1] J. Judge, unpublished data from this laboratory, 1970.

Carcass fat Carcass fat was determined by a modification of the method of Soderhjelm and Soderhjelm (1949) for fecal fat determination, using Mojohnnier flasks. Carcass fat (%) was calculated as the weight of fat in the aliquot times 100 divided by the weight rat in the aliquot (see moisture determination).

Carcass hydrolysis Rat carcasses were saponified for 16 hours in 10% NaOH with 95% ethanol at a temperature between 75 and 80°, according to the method of Dupont et al. (1972). The solution was funnelled into a 1000 ml volumetric flask and made to volume with 50% ethanol. Ten ml aliquots were removed to 250 ml centrifuge bottles and 20 ml of 50% ethanol was added to each bottle. Fifty ml of petroleum ether (Fisher Scientific, b.p. 37.7-48.9°) was added and the mixture shaken well for 2 minutes. Samples were stoppered and centrifuged at 1000 x g for 5 minutes. Immediately 4 4-ml aliquots were pipetted from the upper petroleum ether phase into 4 12-ml centrifuge tubes and evaporated to dryness under nitrogen.

To each of the 4 evaporated aliquots was added 6 ml acetone-ethanol (1:1) and 3 ml digitonin solution and 1 drop of 10% acetic acid. Precipitation was allowed to continue overnight. Samples were carried through the cholesterol analysis according to Sperry and Webb (1950). Two samples were analyzed colorimetrically and 2 were transferred to scintillation vials for double label counting. Cholesterol digitonide was dissolved and counted according to the procedure used for serum cholesterol digitonide.

Acid steroid-^{14}C For approximately 1/2 of the carcass samples, extraction of neutral steroid-^{14}C was completed with two additional 50 ml petroleum ether extractions. These were discarded and the aqueous lower phase was acidified to pH 2 with concentrated HCl and extracted with 75 ml

chloroform-methanol (2:1). The procedure used was for extraction of fecal acid steroid (Grundy et al., 1965). The rigorous saponification preceding extraction was omitted. Because all samples analyzed for acid steroid-^{14}C yielded negligible quantities of label in this fraction, this extraction was not done for all rats.

Other tissue analyses

In Experiment 2, kidney and heart lipid were extracted using Stadler's modification of Folch et al. (1957). Cholesterol was precipitated with digitonin and analyzed colorimetrically and radiochemically by procedures similar to the procedure used for liver cholesterol analysis.

Statistical Analysis

A Monroe 1350 calculator was used to calculate means and standard errors of means for groups. Data were also computed for analysis of variance for treatments and residual, or for fat, protein and meal pattern tested against residual. The Statistical Analysis System (SAS) of Barr and Goodnight (1971) was used. Differences between individual groups were calculated using the student T test or LSD (least significant difference) for $P<.05$.

RESULTS

In the data which follow, reference to one of three experiments will be made. The reader may wish to refer to Table 3 for a summary of the dietary and meal pattern variables employed in each experiment. It can be generally assumed that failure to report findings for a given variable results because that particular variable was not included in the experiment under discussion.

Data from depleted controls and stock refed animals are expressed in tables in this section. Results from these groups were used for interpretation but will generally not be elaborated on here.

A glossary of commonly used abbreviations is printed prior to the introduction, though an attempt has been made to define each abbreviation used as it occurs in the text.

The parameters considered in the results fall into several major categories: 1) tissue weights; 2) tissue cholesterol concentrations; 3) recovery of ^{14}C-labeled neutral and acid steroid, following intraperitoneal injection of 4-^{14}C-cholesterol as a measure of acute turnover of cholesterol from the rapidly equilibrating pool; 4) recovery of ^{3}H-label in total lipid and digitonin precipitable sterol (DPS), after injection of ^{3}H-acetate, as a relative measure of cholesterol and fatty acid biosynthesis.

The results from body weights, food consumption and food efficiency confirm the findings of Reeves (1971) with similar diets and meal patterns. They are summarized briefly in this section. Tables and figures pertaining to these parameters are included in the Appendix (Tables 34 to 39, Figures 10 to 13).

Body Weights

Within each study, pre-experimental and depleted body weights were statistically similar by design. Mean number of days required to deplete rats were 32, 54 and 32 for Experiments 1, 2 and 3, respectively (Tables 34, 35 and 36). Individual treatment means were statistically similar for this variable within each experiment. In Experiment 2, length of depletion time was 69% greater than that in Experiments 1 and 3. This discrepancy between experiments can be attributed to higher initial weights and a modified depletion procedure for Experiment 2 compared with Experiments 1 and 3.

All variables - fat concentration or source, protein concentration and feeding frequency - significantly influenced weight gained during refeeding.

Food Consumption and Food Efficiency

Food consumption

Concentrations and source of dietary fat, as well as feeding frequency, influenced caloric consumption during the 10 day period of realimentation (Tables 37 and 38, Figures 10 and 12). Differences in dietary protein concentration (P or 4P), however, did not affect food consumption in the same refeeding period (Experiments 1 and 3).

Food efficiency

In every case where protein was increased (P versus 4P) food efficiency increased ($P<.001$, Table 38). In Experiment 1 groups SO utilized food more efficiently than groups OF, but this finding was not confirmed in Experiment 2. When BT was substituted for SO in Experiments 2 and 3, food efficiencies were similar during the entire refeeding period, though BT groups utilized food more efficiently than SO groups when the first 3 days of refeeding were disregarded ($P<.02$, Experiment 2; $P<.05$, Experiment 3, Table 39). Ad libitum fed groups had slightly higher food efficiencies than did MF groups during the 10 day refeeding period, though this difference was significant only in Experiment 3 ($P<.02$, Table 38). The influence of meal pattern on food efficiency was reversed if the first 3 days, during which AL groups gorged their rations, were disregarded. Then meal feeding increased food efficiency. This increase was statistically supported in Experiment 2 ($P<.001$) but not in Experiment 3 (Table 39, Figures 11 and 13).

Serum

Serum cholesterol concentration

Dietary fat Diets containing SO tended to decrease serum cholesterol concentrations compared to low-fat diets (Experiment 1). This decrease was significant only for the comparison between groups 20SO-4P-AL and OF-4P-AL. Serum cholesterol concentrations were 48 and 59 mg/dl with these treatments, respectively (Table 5).

Concentration and source of dietary fat, however, did not influence serum cholesterol concentrations in Experiments 2 and 3 (Tables 6 and 7).

Table 5. Serum and hepatic cholesterol concentrations (Experiment 1)

Treatment	Serum cholesterol[a]		Hepatic cholesterol[b]	
	Free(mg/dl)	Total(mg/dl)	Ester(mg/g)	Total(mg/g)
Depleted control	13±2[c]	39±2	.35±.12	2.44±.37
OF-P-AL	24±3	55±2	1.59±.40	3.19±.60
OF-P-MF	25±2	68±5	.75±.11	2.40±.27
OF-4P-AL	20±1	59±2	.39±.11	2.30±.24
OF-4P-MF	25±1	61±5	.26±.06	2.02±.16
20SO-P-AL	16±1	55±3	1.44±.29	3.16±.90
20SO-P-MF	21±1	62±4	.70±.18	2.45±.10
20SO-4P-AL	13±1	48±2	1.04±.29	2.82±.38
20SO-4P-MF	19±1	59±3	.80±.24	2.67±.71

[a]Serum data are from 5 pooled samples, 2 rats in each pool.

[b]Liver data are from 5 individual animals out of 10 in each treatment.

[c]Mean±SEM.

Table 6. Serum and hepatic cholesterol concentrations (Experiment 2)

Treatment	Serum cholesterol		Hepatic cholesterol	
	Free(mg/dl)	Total(mg/dl)	Ester(mg/g)	Total(mg/g)
Depleted control	14±2[a]	51±6	.19±.10	1.74±.10
OF-P-AL	12±1	46±3	1.00±.13	2.70±.13
OF-P-MF	18±2	58±5	.28±.04	1.76±.05
20SO-P-AL	13±2	45±3	1.04±.15	2.87±.14
20SO-P-MF	17±1	61±3	.51±.11	2.20±.11
20BT-P-AL	13±2	42±3	.83±.12	2.48±.10
20BT-P-MF	19±1	60±2	.58±.07	2.17±.05
Stock-AL	14±2	62±4	.06±.02	1.76±.05
OF	15	52	0.61	2.19
20SO	15	52	0.78	2.55
20BT	16	52	0.71	2.33
AL	13	44	0.96	2.70
MF	18	60	0.44	2.01
Analysis of Variance				
Fat	NS	NS	NS	P<.001
Meal pattern	P<.001	P<.001	P<.001	P<.001

[a]Mean±SEM.

Table 7. Serum and hepatic cholesterol concentrations (Experiment 3)

Treatment	Serum cholesterol		Hepatic cholesterol	
	Free(mg/dl)	Total(mg/dl)	Ester(mg/g)	Total(mg/g)
20SO-P-AL	13±2[a]	55±4	0.88±.07	2.49±.08
20SO-P-MF	15±2	54±4	0.30±.14	1.92±.14
20SO-4P-AL	13±1	43±2	0.41±.08	1.95±.08
20SO-4P-MF	15±2	46±5	0.29±.05	1.88±.08
20BT-P-AL	15±2	44±3	0.78±.15	2.19±.21
20BT-P-MF	15±2	55±5	0.60±.19	2.05±.18
20BT-4P-AL	12±1	45±3	0.19±.05	1.81±.07
20BT-4P-MF	16±2	58±4	0.10±.01	1.68±.06
20SO	14	48	0.46	2.04
20BT	14	52	0.38	1.91
AL	13	48	0.53	2.08
MF	15	53	0.31	1.87
P	14	53	0.64	2.16
4P	14	48	0.25	1.83
Analysis of Variance				
Fat	NS	NS	NS	NS
Meal pattern	NS	P<.07	P<.01	P<.01
Protein	NS	P<.04	P<.001	P<.001

[a]Mean±SEM.

Overall mean serum cholesterol concentrations for OF, 20SO and 20BT groups in Experiment 2 were 51.8, 52.4 and 51.5 mg/dl, respectively. Serum cholesterol concentrations for 20SO and 20BT groups in Experiment 3 were 48.6 and 51.6 mg/dl. None of these differences were significant.

In the third experiment, fat source (SO or BT) influenced the response of serum cholesterol concentrations to other variables. Serum cholesterol concentrations decreased with 4P compared to P when the source of dietary fat was SO ($P<.01$), but not when BT was the source of dietary fat. In the presence of SO, meal pattern did not influence serum cholesterol concentrations. In contrast, this parameter was increased with meal feeding in BT groups ($P<.01$).

Meal pattern Serum cholesterol concentrations were higher in MF rats compared to AL controls (Experiment 1, Table 5). For example, serum cholesterol concentrations were higher in groups OF-P-MF and 20SO-P-MF than in groups fed the corresponding diets ad libitum ($P<.01$). A similar trend toward an increase in serum cholesterol concentration with meal feeding was apparent with the other two diets, but differences failed to reach statistical significance.

In the second experiment, all meal pattern comparisons were statistically different ($P<.01$). Overall mean serum cholesterol concentrations were 44.2 and 59.6 mg/dl for AL and MF groups ($P<.001$). In individual comparisons, meal feeding also resulted in higher serum cholesterol concentrations with BT, but not with SO (Experiment 3, Table 7).

Dietary protein Protein decreased serum cholesterol concentrations in Experiment 3 ($P<.05$, Table 7). In Experiment 1 the trend was similar but differences were not significant statistically (Table 5). The significant decrease in concentration seen with protein in Experiment 3 was somewhat misleading. Protein decreased serum cholesterol concentrations in groups fed SO but not in groups fed BT. In every comparison with SO, protein decreased concentrations of serum cholesterol. A consistent decrease in this parameter was not shown with OF and BT treatments when protein was increased (Experiments 1 and 3).

Serum cholesterol radioactivity

Serum ^{3}H-cholesterol Serum levels of ^{3}H, based on ^{3}H-acetate incorporation into DPS, were too small to give confidence to the data obtained after correction for ^{14}C in samples.

Serum ^{14}C-cholesterol Depleted controls had more ^{14}C-dpm/ml serum than did any refed group (Experiment 2, Tables 8 and 9). Serum cholesterol S.A. (^{14}C-dpm/mg) was also highest in depleted controls, whereas rats refed the stock ration had the lowest ^{14}C/ml serum and the lowest serum cholesterol S.A.

Concentration and source of dietary fat influenced ^{14}C-dpm DPS/ml serum in Experiment 2 ($P<.05$). Groups fed diets OF, 20SO and 20BT had values of 9200, 6900 and 8000, respectively (Table 9). Serum cholesterol S.A. was also influenced by fat source ($P<.01$, Table 9). The SO regimen compared with that containing OF and BT reduced cholesterol S.A.

In the third study, fat source (SO or BT) did not influence either serum ^{14}C-dpm DPS/ml or ^{14}C-cholesterol S.A.

Table 8. Serum DPS[a], ^{14}C-DPS/ml and S.A. (^{14}C-dpm x 10^3/mg DPS)[b]

Treatment	Serum DPS (experiment 2)			Serum DPS (experiment 3)		
	mg/ml	^{14}C-dpm x 10^3/ml	S.A.	mg/ml	^{14}C-dpm x 10^3/ml	S.A.
Depleted control	.51±.06[c]	10.3±1.6	19.8±2.3	-	-	-
OF-P-AL	.46±.03	8.9± .8	19.3±1.8	-	-	-
OF-P-MF	.58±.05	8.8±1.3	16.5±1.9	-	-	-
20SO-P-AL	.45±.03	5.6± .8	13.4±1.3	.55±.04	7.0± .4	12.9± .8
20SO-P-MF	.61±.03	8.6± .9	13.0± .9	.54±.04	5.2± .6	9.3±1.5
20SO-4P-AL	-	-	-	.43±.02	5.3± .7	11.7±1.4
20SO-4P-MF	-	-	-	.46±.05	4.5± .4	10.5±1.0
20BT-P-AL	.42±.03	7.6±1.0	17.9±1.4	.44±.03	6.6±1.1	13.6±2.2
20BT-P-MF	.60±.02	8.3± .6	13.8± .9	.55±.05	6.6± .7	11.9± .8
20BT-4P-AL	-	-	-	.45±.03	5.0± .4	11.1± .6
20BT-4P-MF	-	-	-	.58±.04	6.0± .6	10.5± .9
Stock-AL	.62±.04	5.4± .5	8.9± .8	-	-	-

[a]Digitonin precipitable sterol, cholesterol.

[b]Variable means and analysis of variance in Table 9.

[c]Mean±SEM.

Table 9. Serum DPS[a], ^{14}C-DPS/ml and S.A. (^{14}C-dpm x 10^3/mg DPS): Variable means and analysis of variance

Treatment	Serum DPS (experiment 2)			Serum DPS (experiment 3)		
	mg/ml	^{14}C-dpm x 10^3/ml	S.A.	mg/ml	^{14}C-dpm x 10^3/ml	S.A.
OF	.52	9.2	18.0	-	-	-
20SO	.52	6.9	13.2	.48	5.4	11.1
20BT	.52	8.0	15.8	.52	5.9	11.6
AL	.44	7.3	16.8	.48	5.8	12.2
MF	.60	8.5	14.3	.53	5.5	10.6
P	-	-	-	.53	6.3	12.0
4P	-	-	-	.48	5.2	10.9
Fat	NS	P<.05	P<.01	NS	NS	NS
Meal pattern	P<.001	NS	P<.02	P<.07	NS	P<.05
Protein	-	-	-	P<.05	P<.01	NS

[a]Digitonin precipitable sterol.

Decreased feeding frequency tended to elevate the amount of label recovered in the serum. This was to be expected since cholesterol concentrations had increased with meal feeding. Serum cholesterol S.A., however, was decreased in MF compared with AL groups in Experiments 2 ($P<.02$) and 3 ($P<.05$, Table 9).

Increased protein intake decreased the recovery of ^{14}C-dpm DPS/ml of serum ($P<.02$, Experiment 3). Cholesterol S.A., however, did not decrease similarly (Table 9).

Serum cholesterol ester fatty acid pattern

Low-fat compared with SO diets increased the percentage contribution of the major fatty acids formed from dietary carbohydrate to cholesterol ester fatty acids (CEFA): palmitate (C16), palmitolate (C16:1) and oleate (C18:1) (Table 10). This same comparison resulted in a reciprocal decrease in contribution from the ω6 family of fatty acids: linoleate (C18:2) and arachidonate (C20:4). Changes in CEFA pattern were not seen with increased protein or decreased feeding frequency (Experiment 1, Table 10).

Liver

Hepatic weight and percent lipid

Controls: depleted and stock refed Mean hepatic weights for depleted controls were 6.3 and 7.6 g with 3.7 and 3.2% hepatic lipid (Experiments 1 and 2, Table 11). Rate of hepatic regeneration was highest with the stock diet, but a significant restoration of liver weight occurred with all refeeding treatments.

Table 10. Serum and hepatic cholesterol fatty acid patterns. Percent of total fatty acids (Experiment 1)

Treatment	Tissue	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$
Depleted control	Serum	1±.2[a]	-	13±1.2	2± .7
	Liver	-	-	-	-
OF-P-AL	Serum	1±.1	-	16± .9	23±1.4
	Liver	1±.2	1±.4	25±1.2	28±1.4
OF-P-MF	Serum	1±.2	-	13±1.1	27±2.8
	Liver	2±.6	1±.7	26±1.3	25±2.1
20SO-P-AL	Serum	-	-	11± .4	1± .3
	Liver	1±.4	1±.4	14± .8	-
20SO-P-MF	Serum	-	-	10± .9	1± .3
	Liver	1±.2	1±.5	16±1.2	-

[a]Mean±SEM.

C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:4}$
2± .5	12±1.5	22±1.8	-	48±2.6
-	-	-	-	-
2±1.4	19± .8	12± .5	-	27± .8
3± .5	34±1.0	3± .8	-	4± .7
1± .4	19±1.1	13±1.1	-	26±3.5
3± .3	30±1.9	6±1.4	-	7±1.0
2± .4	5± .6	26±2.2	-	55±2.7
2± .3	15±1.3	50±1.8	-	16±1.7
2± .4	7± .6	29±2.3	-	51±2.0
3± .4	15±1.0	48±1.9	-	16±1.4

Table 11. Hepatic weights and % lipid[a]

Treatment	Liver (experiment 1)[b]		Liver (experiment 2)[c]		Liver (experiment 3)[d]	
	Weight(g)	Lipid(%)	Weight(g)	Lipid(%)	Weight(g)	Lipid(%)
Depleted control	6.3±.1[e]	3.7±.5	7.6±.1	3.2± .2	-	-
OF-P-AL	8.9±.4	6.3±.7	10.2±.1	6.4± .9	-	-
OF-P-MF	9.1±.4	4.4±.2	10.2±.1	3.7± .2	-	-
OF-4P-AL	9.9±.3	4.0±.2	-	-	-	-
OF-4P-MF	9.9±.4	3.9±.2	-	-	-	-
20SO-P-AL	9.7±.4	6.6±.2	9.6±.2	5.7± .4	9.9±.3	6.2±.6
20SO-P-MF	9.2±.2	5.2±.3	9.6±.2	4.2± .3	9.7±.3	3.8±.4
20SO-4P-AL	9.9±.4	4.8±.2	-	-	11.1±.3	4.1±.5
20SO-4P-MF	9.8±.4	4.4±.2	-	-	11.7±.4	3.9±.5
20BT-P-AL	-	-	10.5±.3	7.7±1.1	11.6±.7	7.3±.8
20BT-P-MF	-	-	10.3±.2	5.6± .9	10.9±.3	6.8±.7
20BT-4P-AL	-	-	-	-	12.0±.5	4.3±.5
20BT-4P-MF	-	-	-	-	11.8±.4	3.6±.4
Stock-AL	-	-	14.0±.4	3.6± .2	-	-

[a]Variable means and analysis of variance in Table 12.

[b]Fasted from 5 p.m. to 9 a.m., sacrificed 9 a.m.

[c]Fasted from 5 p.m. to 9 a.m., fed 1 hour, injected with 50μC ^{3}H-acetate, sacrificed 2.5 hours later.

[d]AL allowed food from 5 p.m. to 9 a.m. and MF fasted as in Experiment 2, fed 1 hour, injected with 50 μC ^{3}H-acetate, sacrificed 2.5 hours later.

[e]Mean±SEM.

Dietary fat Groups OF and 20SO had similar hepatic weights and percentages of hepatic lipid in both Experiments 1 and 2. BT compared with low-fat feeding (Experiment 2) and with SO feeding (Experiments 2 and 3) increased liver weights and lipid concentrations (Table 12).

Meal pattern Feeding frequency did not influence hepatic weight in any dietary comparison. In every instance, however, MF groups had decreased hepatic lipid concentrations (Table 11). Mean liver lipid concentrations for AL versus MF groups were 5.5 versus 4.2% ($P<.01$, Experiment 1), 6.6 versus 4.4% ($P<.001$, Experiment 2), and 5.3 versus 4.3% ($P<.01$, Experiment 3, Table 12).

Dietary protein With increased dietary protein, hepatic weights increased in Experiment 3 but not in Experiment 1. In both experiments, however, liver lipid concentration decreased with increased dietary protein ($P<.01$, Experiment 1; $P<.001$, Experiment 3).

Hepatic cholesterol concentration

Dietary fat Level and kind of dietary fat influenced hepatic cholesterol concentration in Experiment 2 ($P<.01$, Table 6). Groups OF, 20SO and 20BT had cholesterol concentrations of 2.2, 2.6 and 2.3 mg/g liver, respectively. Fat source (SO or BT) did not influence hepatic cholesterol concentration in Experiment 3, though SO groups tended to have increased concentrations. These results were similar to those from the second experiment.

Table 12. Hepatic weights and % lipid: Variable means and analysis of variance

Treatment	Liver (experiment 1)		Liver (experiment 2)		Liver (experiment 3)	
	Weight(g)	Lipid(%)	Weight(g)	Lipid(%)	Weight(g)	Lipid(%)
OF	9.5	4.5	10.1	5.0	-	-
20SO	9.8	5.2	9.6	5.0	10.7	4.4
20BT	-	-	10.4	6.6	11.6	5.2
AL	9.8	5.5	10.0	6.6	11.2	5.3
MF	9.5	4.2	10.0	4.4	11.1	4.3
P	9.2	5.6	-	-	10.5	5.9
4P	9.9	4.3	-	-	11.6	4.0
Analysis of Variance						
Fat	NS	P<.01	P<.02	P<.05	P<.01	P<.01
Meal pattern	NS	P<.01	NS	P<.001	NS	P<.01
Protein	P<.01	P<.01	-	-	P<.001	P<.001

Meal pattern Hepatic cholesterol concentrations decreased with meal feeding in all three experiments (Tables 5, 6 and 7). Groups AL compared with MF had concentrations of 2.70 versus 2.01 ($P<.001$, Experiment 2) and 2.08 versus 1.87 ($P<.01$, Experiment 3) mg/g liver.

The decrease in hepatic cholesterol occurred primarily in the esterified fraction. Hepatic cholesterol ester decreased with meal feeding from .94 to .44 mg/g liver ($P<.001$, Experiment 2) and from .53 to .31 mg/g liver ($P<.01$, Experiment 3).

Protein level Increased protein intake decreased hepatic cholesterol concentration in both Experiments 1 and 3. The decrease in hepatic total cholesterol ($P<.001$) was a reflection of the decrease in hepatic cholesterol ester ($P<.001$, Experiment 3, Table 7) in group 4P compared with P.

Hepatic cholesterol radioactivity

Hepatic ^{3}H-cholesterol Animals refed with stock diet incorporated approximately twice as much ^{3}H-acetate into hepatic DPS as did depleted controls. Cholesterol biosynthesis in refed groups other than the stock group did not differ significantly from that of depleted controls (Table 13).

Low-fat groups incorporated less ^{3}H-acetate into DPS than did fat-fed groups ($P<.05$, Experiment 2). Source of dietary fat, however, did not influence cholesterol biosynthesis in either Experiment 2 or 3.

Groups AL and MF incorporated similar amounts of ^{3}H-acetate into DPS (Experiments 2 and 3, Table 13). Likewise, dietary protein concentration did not influence synthesis of cholesterol in the third study.

Table 13. Hepatic ^{3}H-DPS[a] and ^{3}H-DPS as % of total liver lipid: Variable means and analysis of variance

Treatment	Hepatic DPS (experiment 2)		Hepatic DPS (experiment 3)	
	^{3}H-dpm x 10^{3}	% TLL-^{3}H[b]	^{3}H-dpm x 10^{3}	% TLL-^{3}H
OF	7.2	2.6	-	-
20SO	9.6	9.5	30.7	11.2
20BT	10.4	6.6	28.3	9.8
AL	8.6	6.1	29.6	13.0
MF	9.4	5.3	29.4	8.0
P	-	-	29.9	9.8
4P	-	-	29.2	11.2
Depleted control	5.6	8.8	-	-
Stock-AL	16.4	13.8	-	-

Analysis of Variance

Fat	NS	P<.001	NS	NS
Meal pattern	NS	NS	NS	P<.01
Protein	-	-	NS	NS

[a]Digitonin precipitable sterol.

[b]Total liver lipid.

Hepatic ^{14}C-cholesterol Recovery of hepatic ^{14}C-dpm in DPS and cholesterol S.A. in low-fat groups exceded values obtained with high-fat diets ($P<.001$, Table 14). Values for cholesterol S.A. (^{14}C-dpm/mg) were 17,200 for OF and 11,700 and 14,000 for SO and BT. In Experiment 2, hepatic ^{14}C-dpm/mg in DPS was higher with BT than with SO, though this relationship was not seen in Experiment 3 (Table 15).

Decreased feeding frequency lowered hepatic ^{14}C-dpm in DPS in both radiochemical studies ($P<.001$, Experiment 2; $P<.05$, Experiment 3, Figure 3). Cholesterol S.A. was also decreased by meal feeding in Experiment 2 ($P<.002$) and tended to be decreased in Experiment 3 (Table 15).

Increased dietary protein tended to decrease hepatic ^{14}C-dpm in DPS, though cholesterol S.A. was not changed by protein (Table 13).

Hepatic cholesterol ester fatty acid pattern

The combined percentage of C16, C16:1 and C18:1 in CEFA was 84% in OF groups as compared to 30% in SO groups. The sums of C18:2 and C20:4 with low-fat and SO diets were 10 and 65% of hepatic CEFA, respectively. MF compared with AL treatments decreased hepatic cholesteryl oleate ($P<.05$) and tended to decrease cholesteryl palmitate in low-fat groups. Concomitant increases in C18:2 and C20:4 occurred in MF groups, although these increases did not reach the level of statistical significance.

Hepatic fatty acid biosynthesis

Dietary fat Incorporation of ^{3}H-acetate into total lipid (Folch et al., 1957) was assumed to represent primarily incorporation of label into fatty acids. Low-fat diets caused more acetate incorporation into hepatic

Table 14. Hepatic DPS[a], ^{14}C-DPS and S.A. DPS (^{14}C-dpm x 10^3/mg)[b]

Treatment	Hepatic DPS (experiment 2)			Hepatic DPS (experiment 3)		
	mg	^{14}C-dpm x 10^3	S.A.	mg	^{14}C-dpm x 10^3	S.A.
Depleted control	13.6± .8[c]	354±30	23.7±1.6	-	-	-
OF-P-AL	27.1±1.3	463±21	18.8± .6	-	-	-
OF-P-MF	17.8± .5	286±22	15.8±1.3	-	-	-
20SO-P-AL	27.7±1.3	387±49	13.0±1.3	24.6± .3	288±31	11.5±1.1
20SO-P-MF	21.0±1.0	216±17	10.4± .6	18.7±1.4	200±21	8.6±1.8
20SO-4P-AL	-	-	-	21.6± .9	284±26	10.8± .7
20SO-4P-MF	-	-	-	22.0± .9	223±23	10.2±1.2
20BT-P-AL	25.9±1.0	391±20	15.5±1.4	25.4±2.4	305±36	11.9±1.1
20BT-P-MF	22.3± .5	288±25	12.6± .9	22.3±2.0	268±49	11.7± .8
20BT-4P-AL	-	-	-	21.8± .8	222±19	10.8±1.0
20BT-4P-MF	-	-	-	19.8± .7	218±15	10.4± .6
Stock-AL	25.3± .7	228±22	9.5±1.1	-	-	-

[a]Digitonin precipitable sterol, cholesterol.

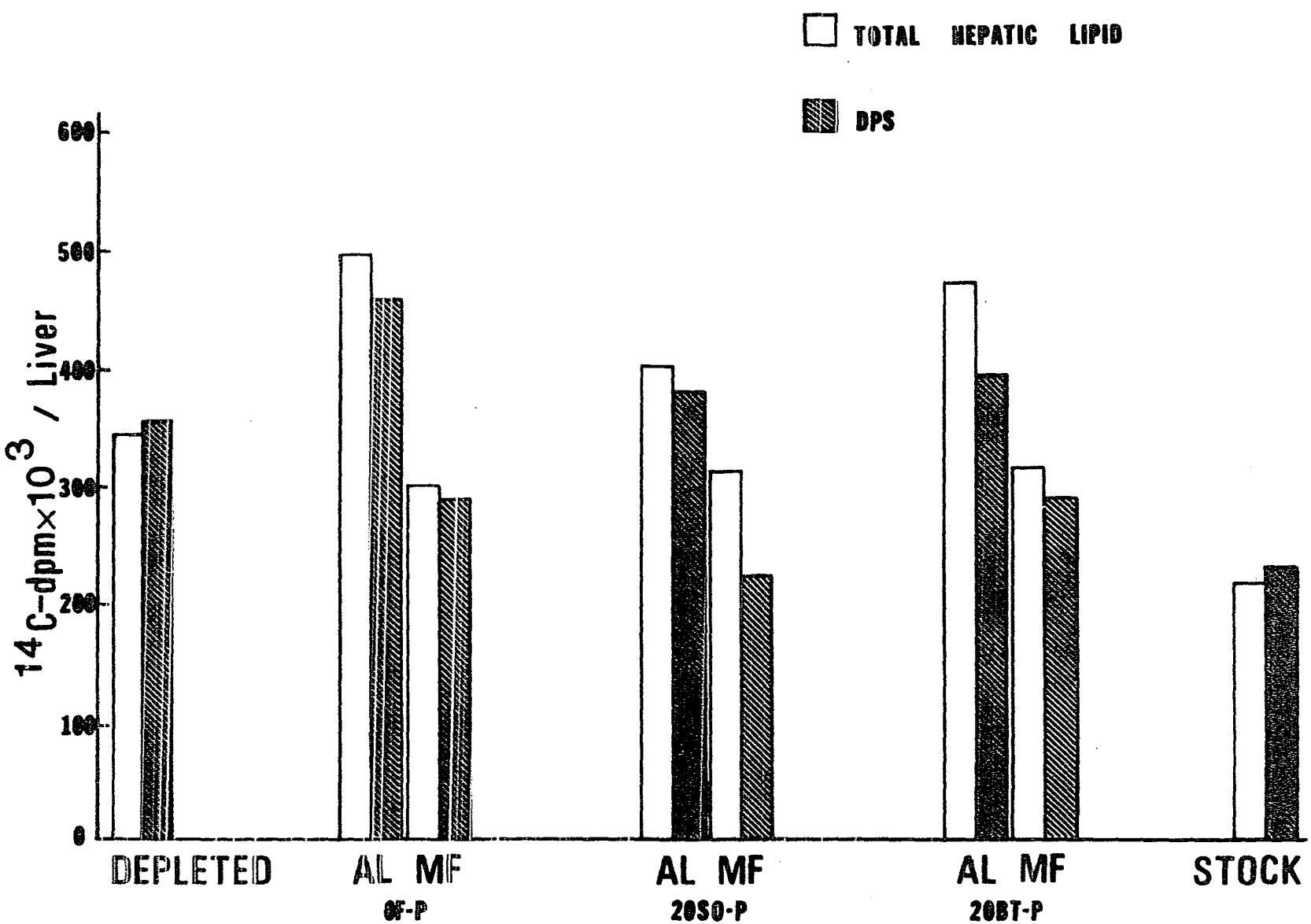
[b]Variable means and analysis of variance for variables are in Table 15.

[c]Mean±SEM.

Table 15. Hepatic DPS[a], ^{14}C-DPS and S.A. (^{14}C-dpm x 10^3/mg DPS): Variable means and analysis of variance

Treatment	Hepatic DPS (experiment 2)			Hepatic DPS (experiment 3)		
	mg	^{14}C-dpm x 10^3	S.A.	mg	^{14}C-dpm x 10^3	S.A.
OF	21.9	363	17.2	-	-	-
20SO	25.5	302	11.7	20.4	248	10.4
20BT	23.2	337	14.0	19.1	246	11.1
AL	27.0	414	15.8	20.8	270	11.2
MF	20.1	266	13.1	18.7	226	10.3
P	-	-	-	21.6	263	11.0
4P	-	-	-	18.3	235	10.6
Analysis of Variance						
Fat	NS	NS	P<.001	NS	NS	NS
Meal pattern	P<.001	P<.001	P<.01	P<.01	P<.05	NS
Protein	-	-	-	P<.001	NS	NS

Figure 3. Hepatic lipid -^{14}C 6 days following intraperitoneal injection of 5 μC 4-^{14}C-cholesterol: total hepatic lipid and DPS (digitonin precipitable sterol). Experiment 2


TOTAL HEPATIC LIPID
DPS
600
500
400
300
200
100
0
^{14}C-dpm$\times 10^3$ / Liver
DEPLETED
AL MF
0F-P
AL MF
20SO-P
AL MF
20BT-P
STOCK

lipids than did those on SO in Experiment 2 ($P<.01$, Table 16), but the increase in lipogenesis seen in Experiment 3 was too small to be significant. Relative incorporation of ^{3}H-acetate into total liver lipid and into DPS is shown in Figure 4 for groups OF, 20SO and 20BT.

Low fat versus fat containing diets were associated with elevated hepatic lipid S.A. ($P<.001$, Experiment 2, Table 17). Activities were 615 versus 330 ^{3}H-dpm x 10^{3}/g hepatic lipid. Hepatic lipid S.A., however, was not influenced by source (SO or BT) of dietary fat (Experiments 2 and 3).

Meal pattern Tritium recovered in hepatic lipid with MF groups compared to AL groups was increased in both radiochemical studies, but the differences were not at the level of significance ($P<.20$, Experiment 2; $P<.10$, Experiment 3, Table 17). Hepatic lipid S.A., however, was elevated with MF versus AL treatments ($P<.001$, Experiment 2; $P<.01$, Experiment 3).

Dietary protein Dietary protein concentrations did not affect lipogenesis (Experiment 3).

Hepatic ^{14}C-conjugated bile acid

In these studies bile acid concentrations were not measured chemically. Interpretation of ^{14}C-bile acid is based on the assumptions 1) that pool size of bile acid is constant for a given weight (Shefer et al., 1969) and 2) that bile acids produced from cholesterol have the same S.A. as hepatic cholesterol. For this reason hepatic cholesterol S.A. is used as the basis of comparison in Table 18.

The ratios of acid steroid-^{14}C to hepatic cholesterol S.A. ranged from approximately 0.5 in depleted controls to more than 2 in the group fed stock ration (Experiment 2). In the same experiment, the ratios in

Table 16. Hepatic lipid, ^{3}H-acetate incorporation and S.A. (^{3}H-dpm x 10^{3}/g lipid)[a]

Treatment	Hepatic lipid (experiment 2)[b]			Hepatic lipid (experiment 3)[c]		
	g	^{3}H-dpm x 10^{3}	S.A.	g	^{3}H-dpm x 10^{3}	S.A.
Depleted control	.25±.02[d]	110± 8	474±42	-	-	-
OF-P-AL	.56±.05	304±41	480±62	-	-	-
OF-P-MF	.38±.03	271±23	729±55	-	-	-
20SO-P-AL	.57±.05	99± 9	203±45	.61±.04	264± 79	416±102
20SO-P-MF	.40±.03	140±11	357±39	.37±.04	469±115	1409±403
20SO-4P-AL	-	-	-	.46±.03	244± 41	574±128
20SO-4P-MF	-	-	-	.46±.02	364± 90	810±210
20BT-P-AL	.82±.14	181±20	250±48	.87±.16	446± 99	594±156
20BT-P-MF	.58±.10	198±23	410±56	.70±.08	250± 38	360± 35
20BT-4P-AL	-	-	-	.52±.08	268± 63	519±126
20BT-4P-MF	-	-	-	.43±.03	478±110	1108±243
Stock-AL	.50±.02	136± 6	274±16	-	-	-

[a]Variable means for analysis of variance are in Table 17.

[b]Injected with 50 mg Na pentobarbitol 2 hours after 50μC ^{3}H-acetate injection, sacrificed at 2.5 hours.

[c]Injected with 25 mg Na pentobarbitol per 100 g body weight 2 hours and 20 min after 50μC ^{3}H-acetate injection, sacrificed at 2.5 hours.

[d]Mean±SEM.

Figure 4. Hepatic lipid - ^{3}H 2.5 hours following injection with 50 µC ^{3}H-acetate. Total hepatic lipid and DPS (digitonin precipitable sterol). Experiment 2

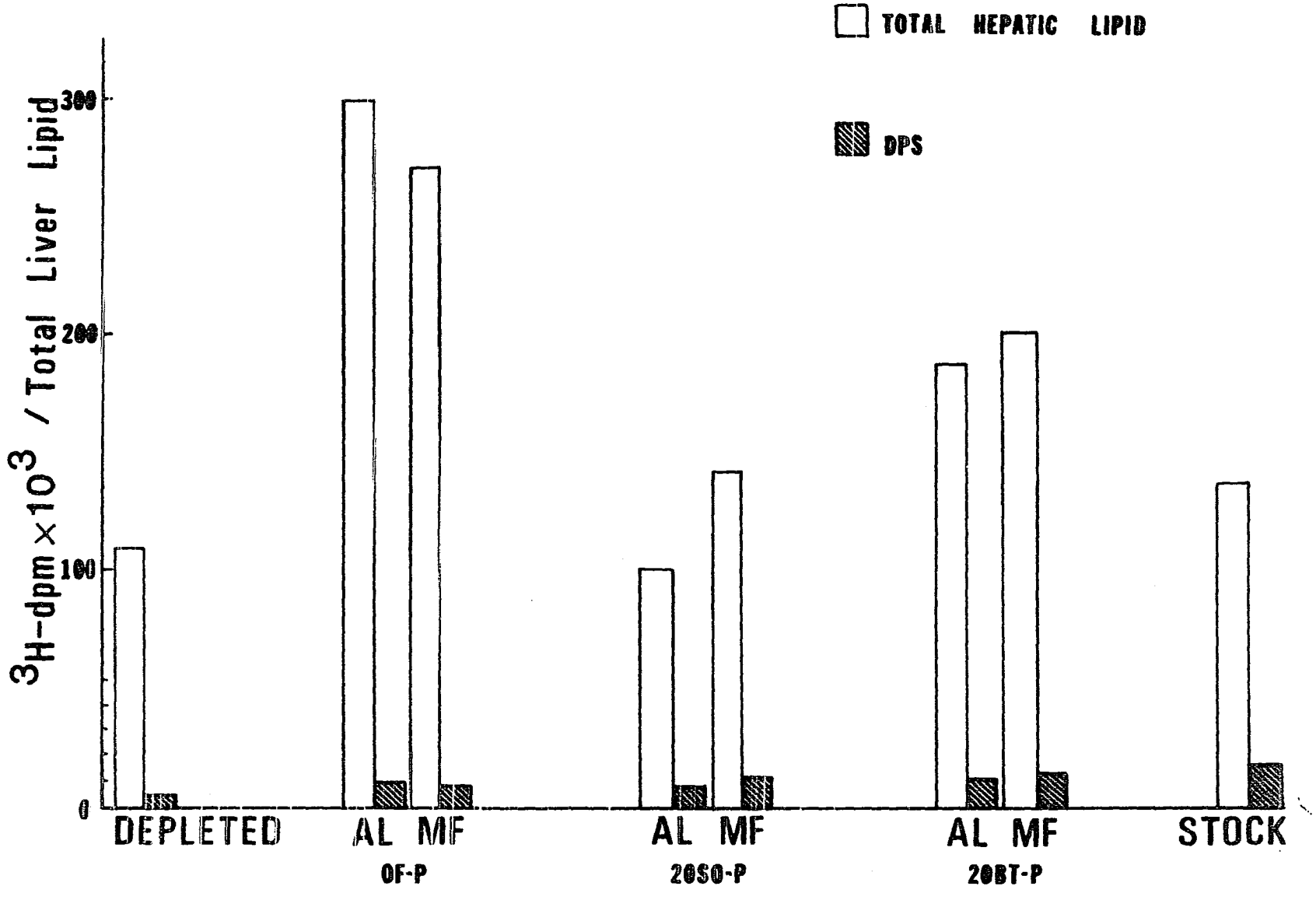

TOTAL HEPATIC LIPID
DPS
^{3}H-dpm×10^3 / Total Liver Lipid
300
200
100
0
DEPLETED
AL MF
OF-P
AL MF
20SO-P
AL MF
20BT-P
STOCK

Table 17. Hepatic lipid, ^{3}H-acetate incorporation and S.A. (^{3}H-dpm x 10^{3}/g lipid): Variable means for analysis of variance

Treatment	Hepatic lipid (experiment 2)			Hepatic lipid (experiment 3)		
	g	^{3}H-dpm x 10^{3}	S.A.	g	^{3}H-dpm x 10^{3}	S.A.
OF	.46	286	615	-	-	-
20SO	.49	120	280	.47	331	787
20BT	.70	190	330	.61	362	669
AL	.64	199	318	.60	298	529
MF	.45	210	523	.48	394	927
P	-	-	-	.64	357	695
4P	-	-	-	.47	338	753
Analysis of Variance						
Fat	P<.01	P<.001	P<.001	P<.01	NS	NS
Meal pattern	P<.01	NS	P<.001	P<.01	NS	P<.01
Protein	-	-	-	P<.001	NS	NS

refed groups other than stock were approximately 1. In Experiment 3, these ratios were decreased and were similar for all groups regardless of experimental variables (Table 18).

Small Intestine

Intestinal[1] cholesterol concentration

Dietary fat More DPS was recovered in intestine of SO than BT groups ($P<.02$, Experiment 3, Table 19). SO tended to increase cholesterol concentrations in Experiment 2, but the differences between SO, OF and BT were statistically not significant.

Meal pattern Intestinal cholesterol concentrations were similar for AL and MF groups (Experiments 2 and 3).

Dietary protein Increased dietary protein increased intestinal cholesterol concentration ($P<.05$, Experiment 3). This increase was associated with BT but not with SO.

Intestinal cholesterol radioactivity

Intestinal ^{3}H-cholesterol Data for ^{3}H-acetate incorporation into DPS are only available for Experiment 3. Data from incorporation of label indicated that more newly synthesized cholesterol was present in the intestine of SO than of BT groups ($P<.01$, Experiment 3). The meal patterns and protein concentrations used resulted in similar values for ^{3}H-dpm in intestinal DPS.

---

[1] Small intestine and contents.

Table 18. S.A. hepatic DPS[a] (^{14}C-dpm x 10^3/mg) and ^{14}C-acid steroid

Treatment	Experiment 2		Experiment 3	
	Hepatic DPS S.A.	Acid steroid ^{14}C-dpm x 10^3	Hepatic DPS S.A.	Acid steroid ^{14}C-dpm x 10^3
Depl ted control	23.7±1.6[b]	13.6±1.6	-	-
OF-P-AL	18.8± .6	17.6±2.6	-	-
OF-P-MF	15.8±1.3	17.9±2.2	-	-
20SO-P-AL	13.0±1.3	10.3±1.0	11.5±1.1	7.7±1.3
20SO-P-MF	10.4± .6	10.2± .5	8.6±1.8	5.3±1.0
20SO-4P-AL	-	-	10.8± .7	6.1±1.5
20SO-4P-MF	-	-	10.2±1.2	5.4± .7
20BT-P-AL	15.5±1.4	16.6±1.3	11.9±1.1	7.8±3.0
20BT-P-MF	12.6± .9	13.6± .8	11.7±1.8	7.3±2.4
20BT-4P-AL	-	-	10.8±1.0	6.1± .4
20BT-4P-MF	-	-	10.4± .6	5.6±1.8
Stock-AL	9.5±1.1	21.9±2.8	-	-
Analysis of Variance				
Fat	P<.001	P<.001	NS	NS
Meal pattern	P<.005	NS	NS	NS
Protein	-	-	NS	NS

[a]Digitonin precipitable sterol.

[b]Mean±SEM.

Table 19. Small intestine weight[a], DPS[b], ^{14}C-DPS, ^{3}H-DPS and S.A. DPS (^{14}C-dpm x 10^3 dpm/mg). Experiments 2 and 3

Treatment	Experiment 2				
	Small intestine weight	Small intestine DPS			
	g	mg	^{14}C-dpm x 10^{3c}	^{3}H-dpm x 10^3	S.A.
Depleted control	5.6±.1[e]	11.0± .4	168±18	-	15.3±.3
OF-P-AL	7.9±.3	15.4± .6	206±26	-	4.9±.6
OF-P-MF	7.2±.4	16.0± .9	166±17	-	4.0±.3
20SO-P-AL	8.2±.2	16.7± .4	180±19	-	4.3±.4
20SO-P-MF	8.1±.3	16.7± .9	164±19	-	3.9±.4
20SO-4P-AL	-	-	-	-	-
20SO-4P-MF	-	-	-	-	-
20BT-P-AL	9.1±.3	15.9± .6	188±18	-	4.5±.4
20BT-P-MF	8.8±.3	15.8± .8	156±13	-	3.7±.3
20BT-4P-AL	-	-	-	-	-
20BT-4P-MF	-	-	-	-	-
Stock-AL	15.2±.6	27.8±1.4	184±30	-	6.6±.9
Analysis of Variance					
Fat	P<.001	NS	NS	-	NS
Meal pattern	NS	NS	P<.05	-	P<.05
Protein	-	-	-	-	-

[a]Small intestine: tissue + contents.

[b]Digitonin precipitable sterol.

[c]Experiment 2, ad libitum fed rats fasted overnight prior to day of sacrifice.

[d]Experiment 3, ad libitum fed rats allowed access to food overnight before day of sacrifice.

[e]Mean±SEM.

Experiment 3				
Small intestine weight	Small intestine DPS			
g	mg	^{14}C-dpm x 10^{3d}	^{3}H-dpm x 10^{3}	S.A.
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
9.2±.7	19.9±1.2	136±15	39± 6	6.9± .7
10.3±.5	20.0±1.0	231±53	46± 5	14.7±2.8
10.0±.5	20.4±0.5	191±54	43± 6	9.4±2.7
10.0±.4	19.8±0.6	261±57	39± 7	13.2±2.8
10.6±.3	17.5±1.0	231±61	73±14	14.5±4.5
10.0±.3	17.1±0.8	241±51	60±12	14.4±3.2
10.6±.5	19.8±0.8	266±45	46± 7	13.8±2.7
11.4±.6	18.5±0.8	230±39	53± 9	12.5±2.1
-	-	-	-	-
Analysis of Variance				
P<.02	P<.02	NS	P<.01	NS
NS	NS	NS	NS	NS
NS	P<.05	NS	NS	NS

Intestinal ^{14}C-cholesterol Neither intestinal ^{14}C-dpm in DPS nor ^{14}C-dpm/mg DPS were affected by level or source of dietary fat (Experiments 2 and 3). Meal feeding compared with ad libitum feeding decreased small intestinal ^{14}C-dpm in DPS in Experiment 2 ($P<.05$). These data were not confirmed in the third experiment; in fact in three out of four comparisons there were numerically large trends in the opposite direction. The apparent inconsistency probably results from the fact that AL groups were fasted overnight in Experiment 2, whereas in Experiment 3 they were allowed access to food throughout the night before sacrifice. Protein level did not influence intestinal ^{14}C-dpm in DPS (Experiment 3, Table 19).

Intestinal conjugated ^{14}C-bile acids

Dietary fat Groups OF had significantly more ^{14}C in conjugated bile acids in the small intestine than did SO and BT groups ($P<.02$, Experiment 2, Table 20). Depleted and stock refed controls received low levels of dietary fat and were comparable to OF groups in this same experiment. In both radiochemical studies, similar amounts of acid-^{14}C were recovered in SO and BT groups with intestinal analysis.

Meal pattern Meal pattern did not influence the amount of ^{14}C-bile acid label recovered at the time of sacrifice (Experiments 2 and 3, Table 20).

Dietary protein Increased concentrations of dietary protein did not alter ^{14}C-dpm recovered in conjugated acid steroids (Experiment 3, Table 20).

Table 20. Small intestine[a] conjugated bile acid - ^{14}C (Experiments 2 and 3)

Treatment	Experiment 2	Experiment 3
	Bile acid ^{14}C-dpm x 10^3	Bile acid ^{14}C-dpm x 10^3
Depleted control	274±22[b]	-
OF-P-AL	278±20	-
OF-P-MF	292±31	-
20SO-P-AL	185±23	186±40
20SO-P-MF	207±31	133±42
20SO-4P-AL	-	123±10
20SO-4P-MF	-	127±18
20BT-P-AL	221±27	124±16
20BT-P-MF	217±15	104±13
20BT-4P-AL	-	147±21
20BT-4P-MF	-	155±26
Stock-AL	295±44	-
Analysis of variance		
Fat	P<.02	NS
Meal pattern	NS	NS
Protein	-	NS

[a]Small intestine: tissue plus contents.

[b]Mean±SEM.

Epididymal Fat Pad

Weights of epididymal fat pad and epididymal lipid

Dietary fat More epididymal lipid was deposited during refeeding by SO compared to low-fat groups ($P<.001$, Experiment 1, Table 21). The amount of epididymal fat was highly correlated with total body lipid in this experiment ($r = .85$, Figure 5). Experiment 2 confirmed that epididymal, and therefore carcass, lipid increased more with SO and BT than with OF ($P<.01$, Table 22a), though fat source did not seem to influence deposition of lipid (Experiment 2).

Meal pattern Meal pattern did not influence lipid deposition in either Experiment 1 or 2, despite lower consumption of calories by MF compared to AL groups ($P<.01$, Experiment 1; $P<.001$, Experiment 2).

Epididymal fatty acid biosynthesis

Dietary fat Epididymal fatty acid synthesis[1] was comparable to hepatic lipogenesis in that groups OF incorporated more acetate into lipid than did groups SO and BT ($P<.001$, Table 22a). Lipid-^{3}H S.A. was also higher in low-fat than in high-fat groups ($P<.001$). As in liver, group 20BT-P-AL had more ^{3}H-acetate incorporation into lipid than did group 20SO-P-AL ($P<.05$, Experiment 2). Lipid S.A. was similar for groups SO and BT, though it tended to increase with BT.

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[1] ^{3}H-acetate incorporated into total lipid (Folch et al., 1957) was not fractionated. Total lipid ^{3}H-label was interpreted as being predominantly from fatty acid synthesis.

Table 21. Carcass and epididymal lipid deposition during refeeding (Experiment 1)

Treatment	Body weight	Body lipid	Body lipid[a] deposited
	g	%	g
Depleted control	298±1[b]	1.8± .2	-
OF-P-AL	345±4	7.6± .3	21±1
OF-P-MF	346±2	7.8± .4	22±1
OF-4P-AL	365±5	6.7± .6	20±2
OF-4P-MF	369±4	8.3±1.7	23±3
20SO-P-AL	366±5	11.5± .3	38±1
20SO-P-MF	358±3	9.4± .4	29±2
20SO-4P-AL	395±6	10.8± .7	38±2
20SO-4P-MF	388±6	11.1± .7	39±3
OF	356	7.6	22
20SO	376	10.6	36
AL	366	8.9	28
MF	365	9.1	28
P	353	8.9	27
4P	378	9.2	30
Analysis of Variance			
Fat	P<.001	P<.001	P<.001
Meal pattern	NS	NS	NS
Protein	P<.001	NS	NS

[a]Lipid deposited (g) = total carcass lipid (including liver and epididymal lipid) - 6.2 g (mean depleted carcass lipid).

[b]Mean±SEM.

Weight deposited as lipid %	Kcal consumed deposited as lipid %	Epididymal lipid deposited g
-	-	-
45±1	27±3	0.9±.1
46±4	34±5	1.4±.1
29±4	27±3	1.3±.1
32±4	32±3	1.4±.4
56±1	42±2	2.4±.2
48±1	35±2	1.6±.3
40±5	41±3	2.0±.5
43±4	44±2	2.7±.4
38	30	1.2
47	40	2.2
42	33	1.6
42	36	1.8
49	34	1.5
36	36	1.8
Analysis of Variance		
P<.001	P<.001	P<.001
NS	NS	NS
P<.001	NS	NS

Figure 5. Weight carcass fat compared with weight epididymal fat (Experiment 1)

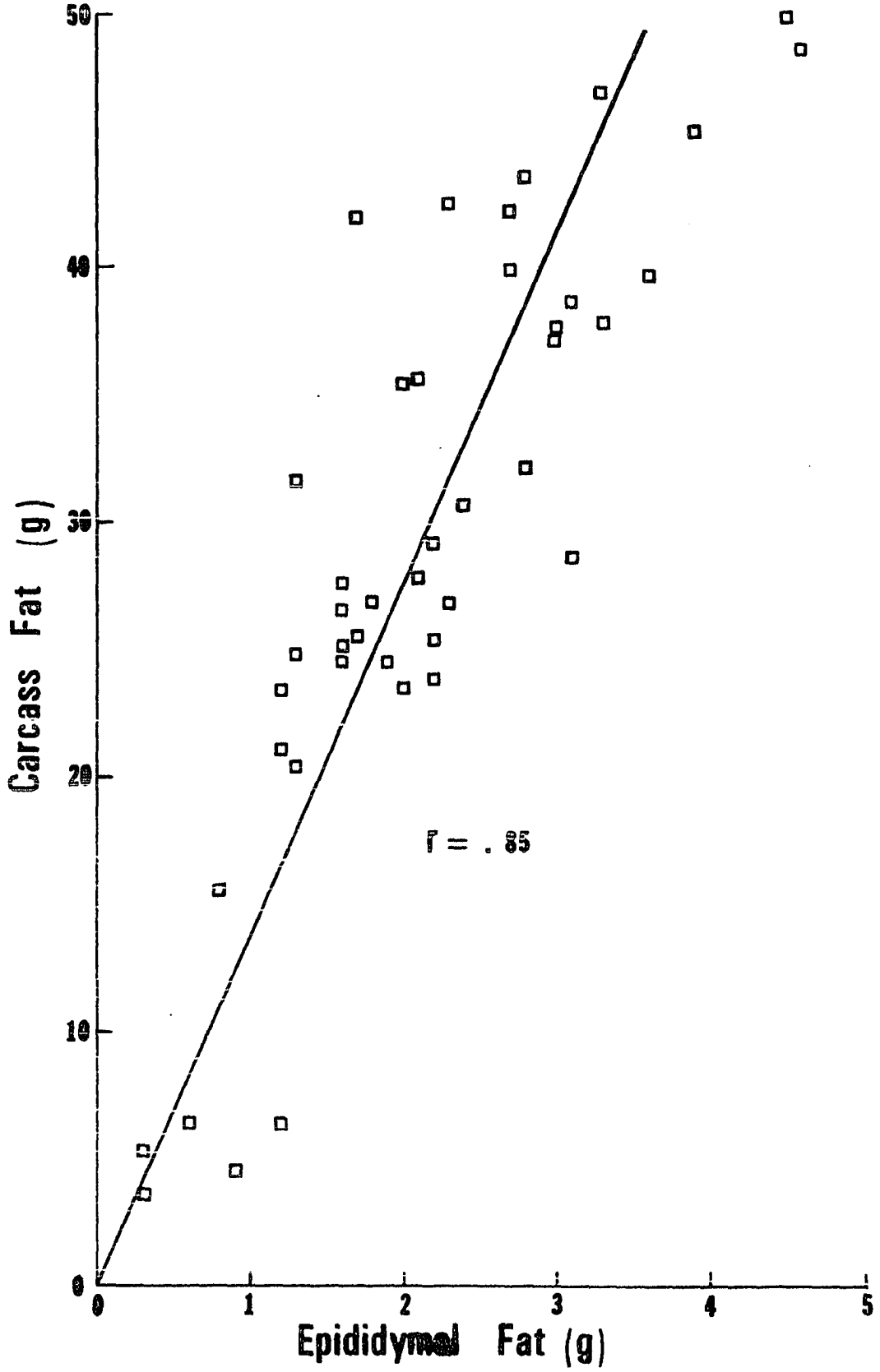
50
40
30
20
10
0
Carcass Fat (g)
r = .85
0
1
2
3
4
5
Epididymal Fat (g)

Table 22a. Epididymal fat pad weight and lipid weight, ^{3}H-acetate incorporation and S.A. (^{3}H-dpm x 10^{3}/g). Experiment 2

Treatment	Epididymal fat pad weight g	Epididymal lipid		
		g	^{3}H-dpm x 10^{3}	S.A.
Depleted control	1.7±.3[a]	.92±.20	6.1±1.0	8.1±1.8
OF-P-AL	3.4±.3	1.86±.21	52.1±8.4	31.2±6.4
OF-P-MF	2.8±.2	1.60±.18	57.5±5.0	40.8±4.9
20SO-P-AL	4.0±.3	2.30±.22	15.1±2.3	7.5±1.4
20SO-P-MF	3.8±.3	2.34±.17	26.4±4.0	11.7±1.8
20BT-P-AL	4.1±.3	2.50±.25	32.3±8.3	9.6± .8
20BT-P-MF	3.9±.4	2.09±.22	29.1±5.8	14.1±2.2
Stock-AL	4.0±.3	2.14±.32	28.5±5.5	13.5±2.2
Analysis of Variance				
Fat	NS	P<.01	P<.001	P<.01
Meal pattern	NS	NS	NS	NS

[a]Mean±SEM.

Meal pattern Feeding frequency did not significantly influence the incorporation of ^{3}H-acetate into epididymal lipid, though meal feeding tended to elevate epididymal lipogenesis ($P<.10$, Table 22a). The lipid-^{3}H S.A., however, was increased in MF compared with AL groups.

Epididymal lipid neutral-^{14}C

Concentration and source of dietary fat did not influence the amount of ^{14}C-cholesterol label[1] recovered in epididymal lipid, but ^{14}C-dpm decreased with meal feeding ($P<.01$, Experiment 2, Table 22b).

Table 22b. Epididymal lipid neutral-^{14}C

Treatment	^{14}C-dpm x 10^{3}
OF-P-AL	32.1±2.2
OF-P-MF	22.1±5.4
20SO-P-AL	26.2±2.1
20SO-P-MF	22.7±2.7
20BT-P-AL	32.4±2.0
20BT-P-MF	21.2±2.6
Analysis of Variance	
Fat	NS
Meal pattern	$P<.01$

[1]Label (^{14}C) in total epididymal lipid (Folch et al., 1957) was assumed to be largely digitonin precipitable as in liver (Figure 3).

Kidney

Kidney weight

Dietary fat Neither concentration nor source of dietary fat influenced kidney weight (Experiment 2, Table 23).

Meal pattern Groups MF compared with groups AL had smaller kidneys ($P<.001$, Table 23). Kidney weights for MF groups were identical with mean weight for depleted controls, whereas AL groups showed increased tissue weight with refeeding.

Kidney cholesterol concentration

Dietary fat Low-fat diets depressed kidney cholesterol concentration compared with diets containing fat ($P<.05$, Table 23), though the source of dietary fat did not influence kidney cholesterol concentration.

Meal pattern Though kidney size was decreased with meal feeding in comparison with ad libitum feeding, kidney cholesterol concentration was not influenced by feeding frequency (Table 23).

Kidney ^{14}C-cholesterol

Dietary fat Concentration and source of dietary fat did not influence the ^{14}C-dpm recovered in kidney DPS, but omitting fat from the diet increased ^{14}C-cholesterol S.A. ($P<.01$).

Meal pattern Kidney ^{14}C-cholesterol was decreased in MF compared with AL groups ($P<.02$). Cholesterol S.A. was similar with both treatments (Table 23).

Table 23. Kidney weight, DPS[a], ^{14}C-DPS and S.A. DPS (^{14}C-dpm x 10^3/mg). Experiment 2

Treatment	Kidney weight g	Kidney DPS		
		mg	^{14}C-dpm x 10^3	S.A.
Depleted control	2.05±.06[b]	7.8±.5	48±4	6.0±.5
OF-P-AL	2.26±.05	7.3±.4	50±5	6.9±.7
OF-P-MF	2.03±.05	7.1±.6	42±6	7.0±.8
20SO-P-AL	2.23±.05	8.0±.5	46±4	5.0±.4
20SO-P-MF	2.03±.05	8.0±.3	40±3	5.0±.3
20BT-P-AL	2.21±.09	8.2±.3	49±4	6.0±.4
20BT-P-MF	2.05±.06	7.6±.4	40±4	5.4±.6
Stock-AL	3.03±.08	10.6±.5	36±4	3.3±.4
Analysis of Variance				
Fat level	NS	P<.05	NS	P<.01
Fat	NS	NS	NS	P<.01
Meal Pattern	P<.001	NS	P<.02	NS

[a]Digitonin precipitable sterol.

[b]Mean±SEM.

Heart

Cardiac weight

Cardiac weight was not influenced significantly by any variable used, though groups MF compared to AL tended to have decreased weights ($P<.10$, Table 24).

Cardiac cholesterol concentration

The cholesterol concentration of heart was not altered by concentration or source of dietary fat. Meal feeding, however, tended to decrease heart cholesterol concentration ($P<.10$, Table 24).

Cardiac ^{14}C-cholesterol

Dietary fat Source but not level of dietary fat influenced ^{14}C-dpm recovered in DPS. Groups SO had the lowest value for ^{14}C-dpm when compared with groups OF and BT ($P<.05$). Cholesterol-^{14}C S.A. was also decreased in SO groups, but the difference was too small to be statistically significant.

Meal pattern Meal feeding decreased ^{14}C-dpm recovered in DPS ($P<.01$) as well as cholesterol S.A. ($P<.05$, Table 24).

Table 24. Heart weight, DPS[a], ^{14}C-DPS and S.A. DPS (^{14}C-dpm x 10^3/mg). Experiment 2

Treatment	Heart weight g	Heart DPS		
		mg	^{14}C-dpm x 10^3	S.A.
OF-P-AL	1.22±.02[b]	1.32±.03	18.9± .7	14.1± .6
OF-P-MF	1.18±.03	1.24±.05	16.0±1.4	13.0±1.2
20SO-P-AL	1.16±.03	1.25±.04	16.3±1.8	13.2±1.4
20SO-P-MF	1.16±.06	1.22±.04	11.6±1.0	10.0±1.0
20BT-P-AL	1.24±.03	1.28±.05	18.8±1.7	14.6±1.1
20BT-P-MF	1.10±.02	1.22±.03	15.5±1.5	12.6±1.2
Analysis of Variance				
Fat level	NS	NS	NS	NS
Fat	NS	NS	P<.05	NS
Meal pattern	P<.10	P<.10	P<.01	P<.05

[a]Digitonin precipitable sterol.

[b]Mean±SEM.

Feces

Fecal ^{14}C-acid[1]

Dietary fat Concentration and source of dietary fat did not influence the quantity of total acid steroid-^{14}C excreted (Experiments 2 and 3, Table 26). When acid-^{14}C excreted was expressed as percent of total excreted steroid-^{14}C, both concentration and source of dietary fat influenced the proportioning of label. Low-fat groups excreted 85% of total label in the acid fraction as opposed to 80 and 71% by groups SO and BT ($P<.001$, Experiment 2). Groups BT compared with groups SO excreted 71 versus 80% (Experiment 2) and 74 versus 82% (Experiment 3) of total labeled steroid in the acid fraction. In both experiments the increase with SO was highly significant ($P<.001$). The difference in proportion between OF, SO and BT is illustrated in Figure 6.

In the third study groups SO consumed less food than groups BT. Fecal bulk appeared to be associated with quantity of diet consumed. Consequently, fecal bile acid fraction was expressed as ^{14}C-dpm/g diet consumed. When excretion data were calculated in this way, groups SO excreted more acid label than did groups BT ($P<.01$, Experiment 3, Table 27, Figure 7). In Experiment 2 fat source did not influence this parameter but this discrepancy is easily explainable on the basis of differences in hepatic cholesterol S.A. with fat source.

---

[1]Fecal ^{14}C-acid (bile acid, acid steroid) by chloroform:methanol extraction following removal of neutral nonsaponifiable lipid with petroleum ether.

Table 25. Fecal acid and neutral ^{14}C-steroid (Experiments 2 and 3)[a]

Treatment	Experiment 2			
	Fecal steroid			
	Acid ^{14}C-dpm x 10^3	Neutral ^{14}C-dpm x 10^3	Total ^{14}C-dpm x 10^3	Acid-^{14}C % total
Depleted control	572± 44[b]	96±12	668± 48	86±1
OF-P-AL	803± 90	156±16	960± 99	84±1
OF-P-MF	718±109	149±25	931±147	85±1
20SO-P-AL	817± 71	210±18	1027± 80	79±1
20SO-P-MF	588± 62	153±17	741± 44	80±1
20SO-4P-AL	-	-	-	-
20SO-4P-MF	-	-	-	-
20BT-P-AL	657± 70	303±21	996± 72	69±2
20BT-P-MF	714± 54	271±42	991± 90	73±3
20BT-4P-AL	-	-	-	-
20BT-4P-MF	-	-	-	-
Stock-AL	1376±148	245±20	1621±200	85±1

[a]Variable means and analysis of variance are in Table 26.

[b]Mean±SEM.

Experiment 3			
Fecal steroid			
Acid ^{14}C-dpm x 10^3	Neutral ^{14}C-dpm x 10^3	Total ^{14}C-dpm x 10^3	Acid-^{14}C % total
-	-	-	-
-	-	-	-
-	-	-	-
777± 96	206±38	984±133	80±1
680±132	170±35	851±161	80±2
621± 69	140±40	762±101	83±2
663± 82	118±14	780± 92	85±1
629± 62	250±91	879± 90	74±7
555± 59	199±34	754± 73	74±3
628± 33	225±17	853± 42	74±1
586± 57	186±17	772± 69	76±1
-	-	-	-

Table 26. Fecal acid and neutral ^{14}C-steroid: Variable means and analysis of variance

Treatment	Fecal steroid							
	Acid steroid ^{14}C-dpm x 10^3		Neutral steroid ^{14}C-dpm x 10^3		Total steroid ^{14}C-dpm x 10^3		Acid-^{14}C % total	
	Exp.2	Exp.3	Exp.2	Exp.3	Exp.2	Exp.3	Exp.2	Exp.3
OF	756	-	153	-	945	-	85	-
20SO	708	681	183	155	891	806	80	82
20BT	686	599	290	212	994	783	71	74
AL	760	661	222	203	997	802	77	78
MF	675	621	192	166	886	787	79	79
P	-	662	-	204	-	830	-	77
4P	-	624	-	168	-	768	-	79
Analysis of Variance								
Fat	NS	NS	P<.001	P<.02	NS	NS	P<.001	P<.001
Meal pattern	NS	NS	NS	NS	NS	NS	P<.02	NS
Protein	-	NS	-	NS	-	NS	-	NS

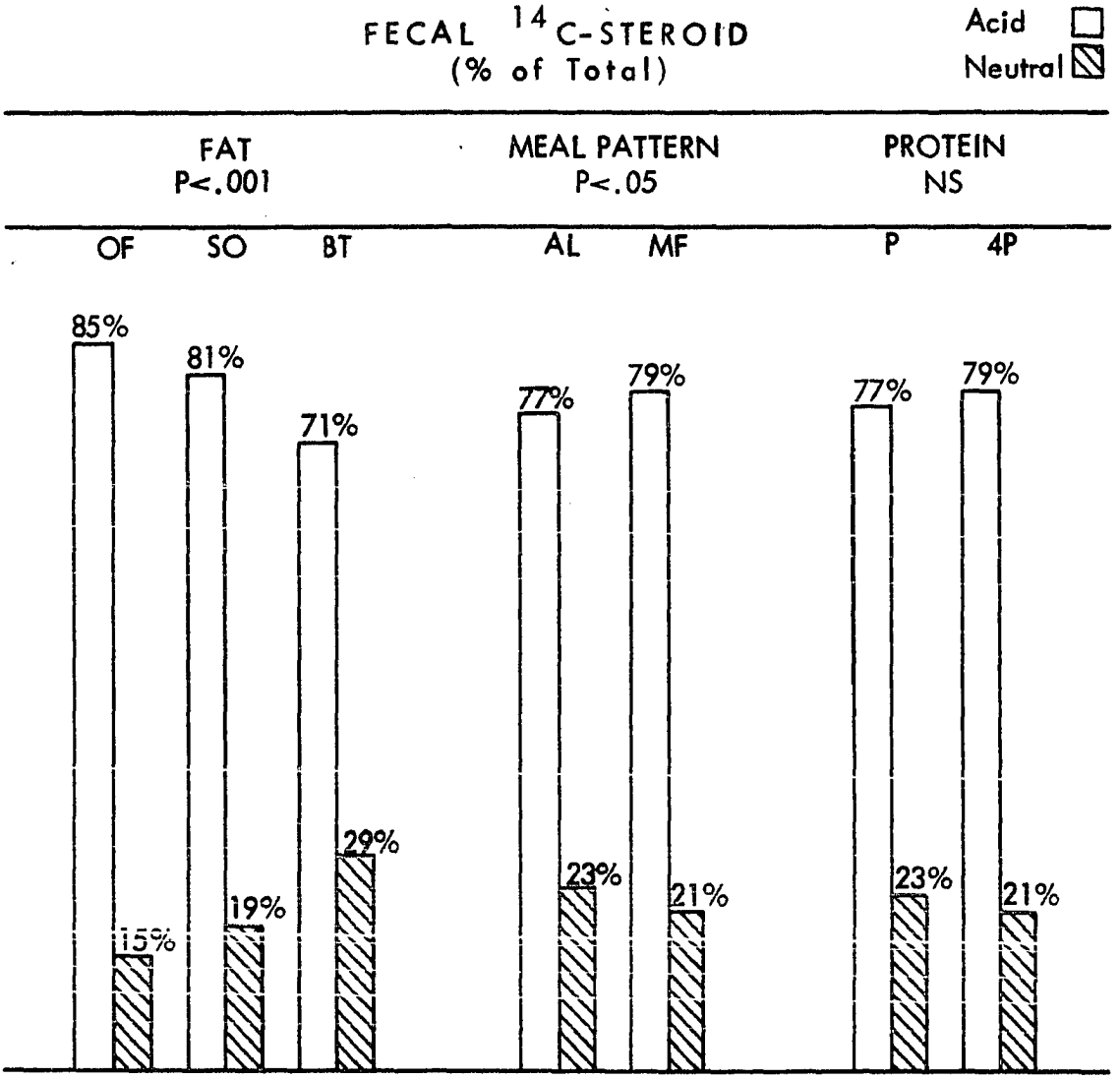

Figure 6. Fecal acid and neutral steroid (% of total excreted steroid ^{14}C-dpm). Experiments 2 and 3

Table 27. Acid and neutral ^{14}C-steroid excreted[a]/g diet consumed

Treatment	Acid steroid excreted ^{14}C-dpm/g diet		Neutral steroid excreted ^{14}C-dpm/g diet	
	Experiment 2	Experiment 3	Experiment 2	Experiment 3
Depleted control	-	-	-	-
OF-P-AL	4590±548[b]	-	718± 97	-
OF-P-MF	4121±462	-	918±137	-
20SO-P-AL	4753±417	3858±550	1201±102	1024±202
20SO-P-MF	3850±180	4414±894	983± 85	1080±201
20SO-4P-AL	-	3479±388	-	789±223
20SO-4P-MF	-	4569±378	-	811± 79
20BT-P-AL	3730±368	3033±202	1627±123	752± 66
20BT-P-MF	4520±428	3033±265	1726±264	1089±168
20BT-4P-AL	-	3150±292	-	1125±114
20BT-4P-MF	-	3233±314	-	1052±135
Stock-AL	4909±643	-	880± 92	-
Analysis of Variance				
Fat	NS	P<.01	P<.001	NS
Meal pattern	NS	NS	NS	NS
Protein	-	NS	-	NS

[a]Large intestine contents were included with excreta.

[b]Mean±SEM.

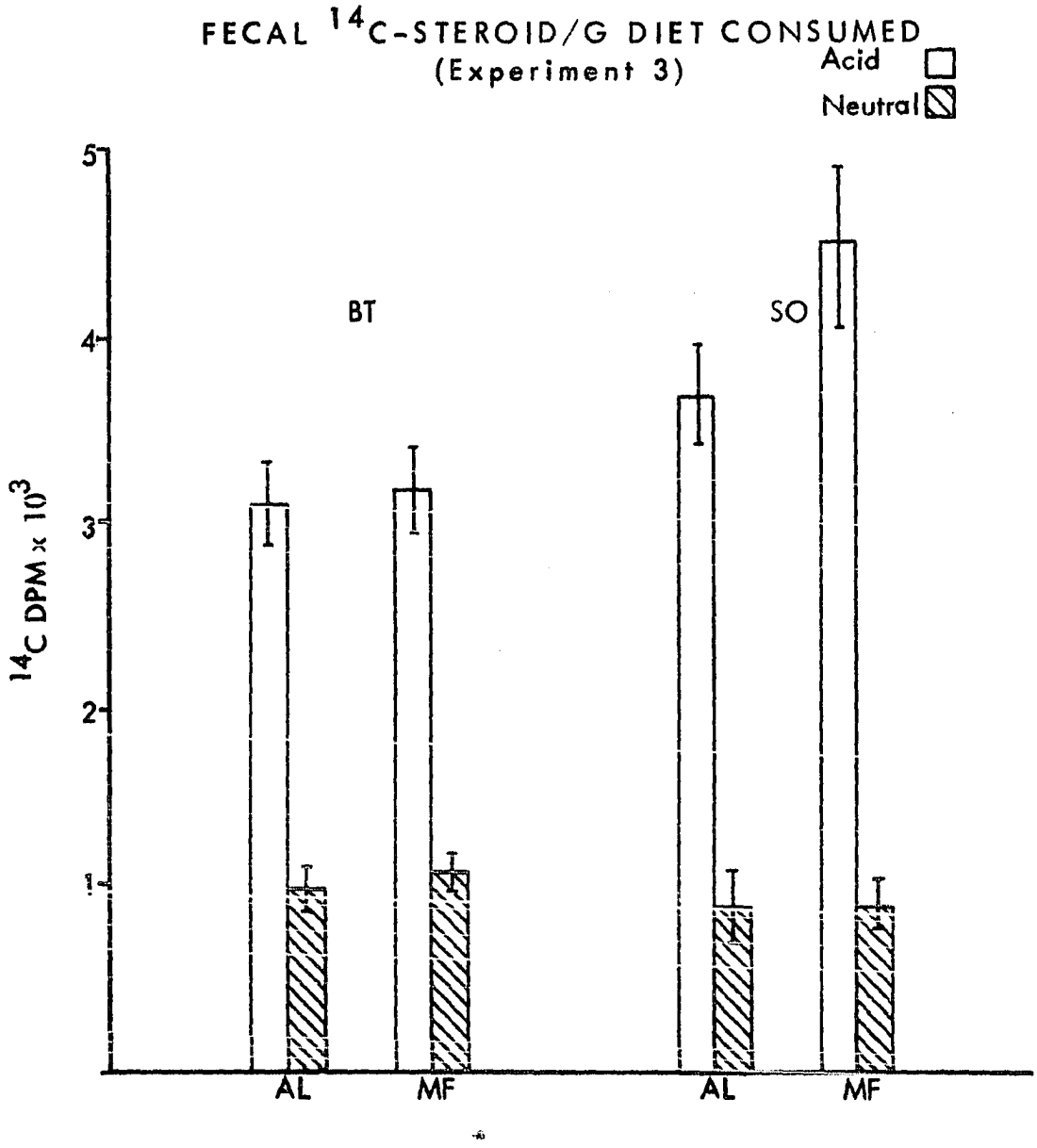

Figure 7. Fecal acid and neutral steroid (^{14}C-dpm/g diet consumed). Experiment 3

Meal pattern AL and MF groups excreted similar amounts of ^{14}C in bile acid in both experiments (Tables 25 and 26). MF groups excreted a larger percentage of label in bile acid than did AL rats in Experiment 2 ($P<.02$) but not in Experiment 3 (Table 26, Figure 6).

Groups MF and AL also excreted similar amounts of ^{14}C-acid/g diet consumed (Table 27) when all groups were included in analysis of variance. When SO was the dietary fat source, however, significantly more acid steroid-^{14}C/g diet was recovered in MF groups compared with AL groups (Figure 7).

Fecal ^{14}C-neutral[1]

Dietary fat Low-fat groups excreted less neutral steroid-^{14}C when compared to SO and BT fed rats (Experiment 2). Source of dietary fat (SO or BT) influenced ^{14}C-neutral excretion in both Experiments 2 and 3. Groups BT excreted more label than did groups SO ($P<.001$, Experiment 2; $P<.02$, Experiment 3). When excreted neutral steroid-^{14}C was expressed per g diet, however, excretion was not altered by dietary fat (Experiment 3, Table 26, Figure 7).

Meal pattern Neither total neutral steroid-^{14}C (Table 26) nor neutral steroid-^{14}C/g diet (Table 27, Figure 7) recovered from feces was altered by meal pattern.

Dietary protein Low and adequate protein diets influenced all parameters of neutral steroid excretion similarly.

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[1]Fecal ^{14}C-neutral (neutral steroid) by petroleum ether extraction from an aqueous solution of basic pH.

Carcass

Carcass[1] cholesterol concentration

Neither total mg carcass cholesterol nor mg cholesterol/100 g body weight were influenced by treatment variables used (Experiment 2, Table 28).

Carcass[1] ^{14}C-cholesterol

Though total neutral steroid-^{14}C was not influenced by any treatment at the level of statistical significance, dietary fat tended to influence the amount of digitonin precipitable-^{14}C. SO groups tended to have less ^{14}C- DPS than did groups OF and BT ($P<.10$), though this difference may be an artifact produced by oxidation of cholesterol during carcass hydrolysis. For whatever reason, groups SO and BT had smaller ratios of ^{14}C in DPS total neutral nonsaponifiable label than did low-fat groups ($P<.02$, Table 28).

Carcass[2] composition

Carcass lipid In Experiment 1, carcasses of 5 rats from each treatment were analyzed for fat and moisture. Fat-free residue (nonlipid, nonwater) gain was calculated by difference.

---

[1]Carcass, as used here, refers to the whole rat minus liver, epididymal fat pad, kidneys, heart, small and large intestines and about 10 ml blood. Data are from carcass hydrolysis in Experiment 2 only.

[2]Carcass refers to the whole rat minus liver, epididymal fat and about 10 ml blood. Data are from Experiment 1 only.

Table 28. Carcass DPS[a], total neutral ^{14}C-steroid and ^{14}C-DPS as proportion of total neutral ^{14}C-steroid (Experiment 2)

Treatment	Carcass DPS			Total neutral steroid C-dpm x 10^6	^{14}C-dpm DPS / ^{14}C-dpm total
	mg	mg/100 g	^{14}C-dpm x 10^6		
Depleted control	395±19[b]	143±6	6.0±.4	15.4± .8	.39
OF-P-AL	415±12	128±4	5.2±.2	10.5± .4	.49
OF-P-MF	398±13	127±4	5.2±.4	10.5± .6	.53
20SO-P-AL	419±14	123±4	4.3±.3	10.0±1.2	.38
20SO-P-MF	407±19	126±6	4.9±.3	10.4± .3	.47
20BT-P-AL	404±10	119±3	5.3±.3	11.2± .5	.48
20BT-P-MF	421±11	129±4	5.4±.4	12.8± .8	.44
Stock-AL	502±12	135±2	3.9±.3	9.2±1.0	.42
OF	406	128	5.2	10.5	.51
20SO	413	125	4.6	10.4	.44
20BT	413	124	5.4	12.0	.46
AL	413	124	5.0	10.8	.46
MF	407	127	5.2	11.2	.48
Analysis of Variance					
Fat	NS	NS	P<.10	NS	P<.02
Meal pattern	NS	NS	NS	NS	NS

[a]Digitonin precipitable sterol.

[b]Mean±SEM.

Dietary fat Carcasses of depleted controls contained 1.8% fat. Refeeding increased % of body fat above that of depleted controls with all treatments (Table 21). Low-fat groups did not deposit[1] as much body lipid as did SO groups ($P<.001$, Table 29). Groups OF and SO deposited 22 and 36 g fat during the refeeding period, respectively. These gains in body fat represent increases of 355 and 590% over mean value of 6.2 g for carcass lipid in depleted controls. In all fat-related parameters calculated - fat as percent body weight, g body fat deposited during refeeding, fat as percent weight gained, percent of kcal consumed deposited as fat - groups SO had higher values than did groups OF ($P<.001$ for all comparisons, Table 21).

Meal pattern Animals deposited practically identical amounts of body fat (Table 21), despite the fact that meal fed animals gained significantly less weight and consumed fewer kcal during the 10 day refeeding period. With the exception of diet 20SO-P, meal feeding led to deposition of more dietary energy as body fat than did ad libitum feeding though overall statistical significance was not obtained with variation in feeding frequency (Table 21).

Dietary protein Although rats fed adequate dietary protein gained about 25% more weight than did rats fed low protein diets, none of the increase in weight was due to body fat (Table 29). Groups P and 4P had similar values for all fat-related parameters calculated (Table 21).

---

[1]Lipid deposited (g) = total carcass lipid (including liver and epididymal lipid) - 6.2 g (mean depleted carcass lipid).

Table 29. Change in carcass composition with realimentation (Experiment 1)

Treatment	Change in body weight g	Deposition during refeeding		
		Lipid[a] g	Water[b] g	Fat free solids[c] g
Depleted control	-	-	-	-
OF-P-AL	47±4[d]	21±1	23±3	5± .9
OF-P-MF	48±2	22±1	23±2	5± .5
OF-4P-AL	67±5	20±2	41±6	9± .9
OF-4P-MF	71±4	23±3	38±4	8±1.4
20SO-P-AL	68±5	38±1	25±4	5±1.1
20SO-P-MF	60±3	29±2	28±2	3±1.0
20SO-4P-AL	97±6	38±2	47±6	12±2.8
20SO-4P-MF	90±6	39±3	46±3	6±1.4
Analysis of Variance				
Fat	P<.001	P<.001	P<.05	NS
Meal pattern	NS	NS	NS	NS
Protein	P<.001	NS	P<.001	P<.01

[a]Lipid deposited (g) = total carcass lipid (including liver and epididymal lipid) - 6.2 g (mean depleted carcass lipid).

[b]Water gain (g) = total carcass water - 201.6 g (mean depleted carcass water).

[c]Fat free solids gained (g) = [autopsy weight (g) - 298 g (mean depleted autopsy weight)] - lipid deposited (g) - water gain (g).

[d]Mean±SEM.

Moisture

Dietary fat Although lipid deposition was the primary reason for differences in weight gain between groups OF and SO, increased amounts of body water were retained with increased fat in groups SO ($P<.05$, Table 29).

Meal pattern Groups AL and MF retained similar quantities of body water during refeeding (Table 29).

Dietary protein Increased dietary protein resulted in increased water retention.

Fat-free solids (FFS)[1]

Dietary fat Rats consuming diets OF and SO deposited similar amounts of fat-free solids during the 10 day refeeding period (Table 29).

Meal pattern Meal feeding decreased regeneration of fat-free solids with SO but not with low-fat diets.

Dietary protein Fat-free residue increased with a change in protein intake from P to 4P ($P<.01$, Table 29). About 80% of weight gained with the higher level of protein was water.

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[1]Fat-free solids = g gain - (g lipid gained + moisture retained).

DISCUSSION

In these studies we have used an experimental model suitable for studying acute changes in lipid metabolism. After severe dietary restriction, rats received ample calories and protein and regained weight rapidly. The short accelerated realimentation will be used to interpret results from these studies. The experimental model employed may mask changes which would be evident with a longer period of refeeding. Acute changes, however, are more easily interpreted with regard to sequences in biochemical events than are changes seen in animals in physiological equilibrium.

The rats were injected with 4-^{14}C-cholesterol 6 days prior to sacrifice. Under these conditions label did not equilibrate with all body cholesterol pools. The rapidly equilibrating cholesterol pool (Goodman et al., 1973), which includes serum and hepatic cholesterol, had the highest concentration of the radiotracer during the 6 days following injection of ^{14}C-cholesterol. Label excreted largely indicated the acute turnover of cholesterol from the rapidly equilibrating pool, primarily from degradation of cholesterol to bile acids.

Approximately 80% of excreted steroid-^{14}C was recovered in the acid steroid fraction of feces in the present study. Other investigators have injected a single dose of 4-^{14}C-cholesterol with similar results over a short period of time (Dupont et al., 1972; Bobek et al., 1973b), despite the fact that only about 50% of fecal steroid is in the acid steroid fraction (Wilson and Siperstein, 1959; Moore et al., 1968). Loss of cholesterol from slowly equilibrating pools (such as muscle and nerve tissue) was

underestimated by the radioanalysis.

In these studies cholesterol biosynthesis has been determined by assessing incorporation of ^{3}H-acetate into DPS 3.5 hours after feeding and 2.5 hours after injection of radiotracer. Acetate label incorporated into neutral lipid minus ^{3}H in DPS has been interpreted as a measure of fatty acid synthesis.

The experimental model used may not have allowed demonstration of differences in cholesterol biosynthesis between variables which would exist under other experimental conditions for several reasons. First, the severely restricted state of the rats prior to realimentation may have altered biochemical priorities for nutrient use. Secondly, changes in acetate pool size may have occurred with meal feeding. In nutritional states such as long-term fasting pool size will increase (Dietschy and Brown, 1974), though it is unlikely that large discrepancies in acetate pool S.A. occur in these studies with repeated short-term fasting. Small increases in pool size with intermittent fasting are probably compensated for by the injection of the same radioactivity into a relatively smaller animal (MF compared to AL).

Thirdly, discrepancies between daily enzymatic activity maxima may occur with these meal patterns (Dugan et al., 1972). Lighting was reversed in all experiments so that the dark part of the cycle occurred between 9 A.M. and 9 P.M. MF groups were allowed access to food only between 9 A.M. and 5 P.M.; AL groups had access to food at all times, but preferred to consume most of their food during the same 8 hour period in which MF groups ate.

New evidence is available on HMG-CoA reductase activities in ad libitum fed rats under normal illumination (Edwards et al., 1972). From these data it seems that AL groups in our study under conditions of reversed illumination would be expected to demonstrate maximal cholesterogenesis close to 2 P.M. In meal fed animals, trained to eat between 9 A.M. and 1 P.M., maximal cholesterogenesis seems to occur at about 6 P.M., approximately 9 hours after feeding (Edwards et al., 1972).

Finally, maximal rates of cholesterogenesis did probably not occur until a few hours after measurements of acetate incorporation into cholesterol were made in this study, 3.5 hours after food consumption (Edwards et al., 1972; Goldfarb and Pitot, 1972). An accurate assessment of the influence of feeding frequency on cholesterogenesis may require measurement of HMG-CoA reductase activity at a number of points throughout a 24 hour period.

Findings reported here confirm those of other investigators who demonstrated that serum cholesterol concentrations increase with limited access to food (Gopalan et al., 1962; Wells et al., 1963; Cohn, 1964; Leveille and Hanson, 1965; Reeves and Arnrich, 1974). A 10 day refeeding period does not consistently allow demonstration of decreased serum cholesterol concentration with polyunsaturated fat or increased protein, though such changes might occur with an extended refeeding period (Jagannathan and Gopalan, 1963; Kenney and Fisher, 1973). After 10 days of refeeding, however, protein decreased serum cholesterol concentrations with SO but not with BT.

Serum cholesterol concentrations are indicative of body cholesterol

metabolism. The following discussion will deal with parameters measuring cholesterol transport within the rapidly equilibrating cholesterol pool, biosynthesis, (re)absorption, degradation and excretion. Changes in these parameters with dietary fat, meal pattern and level of dietary protein will be examined.

Cholesterol Transport

Transport of cholesterol and other lipids within the circulating pool is accomplished by plasma lipoproteins synthesized in hepatic parenchymal cells (Hamilton, 1972). The equilibrium of serum and hepatic cholesterol within the serum and hepatic cholesterol pool has been shown to be altered by a number of dietary factors including dietary fat, protein concentration and feeding frequency.

Data from these studies indicate that transport of cholesterol within the serum-hepatic pool is influenced by degree of saturation of dietary fat, though serum cholesterol concentrations did not decrease with polyunsaturated fat. Increased dietary protein decreased both serum and hepatic cholesterol concentrations. In the presence of polyunsaturated fat, the major decreases occurred with protein, whereas with saturated animal fat dietary protein did not seem to alter serum and hepatic cholesterol concentrations (Table 7).

A shift of serum-hepatic cholesterol equilibrium toward liver has been demonstrated with polyunsaturated fat in rats, with (McGovern and Quackenbush, 1973b) and without (Avigan and Steinberg, 1958) dietary cholesterol supplementation. We have seen neither increased hepatic cho-

lesterol concentrations nor decreased serum cholesterol concentrations consistently in these studies with SO. The inverse correlation between serum and hepatic cholesterol concentrations, however, was consistently higher in SO compared with OF and BT groups ($r = -.66$ versus $r = -.26$ and $r = -.54$).

The present findings and those of Reeves (1971) demonstrated that elevated serum cholesterol concentrations with meal feeding were accompanied by reciprocal decreases in hepatic cholesterol concentrations (Figure 8). In another study, hepatic cholesterol concentrations were decreased with meal feeding in agreement with studies in this laboratory, though serum cholesterol concentrations with ad libitum and meal fed swine were similar (Anderson and Fausch, 1964).

In the present study, elevated serum cholesterol concentrations with meal feeding may result from adaptation to periodic fasting, for 16 of every 24 hours. There is evidence that differences in cholesterol concentrations between meal patterns increase with time. Elevation of serum cholesterol concentrations with meal feeding were more marked at 30 than 10 days in rats fed in this laboratory under model, dietary and meal conditions identical with the present study (Reeves and Arnrich, 1974). Differences between AL and MF fed monkeys in another study were greater at 8 than at 4 weeks (Gopalan et al., 1962).

Data from several sources can be used to speculate on a possible cause of increased serum cholesterol concentration with meal feeding. Fasting is known to elevate plasma free fatty acid concentrations. The concentration of free fatty acid probably regulates in vivo hepatic triglyceride production (Prigge and Grande, 1973). In rats very low density

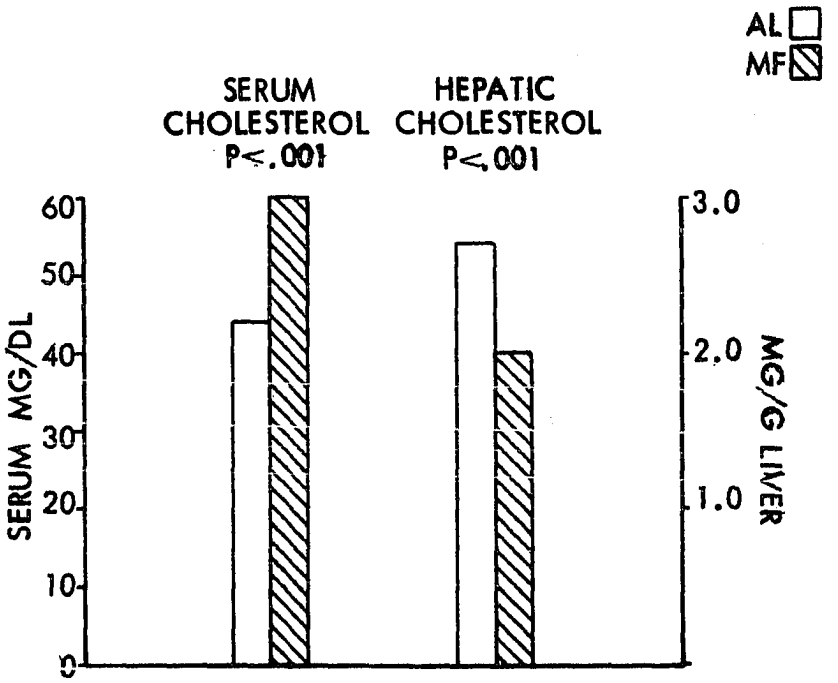

Figure 8. Comparison of serum and hepatic cholesterol concentrations by meal pattern (AL,MF). Experiment 2

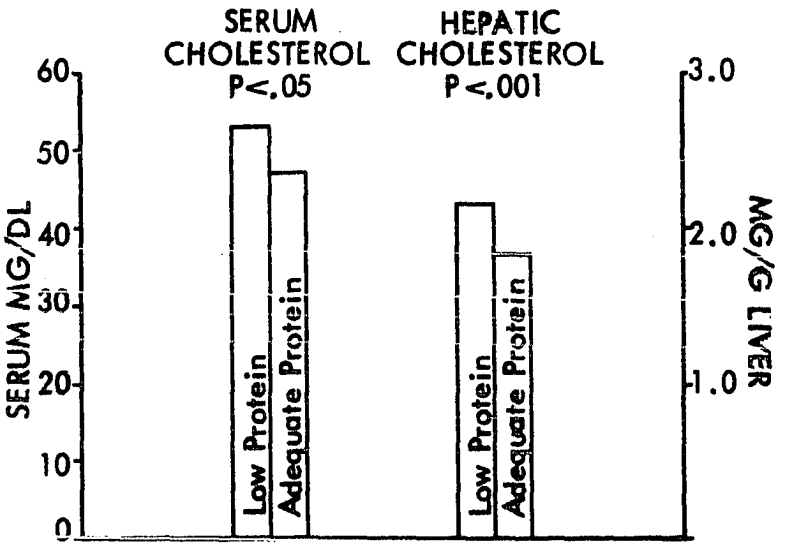

Figure 9. Comparison of serum and hepatic cholesterol concentrations by protein (P,4P). Experiment 3

lipoproteins (VLDL) are secreted by the liver and function in triglyceride transport from the liver. Experimental evidence supports the idea that rate of synthesis and secretion of triglyceride by the liver is increased with meal feeding. In the first study about 25% more lipid was recovered with meal feeding than with continuous eating. Hypertriglyceridemia has been shown to occur in man with decreased feeding frequency (Wadhwa et al., 1973). VLDL require the inclusion of phospholipid and cholesterol for stability (Goh and Heimberg, 1973). Elevated serum cholesterol concentrations with meal feeding may be secondary to elevated serum triglyceride formation in response to free fatty acid concentrations.

Though proportioning of serum-hepatic cholesterol was altered by meal pattern, radiochemical evidence from the present studies suggests that serum and liver cholesterol were in close equilibrium: serum and hepatic cholesterol S.A. within each treatment were almost identical (Tables 8 and 14). Two mechanisms which should be considered in the underlying cause for decreased S.A. with meal feeding are dilution of label by increased biosynthesis and dilution by mixing with cholesterol of relatively lower S.A. cholesterol pools. Radiochemical evidence from these studies precludes elevation in hepatic or intestinal cholesterogenesis with meal feeding (Table 33).

Reduced cholesterol S.A. with meal feeding may, then, result from shifts of cholesterol of low S.A. into the circulating cholesterol pool. Acute labeling with ^{14}C-cholesterol in these studies resulted in cholesterol S.A. greater in liver and serum than in pools which are not in rapid equilibrium. A shift of cholesterol from these tissue pools to the rapidly

equilibrating pool would account for the decreased cholesterol S.A. observed in serum and liver. In all cases but one, ^{14}C recovered in neutral steroid and cholesterol S.A. in intestine, kidney, heart and epididymal lipid were decreased by meal feeding (Experiment 2, Table 30).

The combined hepatic and serum cholesterol values show an apparent decrease in pool size in groups MF compared with groups AL, except when protein intake is increased (Table 31). The data may help to explain the loss of the inverse relationship between serum and hepatic cholesterol concentration with increased protein, though serum cholesterol concentrations continue to be elevated with meal feeding in these groups. Hepatic cholesterol concentrations, specifically the concentration of hepatic cholesterol ester, were elevated after low protein diets. These results suggest impaired transport of cholesterol from the liver. Increases in dietary protein decreased hepatic cholesterol concentration as well as serum cholesterol concentration in Experiment 3 (Figure 9).

Though the findings above may indicate impaired transport from the liver, at least one aspect of hepatic metabolism of cholesterol esters seems normal. Impaired hepatic function has been suggested to result in entrapment of serum cholesterol ester in liver (Fex and Wallinder, 1973). Decreased hepatic weight after depletion is justification to suspect decreased hepatic function. During the 10 day refeeding period, liver size increased from 33 to 100% over the depletion value (Table 11). Cholesterol ester patterns, however, indicated normal function at the end of the feeding period: hepatic and the corresponding serum CEFA patterns were distinctly different (Table 10). Hepatic CEFA patterns appeared to be in-

Table 30. Neutral ^{14}C-steroid and specific activities DPS[a]

Meal pattern	Liver		Small intestine		Kidney		Heart		Epididymal fat[b]
	^{14}C-dpm x 10^3	S.A.	^{14}C-dpm x 10^3	S.A.	^{14}C-dpm x 10^3	S.A.	^{14}C-dpm x 10^3	S.A.	^{14}C-dpm x 10^3
AL	414	15.8	192	4.6	48	6.0	18	14.0	30
MF	266	13.1	162	3.9	40	5.8	14	11.9	22
	P<.001	P<.01	P<.05	P<.05	P<.02	NS	P<.01	P<.05	P<.01

[a]Digitonin precipitable sterol.

[b]Specific activity data not available.

Table 31. Estimate of total cholesterol present in serum and liver

Treatment	Experiment 1[a]		
	Serum(mg)[b]	Liver(mg)	Serum & liver(mg)
Depleted control	3.9	15.4	19.3
OF-P-AL	6.6	28.4	35.0
OF-P-MF	8.2	21.8	30.0
OF-4P-AL	7.1	22.8	29.9
OF-4P-MF	7.3	20.0	27.3
20SO-P-AL	6.6	31.2	37.8
20SO-P-MF	7.3	22.5	29.8
20SO-4P-AL	6.2	27.9	34.1
20SO-4P-MF	7.7	26.2	33.9
20BT-P-AL	-	-	-
20BT-P-MF	-	-	-
20BT-4P-AL	-	-	-
20BT-4P-MF	-	-	-
Stock-AL	-	-	-

[a]Serum values from 5 pooled samples, 2 rats per pool; liver values are from 5 individual animals of 10, therefore combined serum and liver values are an estimate.

[b]Assuming 3.3 ml serum/100 g body weight.

Experiment 2			Experiment 3		
Serum(mg)[b]	Liver(mg)	Serum & liver(mg)	Serum(mg)[b]	Liver(mg)	Serum & liver(mg)
5.2	12.3	17.5	-	-	-
5.6	27.5	33.1	-	-	-
6.6	18.0	24.6	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
5.5	27.7	33.2	7.2	24.6	31.8
7.4	21.1	28.5	7.0	18.7	25.7
-	-	-	5.6	21.6	27.2
-	-	-	6.0	22.0	28.0
5.2	26.0	31.2	5.7	25.4	31.1
7.4	22.6	30.0	7.2	22.3	29.5
-	-	-	5.8	21.8	27.6
-	-	-	7.5	19.8	27.3
8.8	24.6	33.4	-	-	-

Table 32. The influence of fat source on hepatic and intestinal cholesterogenesis (Experiment 3)

	Hepatic DPS ^{3}H-dpm x 10^3	Intestinal DPS ^{3}H-dpm x 10^3
SO	31	42
BT	28	57
	NS	P<.01

Table 33. The influence of feeding frequency on hepatic and intestinal cholesterogenesis (Experiment 3)

	Hepatic DPS ^{3}H-dpm x 10^3	Intestinal DPS ^{3}H-dpm x 10^3
AL	29.6	49.4
MF	29.4	49.3
	NS	NS

fluenced primarily by availability of fatty acids. For example, the predominant fatty acid in hepatic cholesterol esters with SO diets was C18:2, whereas with low-fat diets fatty acid patterns reflected newly synthesized fatty acids, with increased esterification to C16, C16:1 and C18:1.

Esterification of cholesterol in serum is catalyzed by serum lecithin cholesterol acyltransferase (Glomset, 1968). The pattern of CEFA in serum after low-fat and fat diets reflected the specificity of this enzyme for C20:4 esterification of cholesterol (Sugano et al., 1969). The species of fatty acids available due to either biosynthesis or dietary intake were

also factors in the pattern of serum CEFA: an increased proportion of C18:2 and C20:4 was esterified to cholesterol when SO diets were substituted for low-fat diets. These cholesterol esters resemble the hepatic CEFA pattern and may originate, at least in part, in the liver (Gidez et al., 1967).

Hepatic cholesterol esters had similar fatty acid patterns in AL and MF groups though hepatic cholesterol ester concentrations were significantly increased with ad libitum feeding (Table 10).

Cholesterol Biosynthesis

Results reported here indicate elevated hepatic cholesterogenesis with high fat diets compared to low fat diets (Table 13). They are in agreement with those of Linazasoro et al. (1958) and Hill et al. (1960). Under conditions of controlled feeding, other investigators have also found elevated hepatic cholesterogenesis with fat (Bortz, 1967; Goldfarb and Pitot, 1972). Increased rates of cholesterol biosynthesis with high fat diets may be related to elevated cellular levels of NADPH. In adipose tissue of rats, high fat diets appear to cause excess production over utilization of reducing equivalents (Zaragoza, 1974). Maximal activation of HMG-CoA reductase occurred after preincubation with NADPH (Tormanen et al., 1975). These finding suggest that elevated specific activity HMG-CoA reductase may be formed with the challenge of high fat diets.

The observation that increased unsaturation of dietary fat does not elevate hepatic cholesterogenesis (Table 32) is surprising in view of the fact that most studies have indicated elevated cholesterol biosynthesis

with substitution of polyunsaturated fats for monounsaturated and saturated fat sources (Wood and Migicovsky, 1958; Carroll, 1959; Boyd, 1962; Merrill, 1969; Tria et al., 1971; Dupont et al., 1972). More recently, however, Mathias[1] failed to demonstrate a difference in cholesterol biosynthetic rates with fats identical to those used in the present study.

The level of dietary fat may determine differences in rates of hepatic cholesterogenesis when saturation of dietary fat is a variable. One might speculate that with consumption of diets containing 20% fat, as used in the present study, NADPH would cease to be limiting for maximal activation of HMG-CoA reductase. Reports that increased concentrations of NADPH occur with high fat diets, and the suggestion that excess NADPH can maximally activate HMG-CoA reductase (Zaragoza, 1974; Tormanen et al., 1975), would support this speculation.

The assumption has been made that most ^{3}H recovered in DPS in the small intestine is from intestinal[2] synthesis of cholesterol. The rate of intestinal cholesterogenesis was elevated with a highly saturated compared with a highly polyunsaturated fat source (Table 32). Intestinal concentrations of DPS were also increased with polyunsaturated fat diets compared to those containing a more saturated fat source. The combination of elevated cholesterol concentration with decreased intestinal cholesterogenesis suggests that negative feedback inhibition of cholesterol biosynthesis may have occurred with polyunsaturated fat diets. The data are consistent with

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[1] M. Mathias, Colorado State University, personal communication, 1975.

[2] Small intestine.

the pronounced reduction of intestinal HMG-CoA reductase activity seen when sterol concentration within the small intestinal crypt cells was increased (Shefer et al., 1973). In addition, intestinal cholesterogenesis has been depressed in other situations in which intestinal concentrations of cholesterol were increased (Cayen, 1971; Chevallier and Lutton, 1973).

The apparent increase in intestinal compared with hepatic cholesterogenesis in these studies (Table 32) is probably a reflection of differences between intestinal and hepatic HMG-CoA reductase activity maxima. Optimal cholesterogenesis occurs up to 6 hours earlier in small intestine compared with liver (Edwards et al., 1972).

Data from the present study support neither decreased nor increased rates of hepatic cholesterogenesis with meal feeding. Other reports suggest that restricted access to food may (Dupont and Lewis, 1963; Dupont, 1965) or may not (Cockburn and Van Bruggan, 1959) elevate cholesterogenesis. These studies, though, were completed before the discovery by Hamprecht et al. (1969) that cholesterol biosynthesis in ad libitum fed rats was maximal at mid-night. In contrast to most investigations, light and feeding schedules were controlled in the present study to optimize measurement of cholesterogenesis. Under these conditions hepatic and small intestinal cholesterogenesis were identical for AL and MF rats 3.5 hours after feeding (Table 33). Though 3.5 hours after feeding was probably less than optimal for measurement of rates of cholesterogenesis, there is no reason to suspect that the data are not correct for the conditions of these experiments. They indicate no trend in biosynthesis with meal feeding in either direction.

Cholesterol biosynthesis appeared to be identical for low and adequate (4 and 17% of kcal, respectively) protein intakes. The stock ration was high in protein (about 25% of kcal) and was associated with elevated cholesterogenesis 3.5 hours after food was consumed (Table 13). High dietary protein (25 versus 12% of kilocalories) has been associated with increased hepatic and intestinal cholesterogenesis (Yeh and Leveille, 1972); however, stock rations in general contain more fiber than do semisynthetic diets and have been associated with increased hepatic cholesterogenesis.

A number of natural fibers, including saponins, are present in our stock ration. Saponins seem to interfere in (re)absorption of cholesterol from the gut (Cheeke, 1971). The elevation in synthesis seen 3.5 hours after feeding stock diet may indicate that HMG-CoA reductase is induced by a mechanism different from that operating in the induction of HMG-CoA reductase by fat or protein. Goldfarb and Pitot (1972) have postulated that cholestyramine, a sequesterant of bile acid, and dietary fat induced HMG-CoA reductase by different mechanisms. They found early elevation of hepatic HMG-CoA reductase activity with cholestyramine while stimulation of activity by fat occurred later. Binding of cholesterol in the gut by dietary fibers could perhaps increase hepatic cholesterogenesis by a mechanism similar to cholestyramine treatment.

Cholesterol (Re)absorption

Excreta have consistently contained less ^{14}C-neutral steroid and decreased ratios of neutral to total steroid-^{14}C when SO was substituted for BT. Increased reabsorption of cholesterol with substitution of polyunsaturated fat for more saturated fat sources has been suggested to explain observed increases in cholesterol half-life with polyunsaturated fat (Bloomfield, 1964; Iritani and Nogi, 1974).

On the other hand, smaller losses of ^{14}C-neutral steroids from the rapidly equilibrating pool with polyunsaturated versus saturated fat could occur with slowed rate of enterohepatic circulation. The bile acid pool is circulated between liver and gut about 12 times/day (Shefer et al., 1969) and with each recycling a small amount of labeled hepatic cholesterol in bile is lost to excreta. Investigators have found decreased hepatic secretion of bile acids (McGovern and Quackenbush, 1973c) and slower intestinal motility (McGovern and Quackenbush, 1973b) with SO compared to BT, the dietary fats used in the present studies. These findings suggest that substitution of SO for BT slows the rate of enterohepatic circulation, and, therefore, decreases loss of neutral steroid from the rapidly equilibrating pool.

On the assumption that a decreased rate of enterohepatic circulation accounts for decreased fecal loss of neutral steroid label, it appears that the rate of enterohepatic circulation in MF rats was slowed in comparison to AL groups. In both radiochemical studies, total neutral steroid excretion tended to decrease with meal feeding ($P<.09$, Experiment 2; $P<.15$, Experiment 3, Tables 25 and 26). If enterohepatic circulation, however, was

slowed by meal feeding, the effect may have been related to quantity of diet consumed. All difference between groups MF and AL with respect to neutral steroid-^{14}C excretion, in both studies, was negated by expression of excreted neutral steroid label on the basis of unit consumption of diet (Table 27, Figure 7).

Dietary protein level did not influence cholesterol absorption in studies with chicks (Kenney and Fisher, 1973). Excretion data from the present studies with low and adequate protein concentrations are consistent with these findings (Tables 25 and 26). A tendency toward increased cholesterol and bile acid reabsorption, however, may be indicated by the trend ($P<.16$) toward decreased neutral steroid-^{14}C in excreta with 4P compared to P diets, especially with dietary SO. Excretion of neutral steroid label in BT groups was not influenced by protein, suggesting that animals have amply recovered from their previous protein deprivation. A synergism between polyunsaturated fat and protein may serve to increase reabsorption of cholesterol in the present study.

Cholesterol Degradation

It is generally accepted that polyunsaturated fats increase cholesterol degradation to bile acids. Direct evidence for this statement comes from analysis of bile obtained by bile duct cannulation following portal administration of ^{3}H-cholesterol (McGovern and Quackenbush, 1973c). Indirect evidence comes from excretion of acid steroid and from measurements of cholic acid half-life (Antonis and Bersohn, 1962; Gordon et al., 1964; Lindstedt et al., 1965; Moore et al., 1968; Connor et al., 1969). Data

presented here further support that cholesterol degradation is increased with polyunsaturated fat. Substitution of SO for BT tended to increase fecal bile acid-^{14}C in Experiment 3 ($P<.13$, Table 26). Since there were differences in food consumption between rats fed SO and BT, fecal bile acid-^{14}C was expressed as ^{14}C-dpm/g diet consumed. Equalization of label excreted on the basis of food consumption showed that SO significantly increased acid steroid excretion (Table 27; Figure 7).

Total fecal steroid-^{14}C was fractionated into acid and neutral steroids. The increased proportion of acid steroid label excreted with substitution of SO for BT in the radiochemical studies (Figure 6) is another indirect support for increased degradation of cholesterol with SO. This significant increase in proportion of bile acid-^{14}C with SO, however, also reflects decreased loss of neutral steroid ^{14}C-label.

Finally, there is indirect evidence from approximate calculations of bile acid S.A. which suggests that polyunsaturated fat increases the degradation rate of cholesterol. Assuming a theoretical bile pool size of 14 mg/100 g body weight (Shefer et al., 1969) and by dividing this value into intestinal steroid-^{14}C a figure for bile acid S.A. was obtained. Assuming that the S.A. of bile acid was influenced by the S.A. of its precursor cholesterol, the calculated value was expressed relative to hepatic cholesterol S.A. For example, group 20SO-P-AL had a mean weight of 373 g, combined hepatic and intestinal acid steroid label of 193.7×10^3 ^{14}C-dpm, and hepatic cholesterol S.A. of 11.5×10^3 ^{14}C-dpm. Therefore:

$$\text{Bile acid pool size} = (14 \times 3.73) = 52.2 \text{ mg}$$

$$\text{Bile acid S.A.} = \frac{193.7}{52.2} = 3.71$$

$$\text{Bile acid S.A./hepatic cholesterol S.A.} = \frac{3.71}{11.5} = .324$$

Data from other groups were calculated in the same way. Ratios of bile acid S.A. over hepatic cholesterol S.A. were:

20SO-P-MF	.323
20BT-P-AL	.207
20BT-P-MF	.185

The data indicate relatively higher bile acid S.A. with 20SO-P compared with 20BT-P diets. Kritchevsky et al. (1974) have interpreted increased bile acid S.A. as a measure of bile acid synthesis. If this is correct then the calculations would indicate that SO increased bile acid synthesis.

The enterohepatic circulation of bile acids may have influenced the rate of cholesterol degradation with polyunsaturated fat diets. Bile acid flux (mg/100 g body weight/hour) is important in maintaining feedback inhibition of bile acid synthesis (Shefer et al., 1969). Decreased bile acid flux might increase hepatic bile acid synthesis due to loss of negative feedback inhibition. Reduced rates of enterohepatic circulation of bile acids have been demonstrated with SO compared to BT (McGovern and Quackenbush, 1973b). These are the same fat sources used in the present study. Decreased intestinal motility with SO diets (McGovern and Quackenbush, 1973b) may contribute to the mechanism by which the rate of enterohepatic circulation is decreased.

Data related to acid steroid-^{14}C excretion in the present studies generally support the hypothesis that feeding frequency does not influence cholesterol catabolism. Though acid steroid-^{14}C excretion tended to be decreased with meal feeding in the first radiochemical study, hepatic cho-

lesterol S.A. was also decreased significantly with meal feeding (Tables 26 and 14). Thus the assumption was made that bile acid synthesized from cholesterol in the liver and excreted in feces was also of reduced S.A., and therefore, that ^{14}C-label recovered underestimated bile acid excretion in meal fed rats. In Experiment 3, hepatic cholesterol S.A. was not influenced by feeding frequency and neither was excretion of total acid steroid label in agreement with Bobek et al. (1972, 1973a).

The proportion of total steroid-^{14}C excreted in the acid steroid fraction increased slightly with meal feeding ($P<.05$, Figure 6). This small increase in proportion of acid steroid may be a reflection of decreased neutral label excreted by MF animals over the six day period after injection.

It appears that meal feeding does not influence synthesis of bile acids, except possibly with polyunsaturated fat feeding. Since MF groups consumed significantly less food than did AL groups, the excretion of label in the third study was expressed relative to food consumed (^{14}C-dpm excreted/g diet consumed). Based on these calculations meal pattern had no effect on these parameters with BT diets. When SO was substituted for BT, however, meal feeding significantly elevated acid steroid-^{14}C excretion (Table 27, Figure 7). An increase in label excreted per unit diet consumed is interpreted to indicate increased catabolism of labeled cholesterol.

Yeh and Leveille (1973) concluded that high dietary protein increased excretion of cholesterol and bile acids in the feces. In the present study high protein diets (except the stock ration) have not been fed, but

when low and adequate protein levels were compared, there was no indication that cholesterol turnover increased with dietary protein. Overall excretion of labeled acid and neutral steroid with low and adequate protein diets was similar (Table 26). In fact there was a tendency for adequate protein to decrease acid steroid excretion with SO diets. Rats used in these studies were severely depleted of dietary protein prior to realimentation. Experiments from our laboratory with similarly treated animals had shown that nitrogen retention during the 10 day realimentation period increased progressively as protein intake increased from P to 4P (Chang, 1971). This was evidence, that even with liberal dietary protein, repletion was incomplete after 10 days of refeeding. The excretion response seen with the increased protein after 10 days may be affected by preferential use of protein for lipoprotein synthesis to facilitate absorption. The pattern of excretion might be expected to change with prolonged periods of refeeding as rats become repleted with respect to this nutrient.

Recovery of fecal bile acid-^{14}C was twice as high with stock diet compared to semisynthetic diets. The results are not surprising in view of reports that stock diets, compared to semisynthetic diets, increased excretion of acid steroid and enhanced cholesterol 7 α-hydroxylation (Antonis and Bersohn, 1962; Jacobson et al., 1973; Balmer and Zilversmit, 1974; Kritchevsky et al., 1974 and Johansson, 1970).

Carcass Composition

Carcass analysis data demonstrated that body weight and all parameters of lipid deposition calculated increased ($P<.001$) when high fat diets were substituted for low-fat rations (Table 21). These findings confirm results obtained in a long term study with young rats, in which high-fat and grain rations were compared (Schemmel et al., 1970; Schemmel et al., 1972). A small increase in body water ($P<.05$) accompanied lipid deposition with high fat diets, but the fat-free solids (FFS) were not altered by fat feeding.

After 10 days of realimentation, body composition was not affected by meal pattern (Table 21) despite a significant reduction in food consumption by MF groups (Table 38). The excellent correlation between carcass and epididymal lipid ($r = +.85$, Figure 5) justifies certain deductions from earlier experiments in which analyses had been limited to epididymal lipid (Reeves, 1971). In those experiments the refeeding period had been extended from 10 to 30 days. Reeves' data suggest that meal feeding increases body fat compared to the ad libitum regimen, if adequate time is given for recovery.

Cohn and Joseph (1968) suggested that changes in body fat composition with decreased periodicity of food intake were contingent on the consumption of at least 80% of kcal consumed by ad libitum fed controls. In experiments in this laboratory, MF rats met this criterion with what appeared to be increased carcass fat (Reeves, 1971).

Increased dietary protein concentration increased weight gain during realimentation. Weight gain represented mostly fat free solids (FFS) accom-

panied by a large increase in body water. Earlier investigations in this laboratory had demonstrated increased nitrogen retention with 4P versus P rations after 10 days of refeeding (Chang, 1971). These results are consistent with the assumption made here that the FFS fraction is largely protein in nature.

Lipogenesis

It is well documented that lipogenesis is decreased by high fat rations. Data from this study are in agreement with those of others indicating that dietary fat suppresses lipogenesis (Experiment 2, Tables 17 and 22). Fat source did not consistently influence lipogenesis as measured by acetate incorporation into fatty acids in the present study. Substitution of BT for a polyunsaturated fat increased hepatic and epididymal lipogenesis (Experiment 2) in agreement with Dupont et al. (1972). The same trend toward increased hepatic lipogenesis with the saturated fat source was shown in the third experiment.

Incorporation of ^{3}H-acetate into hepatic and adipose lipid was increased by meal feeding, but not to the level of statistical significance. There is a possibility that secretion of newly synthesized fat from the liver is altered by meal pattern so that the relative differences in lipogenic capacity are lost. Reeves (1971), however, did not find large increases in NADPH generating enzyme systems or in citrate cleavage enzyme (indicators of lipogenesis) with the same meal patterns as used in these studies. The enzymatic data (Reeves, 1971) combined with radiochemical data suggest that lipogenesis is not greatly elevated with the meal pattern

used here since animals are not acutely challenged with food. Investigators who have restricted access to food to shorter periods of time have found increased lipogenesis with decreased feeding frequency (Allee et al., 1972), though more recently other investigators have reported similar lipogenic responses in trained meal eaters compared to ad libitum consumers in response to a single meal (Baker and Huebotter, 1973).

Meal feeding seemed to increase the proportion of total fatty acid synthesized in epididymal tissue, as representative of adipose tissue. Rough calculations of adipose contribution to lipogenesis were made, assuming similar lipogenic rates in all adipose tissue sites, though this is probably an oversimplification (Anderson et al., 1972; Pothoven and Beitz, 1973). The calculations of adipose tissue contribution to lipogenesis were made by multiplying weight of adipose tissue (Experiment 1, Table 21) by the S.A. of epididymal lipid (Experiment 2, Table 22) and comparing the figures obtained with total hepatic lipid-^{3}H. These calculations indicated that up to 81% of total lipogenesis occurred in adipose tissue in meal fed rats in the present study. Our data indicate an increase in adipose contribution to lipogenesis with meal feeding. A further decrease of the meal period from 8 to 2 hours, to conform with the model of Allee et al. (1972), might be expected to further increase the proportion of lipid synthesized by adipose tissue.

Hepatic lipogenesis did not decrease when dietary protein was increased from low to adequate concentration (Table 17). These data are not in agreement with reports that lipogenesis decreases with substitution of high for moderate concentrations of dietary protein (Yeh and Leveille,

1969; Govind et al., 1973). Diets used here, however, contained 20% fat as compared to 5 and 15% fat in the other studies. The effect of dietary protein in decreasing lipogenesis may be overcome by large amounts of dietary fat. In addition, the reciprocal decrease in dietary carbohydrate with increased protein, rather than increased dietary protein alone, may account for the decreased rate of lipogenesis.

Summary

In summary, the major difference seen in cholesterol metabolism with meal pattern alteration was a shift of equilibrium between serum and hepatic cholesterol concentrations toward the serum with meal feeding. These findings were consistent with the suggestion from Reeves (1971) that increases in serum cholesterol with meal feeding were accompanied by decreases in hepatic cholesterol concentrations. No evidence was found for differing rates of hepatic and intestinal cholesterol biosynthesis with meal pattern. The loss of ^{14}C-cholesterol was slightly decreased with meal feeding. Loss of steroid from the rapidly equilibrating pool, however, may have been underestimated by a more dilute bile acid precursor pool. The decreased excretion of label was attributed to dilution from other body cholesterol compartments. This statement is based on the fact that hepatic cholesterol S.A. was decreased by meal feeding in both Experiments 1 and 2.

There was indirect evidence based on expression of excreted bile acid-^{14}C/g diet consumed that bile acid synthesis was increased by polyunsaturated fat in meal fed animals though the total amount of label in the excreta did not reflect such a change.

Alterations in a number of parameters seen with polyunsaturated fat diets in this study can be reasonably explained on the basis of increased rates of cholesterol degradation to bile acids and decreased enterohepatic circulation of bile acids. These findings confirm those of other investigators.

This study did not indicate that changes with increases in polyunsaturation were accompanied by increased rates of cholesterol biosynthesis. Other investigators, however, have generally reported increased turnover as a combination of elevated biosynthetic and degradative rates of cholesterol. The model used in the studies also did not allow consistent demonstration of decreased serum cholesterol concentration with polyunsaturated fat. These studies may indicate that changes in cholesterol degradation precede other changes in cholesterol metabolism. Or increased biosynthesis may have occurred with SO feeding though ^{3}H-acetate incorporation into DPS 2.5 hours after food consumption did not indicate such change.

Increased dietary protein in this study did decrease serum and hepatic cholesterol concentration. These decreases were not reflected in increased cholesterol biosynthesis and degradation. Since rats fed higher protein grew more rapidly, the apparent decrease in serum-hepatic cholesterol pool size may have been utilized in increased tissue restoration. Rats in a physiological steady state or rats fed higher protein might have demonstrated increased rates of cholesterol turnover with the same alteration in serum-hepatic cholesterol pool size.

It would have been useful to study cholesterol biosynthesis by measurements of HMG-CoA reductase activity and $^{3}H_2O$ incorporation into DPS,

throughout the diurnal cycle, with regard to both meal pattern and dietary fat. Labeled water overcomes differences in measurement of biosynthetic rates due to inequalities in precursor pool size.

SUMMARY AND CONCLUSIONS

Three experiments were designed to explore the influence of feeding frequency and dietary fat or protein on cholesterol metabolism in adult rats. The model used in these studies had been developed in this laboratory to study rapid lipogenesis in adult rats recovering from severe undernutrition. Adult male rats were restricted to 60% of their original weight using a diet with negligible quantities of dietary fat and protein. Depletion was followed by a 10 day realimentation with diets containing 2 or 40% of kcal as fat (SO or BT) and 4 or 17% of kcal as protein. This depletion-refeeding model has proven useful in measurements of acute changes of cholesterol metabolism. In these studies, all rats began in the identical nutritional state and rapidly responded with alterations in cholesterol transport to dietary and feeding frequency variables.

The first experiment confirmed data obtained earlier in this laboratory (Reeves, 1971). It indicated elevated serum cholesterol concentrations in rats consuming their daily allotment within an 8 hour period per day (MF) compared with controls allowed unlimited access to food (AL). Data from this experiment further confirmed that the increase in serum cholesterol concentration with meal feeding was accompanied by a reciprocal decrease in hepatic cholesterol concentration. Serum cholesterol concentrations were not affected by dietary polyunsaturated fat or dietary protein, though such alterations had been reported by other investigators.

It is generally accepted that shifts in serum cholesterol concentrations with changes in dietary patterns are the result of alterations in one or more of the following factors: cholesterol equilibria between

tissues, rate of cholesterol absorption, rate of cholesterol biosynthesis and (or) rates of degradation and excretion of cholesterol and its metabolites. Furthermore, alterations in cholesterol metabolism could occur prior to, and result in, altered serum cholesterol concentrations. Demonstration of differences in cholesterol metabolism with polyunsaturated fat and increased dietary protein might indicate the mechanisms by which serum cholesterol concentrations are altered in rapidly changing physiological states.

Following the first experiment, parameters of in vivo cholesterol metabolism were evaluated in two further studies using acute labeling with 4-^{14}C-cholesterol and ^{3}H-acetate. These radiotracers were injected once intraperitoneally during the period of realimentation. Labeled cholesterol was injected 6 days prior to sacrifice to measure acute cholesterol turnover. On the day of sacrifice, animals were injected with ^{3}H-acetate following a 1 hour meal to obtain a relative measure of cholesterol biosynthesis 3.5 hours after food consumption.

Lighting, food consumption and, therefore, diurnal maxima for cholesterol biosynthesis and degradation were reversed in these studies. Adaptation to reversed lighting and food consumption patterns began during the period of dietary restriction between 32 and 54 days prior to realimentation.

Distribution of ^{14}C-label in bile acids and neutral steroids, and cholesterol S.A. in serum, liver and small intestine were altered by the variables used in the radiochemical studies. These alterations were taken as evidence of differences in overall cholesterol metabolism, though the

mechanisms underlying these metabolic changes remain a matter of speculation. Combined chemical and radiochemical data suggest that alterations occurred primarily in plasma and enterohepatic cholesterol transport and in hepatic cholesterol degradation to bile acids. The primary influence of meal pattern upon cholesterol metabolism seems to be the alteration of the equilibrium between serum and hepatic cholesterol. A shift of cholesterol toward the plasma compartment with meal feeding may contribute to decreased labeling of cholesterol pools which equilibrate less rapidly than the serum-hepatic cholesterol pool. At the same time, the S.A. of cholesterol in the rapidly equilibrating pool could be decreased by dilution of label with cholesterol of low S.A. from other compartments.

In addition, changes in excretion of neutral steroids suggested that enterohepatic circulation was decreased in proportion to consumption of diet. Decreased food consumption in meal fed rats was accompanied by a proportional decrease in excretion of neutral steroid-^{14}C. Neither cholesterol biosynthesis nor degradation seemed to be influenced by meal pattern.

Polyunsaturated fat in the diet appeared to enhance primarily the degradation of cholesterol. Several indirect pieces of evidence from the present study support this conclusion. These findings are in agreement with those of a number of investigators, but are noteworthy in that they seem to precede changes in serum and hepatic cholesterol concentrations which often occur with polyunsaturated fat feeding. Though small increases in the hepatic cholesterol ester fraction occurred in animals fed polyunsaturated fat, it is doubtful that the hepatic cholesterol ester concentration influenced bile acid synthesis. The mechanism by which polyunsatu-

rated fats increase cholesterol degradation rates is open to further study.

The excretion of neutral steroid label decreased with polyunsaturated compared to saturated fat. These results suggest that enterohepatic circulation was slowed with polyunsaturated fat, consistent with another report (McGovern and Quackenbush, 1973c).

Finally in the model used here, polyunsaturated fat did not markedly influence equilibrium within the serum-hepatic cholesterol pool as has been claimed (Avigan and Steinberg, 1958). We found the highest inverse correlation between serum and hepatic cholesterol concentrations, however, following polyunsaturated fat diets.

Protein did not appear to increase turnover of cholesterol in these studies. In fact we found decreased losses of label in excreta with increased dietary protein in 2 of 4 comparisons. These findings suggest that adult animals recovering from protein and calorie malnutrition selectively utilized available protein. During the 10 day period animals were still actively undergoing repletion of lost body protein stores, as has been shown in an earlier report from this laboratory (Chang, 1971). The challenge of large quantities of dietary fat in rats during realimentation may have resulted in preferential use of available protein for lipoprotein formation by the gut.

Data from the present studies are consistent with the following hypotheses: 1) enhanced serum cholesterol and triglyceride levels occur as an adaptation to periodic fasting; 2) the requirement of the liver for cholesterol to stabilize secreted VLDL (very low density lipoproteins) may cause mobilization of cholesterol from less rapidly equilibrating choles-

terol pools as well as increases in serum cholesterol; 3) transfer of cholesterol from the liver may be impaired with low protein diets and possibly with polyunsaturated fat.

The overall turnover of cholesterol within the rapidly equilibrating pools did not seem to be influenced by meal patterns and the various diets used in these studies. The single exception was the stock ration, which produced marked increases in both cholesterol biosynthesis and degradation in comparison to semisynthetic diets. Excretion of cholesterol and its metabolites differs from other factors in cholesterol metabolism in that it is not an active process. Excretion of cholesterol is subject to a number of influences including alterations in cholesterol metabolism by changes in transport, absorption, synthesis and degradation. Radiochemical data of excretion from these studies demonstrate that, despite seeming differences in cholesterol metabolism, total loss of rapidly equilibrating cholesterol is remarkably similar with a number of variables including both feeding frequency and dietary composition.

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APPENDIX

Table 34. Body weights and days to depletion (Experiment 1)

Treatment	Mean body weights			Recovery %	Days required to deplete
	Initial g	Depleted g	Refed g		
Depleted control	472±2	298±1	-	-	32±2
OF-P-AL	471±2	297±1	346±3	28	32±2
OF-P-MF	468±2	297±1	340±4	25	32±1
OF-4P-AL	468±2	299±1	368±4	41	34±2
OF-4P-MF	466±2	300±1	364±4	39	33±2
20SO-P-AL	467±1	298±1	364±5	39	33±2
20SO-P-MF	471±2	298±1	362±4	37	32±1
20SO-4P-AL	469±2	298±1	389±4	53	32±2
20SO-4P-MF	467±1	298±1	384±4	51	32±1
OF	468	298	355	-	32
20SO	469	298	374	-	32
AL	469	298	366	-	32
MF	468	298	363	-	32
P	469	298	353	-	32
4P	468	299	376	-	33
Analysis of Variance					
Fat	NS	NS	P<.001	-	NS
Meal pattern	NS	NS	NS	-	NS
Protein	NS	NS	P<.001	-	NS

Table 35. Body weights and days to depletion (Experiment 2)

Treatment	Mean body weights			Recovery %	Days required to deplete
	Initial g	Depleted g	Refed g		
Depleted control	491±3	301±1	-	-	52±1
OF-P-AL	489±3	298±1	354±2	29	56±1
OF-P-MF	488±2	299±1	346±2	25	54±2
20SO-P-AL	489±2	298±1	369±3	37	56±2
20SO-P-MF	488±7	298±1	361±3	33	54±2
20BT-P-AL	487±2	301±1	375±3	40	56±3
20BT-P-MF	502±7	298±1	365±5	33	56±2
Stock-AL	493±3	296±2	423±4	64	54±2
OF	488	299	350	-	55
20SO	489	298	365	-	55
20BT	495	300	369	-	56
AL	489	299	365	-	56
MF	492	299	356	-	55
Analysis of Variance					
Fat	NS	NS	P<.001	P<.001	NS
Meal pattern	NS	NS	P<.001	P<.001	NS

Table 36. Body weights and days to depletion (Experiment 3)

Treatment	Mean body weights			Recovery %	Days required to deplete
	Initial g	Depleted g	Refed g		
20S0-P-AL	473±7	293±3	373±8	44	32±2
20S0-P-MF	465±3	300±1	356±6	34	32±1
20S0-4P-AL	465±4	297±2	389±3	55	32±1
20S0-4P-MF	469±4	300±2	377±4	46	33±1
20BT-P-AL	471±5	297±2	383±6	49	33±1
20BT-P-MF	473±4	298±1	368±5	40	31±2
20BT-4P-AL	468±4	299±1	407±4	65	32±1
20BT-4P-MF	467±3	298±1	387±7	53	33±2
20S0	468	298	375	-	32
20BT	467	299	387	-	32
AL	469	297	390	-	32
MF	466	300	373	-	32
P	468	298	371	-	32
4P	467	298	389	-	32
Analysis of variance					
Fat	NS	NS	P<.01	P<.001	NS
Meal pattern	NS	NS	P<.001	P<.001	NS
Protein	NS	NS	P<.001	P<.001	NS

Table 37. Food intakes and food efficiencies[a]

Treatment	Experiment 1[b]		Experiment 2[b]		Experiment 3[c]	
	Food intake kcal/day	Food efficiency g gain/ 100 kcal	Food intake kcal/day	Food efficiency g gain/ 100 kcal	Food intake kcal/day	Food efficiency g gain/ 100 kcal
OF-P-AL	71±3[d]	9.0±.3	80±1	9.1±.3	-	-
OF-P-MF	57±3	8.0±.5	63±1	8.5±.2	-	-
OF-4P-AL	71±2	12.0±.6	-	-	-	-
OF-4P-MF	62±2	10.9±.5	-	-	-	-
20SO-P-AL	84±4	9.4±.3	88±3	9.3±.2	96±4	7.9±.3
20SO-P-MF	72±3	8.4±.3	73±3	8.8±.2	74±5	7.4±.5
20SO-4P-AL	88±2	12.0±.2	-	-	83±1	11.3±.3
20SO-4P-MF	79±2	11.4±.5	-	-	74±4	10.6±.3
20BT-P-AL	-	-	91±3	9.4±.3	99±4	8.7±.5
20BT-P-MF	-	-	74±3	8.8±.3	85±4	8.1±.5
20BT-4P-AL	-	-	-	-	96±4	11.3±.3
20BT-4P-MF	-	-	-	-	86±4	10.0±.4

[a]Variable means and analysis of variance are in Table 39.

[b]After 9 days of refeeding.

[c]After 10 days of refeeding.

[d]Mean±SEM.

Table 38. Food intakes and food efficiencies: Variable means and analysis of variance

Treatment	Experiment 1[a]		Experiment 2[a]		Experiment 3[b]	
	Food intake kcal/day	Food efficiency g gain/100 kcal	Food intake kcal/day	Food efficiency g gain/100 kcal	Food intake kcal/day	Food efficiency g gain/100 kcal
OF	63	8.6	68	9.2	-	-
20SO	78	9.4	80	9.0	81	9.5
20BT	-	-	78	9.5	91	9.8
AL	73	8.9	86	9.2	92	10.0
MF	68	9.0	66	9.2	79	9.3
P	69	7.7	-	-	88	8.0
4P	71	10.3	-	-	84	10.9
Analysis of Variance						
Fat	P<.001	P<.01	P<.01	NS	P<.001	NS
Meal pattern	P<.01	NS	P<.001	NS	P<.001	P<.02
Protein	NS	P<.001	-	-	NS	P<.001

[a]After 9 days of refeeding.

[b]After 10 days of refeeding.

Table 39. Food efficiencies for refeeding period minus initial two days (Experiments 2 and 3)

Treatment	Food efficiency g gain/100 kcal	
	Experiment 2[a]	Experiment 3[b]
OF-P-AL	5.6±.3[c]	-
OF-P-MF	6.8±.3	-
20SO-P-AL	5.8±.4	5.2±.6
20SO-P-MF	6.9±.3	5.6±.5
20SO-4P-AL	-	8.4±.5
20SO-4P-MF	-	8.9±.7
20BT-P-AL	6.7±.3	6.2±.6
20BT-P-MF	7.5±.3	6.9±.6
20BT-4P-AL	-	8.8±.4
20BT-4P-MF	-	9.4±.6
Analysis of Variance		
Fat	P<.02	P<.05
Meal pattern	P<.001	NS
Protein	-	P<.001

[a]Refeeding days 3-9.

[b]Refeeding days 3-10.

[c]Mean±SEM.

Figure 10. Food consumption in kcal/2 days of the 10 day realimentation period. Food consumption plotted by diet (Stock, OF-P, 20SO-P, 20BT-P) and meal pattern (AL, MF). Experiment 2

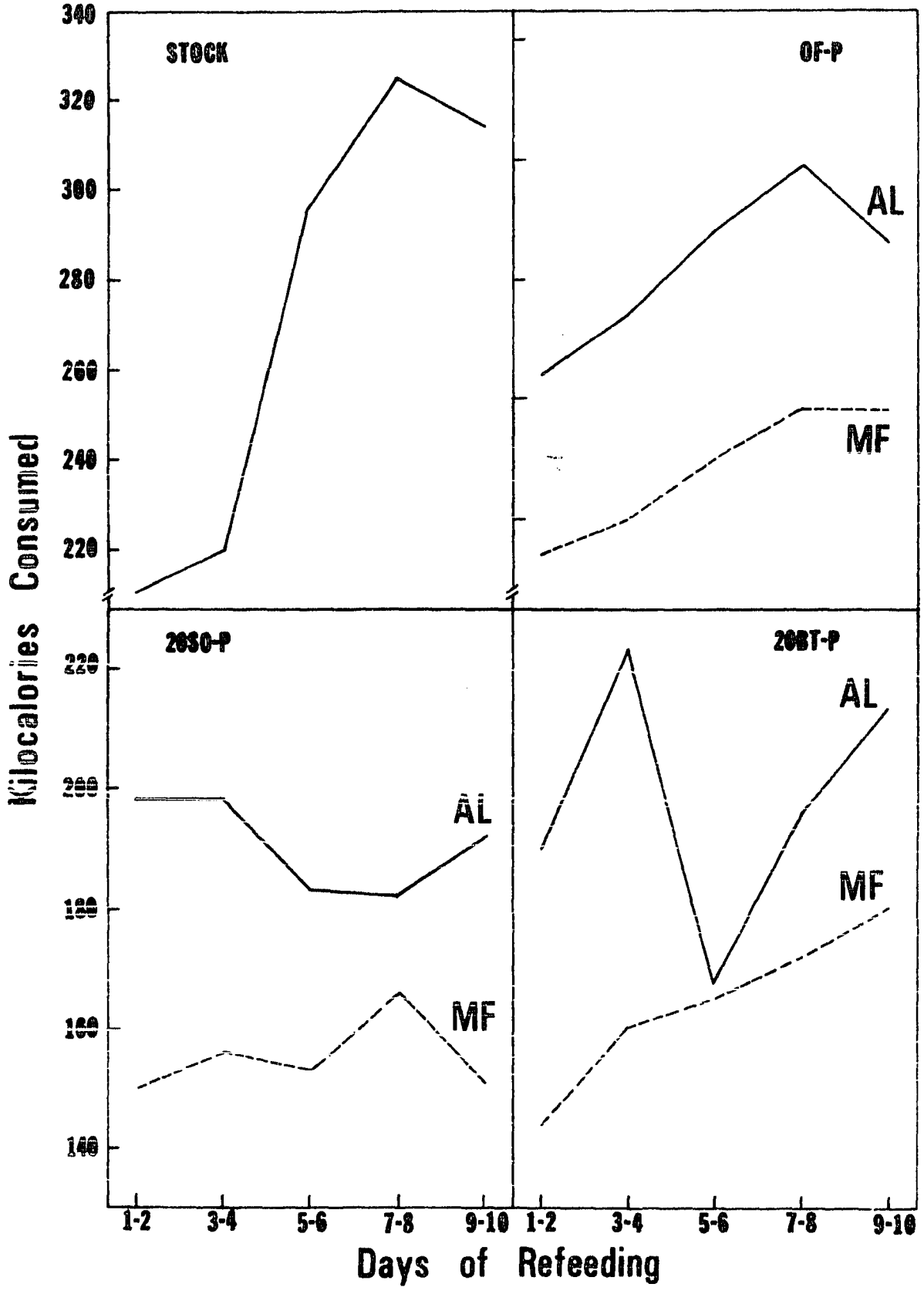

STOCK
OF-P
AL
MF
26SO-P
AL
MF
26BT-P
AL
MF
340
320
300
280
260
240
220
220
200
180
160
140
1-2
3-4
5-6
7-8
9-10
Kilocalories Consumed
Days of Refeeding

Figure 11. Food efficiency (g gain/100 kcal)/2 days of the 10 day realimentation period. Food efficiency plotted by diet (Stock, OF-P, 20SO-P, 20BT-P) and meal pattern (AL, MF). Experiment 2

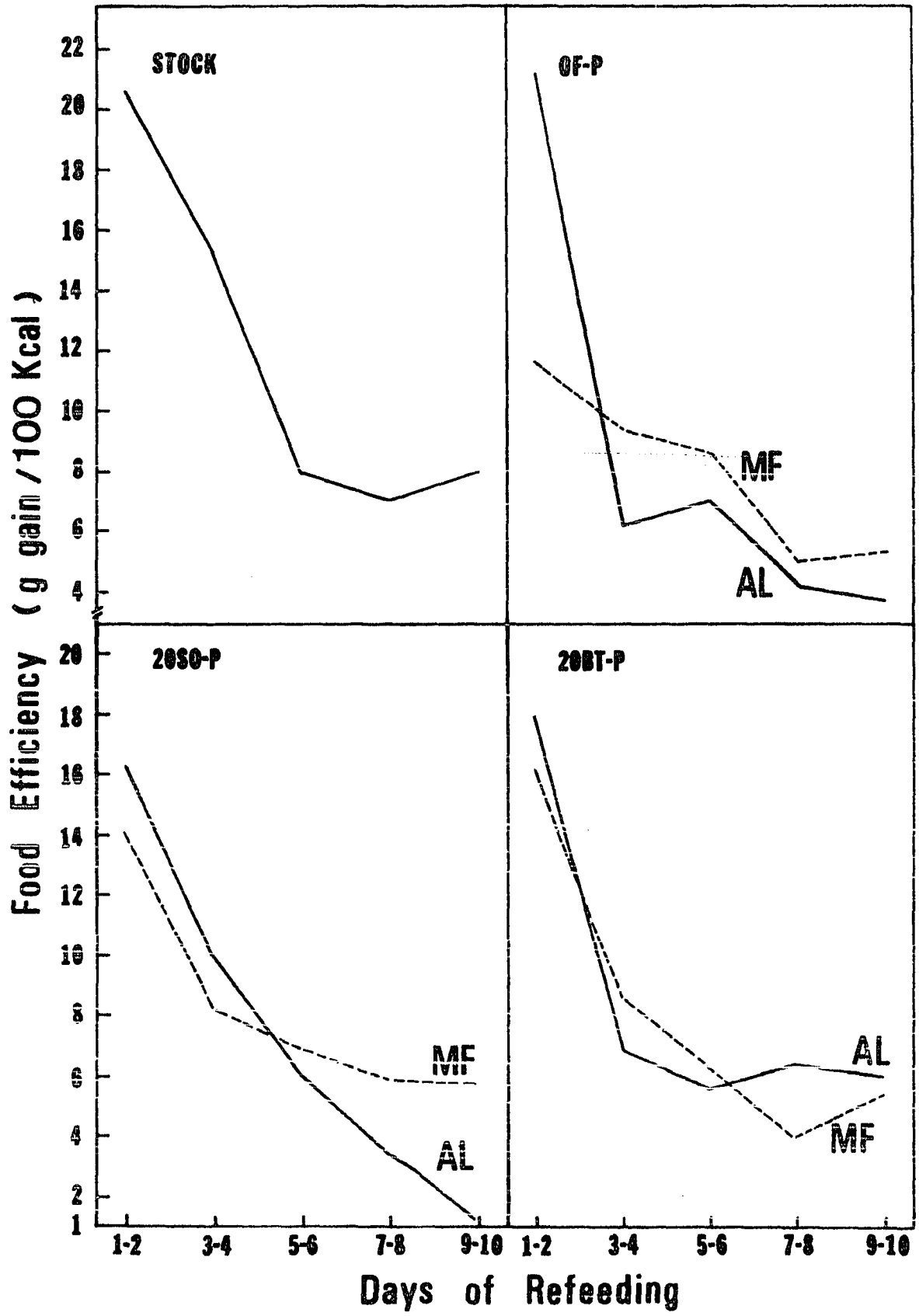

STOCK
OF-P
20SO-P
20BT-P
MF
AL
Food Efficiency (g gain / 100 Kcal)
Days of Refeeding
1-2
3-4
5-6
7-8
9-10

Figure 12. Food consumption in kcal/2 days of the 10 day realimentation period. Food consumption plotted by diet (20SO-P, 20SO-4P, 20BT-P, 20BT-4P) and meal pattern (AL, MF). Experiment 3

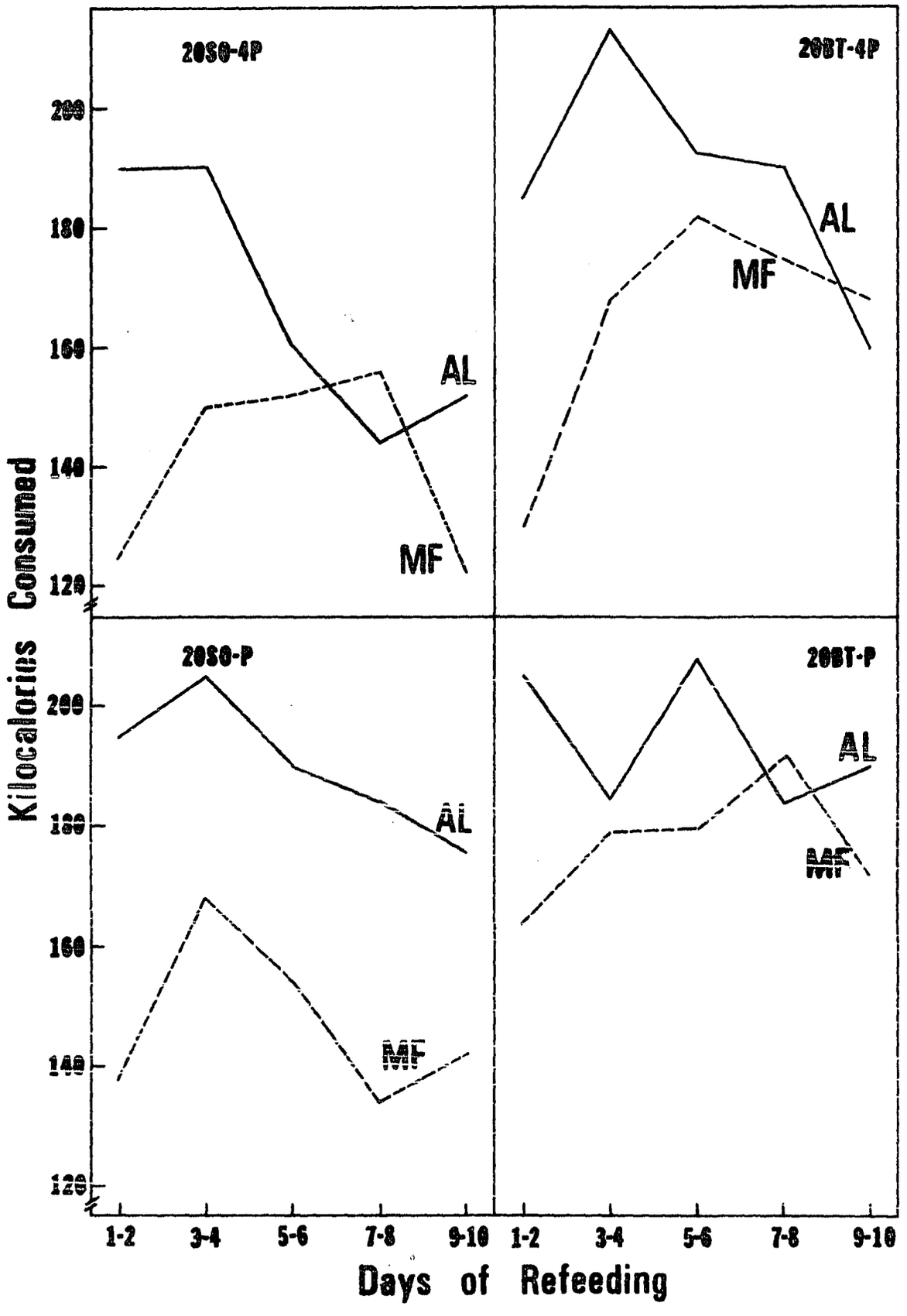
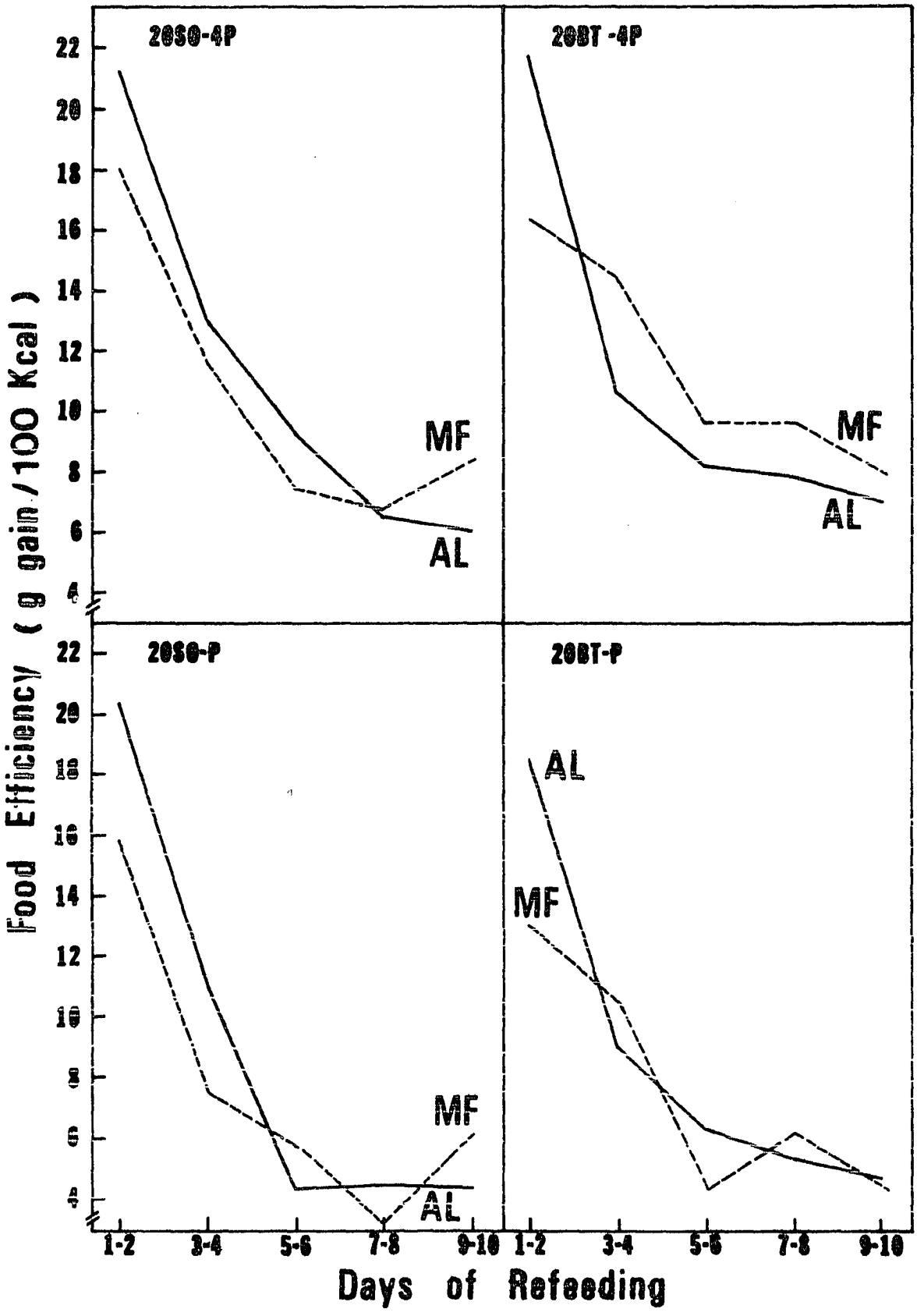

20S0-4P
20BT-4P
20S0-P
20BT-P
AL
MF
Kilocalories Consumed
200
180
160
140
120
1-2
3-4
5-6
7-8
9-10
Days of Refeeding

Figure 13. Food efficiency (g gain/100 kcal)/2 days of the 10 day realimentation period. Food efficiency plotted by diet (20SO-P, 20SO-4P, 20BT-P, 20BT-4P) and meal pattern (AL, MF). Experiment 3


20SO-4P
20BT-4P
20SO-P
20BT-P
MF
AL
Food Efficiency (g gain / 100 Kcal)
Days of Refeeding
1-2
3-4
5-6
7-8
9-10