cases is of a different order of magnitude. Considerations such as these may be the basis of the finding in the present work that, although a reversible antimetabolite such as 2-methyl-MVA showed very high activity in the *L. acidophilus* assay, it was unable to inhibit ergosterol synthesis in yeast. On the other hand, an irreversible analog such as 2-methyl-DMVA was a potent inhibitor of ergosterol synthesis even though it was 100 times less active than 2-methyl-MVA in the *L. acidophilus* assay. One of the aims in the design of the present series of analogs was to realize such irreversible antimetabolites. The experimental findings may be taken to indicate the importance of obtaining irreversible antagonists.

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Interrelationship of Sterol and Fatty Acid Biosynthesis in Rat Liver Slices as Related to Dietary Lipid

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In vitro studies on cholesterol and fatty acid biosynthesis were made in liver slices from rats maintained on four levels of dietary fat. With [1-^{14}C]acetate as precursor, cholesterol biosynthesis was found to be directly related to the fat content of the diet, whereas an inverse relationship was found in lipogenesis. In a more detailed study on the sterol biosynthetic pathway with [2-^{14}C]mevalonate, the incorporation of this precursor into isoprenols, squalene, and cholesterol was determined. Sterol biosynthesis from mevalonate was found to be related to the amount of dietary lipid. The incorporation of the precursors into sterol was found to be directly related to the hepatic sterol levels.

Within recent years the effect of dietary lipids on cholesterol metabolism has been of great interest (Portman and Stare, 1959). However, only a few reports have considered the relationship of dietary fat to endogenous cholesterol metabolism. Endogenous cholesterol was found to accumulate in the livers of male rats fed a fat-free diet, whereas the administration of appropriate amounts of fat led to normal levels (Alfin-Slater *et al.*, 1954). On the other hand, in both the rat (Klein, 1959) and the rabbit (Diller *et al.*, 1961) endogenous cholesterol levels in the liver and blood are directly related to the concentration of fat in the diet.

If the animals in these experiments were in a steady

TABLE I
SERUM AND HEPATIC STEROL CONCENTRATIONS AND FECAL STEROL EXCRETION[a]

Fat Content of Diet (%)	Serum Cholesterol[b]		Hepatic Cholesterol[c]		Fecal Sterol[d] Excreted
	Free	Total	Free	Total	
0	32 ± 2.2	72 ± 6.2	2.21 ± 0.12	2.50 ± 0.12	6.8
5	36 ± 2.1	91 ± 3.1	2.27 ± 0.17	2.53 ± 0.13	8.5
10	34 ± 1.5	90 ± 5.0	2.42 ± 0.09	2.97 ± 0.15	12.2
20	36 ± 1.8	111 ± 8.6	2.56 ± 0.06	4.56 ± 0.24	16.5

[a] Methods for the isolation and determination are described in Experimental Procedures. Data listed are mean values with standard error of mean. [b] mg/100 cc. [c] mg/g of wet liver. [d] mg/rat/day. Based on excretion from three rats from each group for three days.

state, the accumulation of endogenous cholesterol under these dietary conditions may be attributed to a greater rate of cholesterol biosynthesis than excretion. However, cholesterol synthesis in the liver is suppressed when hepatic cholesterol is elevated by cholesterol supplementation of diets (Gould *et al.*, 1953; Tomkins *et al.*, 1953). It was therefore of interest to determine the effect of endogenous cholesterol and dietary fat on sterol biosynthesis.

In vitro studies on cholesterol and fatty acid biosynthesis in liver slices from rats maintained on diets with various levels of dietary fat are reported. [1-^{14}C]-Acetate was used to establish the effect of these diets on cholesterol and fatty acid biosynthesis. A more detailed study on the sterol biosynthetic pathway was made with [2-^{14}C]mevalonate. In addition, the interrelationship of cholesterol biosynthesis and hepatic cholesterol concentration was determined.

EXPERIMENTAL PROCEDURES

Animals and Diets.—Male Holtzman rats weighing about 100 g were housed in screen-bottomed cages and were allowed diet and water *ad libitum*. Animals were assigned at random into four groups of six rats each. The basic diet consisted of 20% vitamin-test casein, 10% cellulose, 35% starch, 31% sucrose, essential inorganic salts, and vitamins. Diets containing either 5%, 10%, or 20% lipid were prepared by substituting safflower oil for the starch of the basic diet on a weight-for-weight basis. Animals were maintained on the dietary regimens at least four weeks before experimental use.

Incubation of Liver Slices with Precursors.—The animals were sacrificed by decapitation and the livers were immediately removed and placed on chopped ice. Liver slices *ca.* 0.5 mm thick were prepared with a Stadie-Rigg microtome; 500-mg portions of each liver were placed into each of two flasks. The flasks contained 5 ml of Krebs-Ringer phosphate buffer and either 10 μmoles [1-^{14}C]acetate or *dl*-[2-^{14}C]mevalonate. The specific activity of the [1-^{14}C]acetate was 40,600 dpm/μmole and that of the [2-^{14}C]mevalonate was 41,819 dpm/μmole. The flasks were gassed with 95% O_2 and 5% CO_2 and were shaken on a Dubnoff metabolic bath at 37.5° for 2 hours. At the end of the incubation period, the reaction mixture in each flask was poured into 10 ml of alcohol-acetone (1:1), and the flask was rinsed with a fresh 10-ml portion of alcohol-acetone. The combined mixture was then heated to a rolling boil to extract lipids. Upon cooling to room temperature, denatured protein was removed by centrifugation.

Isolation of Labeled Products.—Aliquots of the alcohol-acetone extracts were saponified with KOH for 1 hour at 40°. The 3β-hydroxy steroids from the acetate and the mevalonate incubations were isolated as the digitonides according to the method of Sperry and Webb (1950). To determine the incorporation of [1-^{14}C]-acetate into fatty acids, nonsaponifiable lipids were removed by extraction with petroleum ether. The aqueous phase was then acidified with sulfuric acid and was reextracted with petroleum ether to remove the fatty acids. The petroleum ether extracts were subsequently back-washed with a solution of cold acetate to reduce the counts from [1-^{14}C]acetate (Coniglio and Bell, 1957). [2-^{14}C]Mevalonate extracts were saponified and subsequently extracted with petroleum ether to remove the nonsaponifiable components, which included squalene and sterol. These latter were separated by alumina chromatography (Loud and Bucher, 1958). Prenols and prenoic acids were isolated from the aqueous phase according to the method of Goodman and Popjak (1960).

Determination of Radioactivity.—With the exception of the sterol digitonides, the solvents of each lipid fraction were removed *in vacuo*. The sterol digitonides were dissolved in a small volume of acetic acid. All fractions were soluble in Diatol and were counted with a Packard Tri-Carb liquid scintillation counter. A sufficient number of counts was taken to reduce the statistical error of counting to less than 5%. [^{14}C]-Toluene was used as an internal standard to correct for quenching effects.

Sterol Determination.—A portion of unincubated liver was homogenized with a Potter-Elvehjem homogenizer. Cholesterol determinations by the method of Sperry and Webb (1950) were made upon suitable aliquots of an alcohol-acetone (1:1) extract of the liver homogenate and the serum. Sterols were extracted from the feces with alcohol in a Soxhlett extractor. Fecal 3β-hydroxysteroids were determined by the method of Vahouny *et al.* (1960). Cholesterol was used as a standard in all determinations.

Statistics.—Variance of the mean (V) was calculated by the formula $\frac{\Sigma d^2}{n(n-1)}$; standard error of the mean was $\sqrt{V}$. The "t" values were determined by the formula $\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{V_1 - V_2}}$.

RESULTS

The effect of dietary lipid on tissue sterol levels and fecal sterol excretion is shown in Table I. The cholesterol content of the liver and serum was clearly dependent upon the dietary lipid concentrations. There was no evident relationship between serum-free cholesterol and dietary lipid. However, a moderate increase ($P = <0.05$) in hepatic-free cholesterol was found when the fat-free diet was supplemented with 20% fat. Inasmuch as the total cholesterol values include the free and esterified fraction, an elevated esterified fraction accounted for the increased total cholesterol levels

of these tissues. Fecal sterol excretion was also related to the dietary lipid levels. The alteration in total cholesterol levels and fecal sterol excretion was not proportional to the increase in dietary lipid.

The incorporation of [1-^{14}C]acetate into sterol and fatty acids in rat liver slices was modified by the lipid content of the diet (Table II). The synthesis of 3β-hydroxysteroids from [1-^{14}C]acetate was lowest in the liver slices from rats that were fed either a fat-free or 5% fat supplemented diet. Sterol biosynthesis was greatly enhanced (P = <0.05) in rats that were fed a 20% fat diet. An intermediate level of acetate incorporation into sterol was found with 10% fat supplemented diets. Hepatic fatty acid synthesis was remarkably sensitive to the lipid content of the diet. Upon the addition of only 5% fat to the fat-free diet, the incorporation of [1-^{14}C]acetate into fatty acids was reduced (P = <0.1). A minimal lipogenesis (P = <0.05) was found with 20% fat in the diet. These data clearly reveal that a reciprocity exists between fatty acid and sterol biosynthesis as a function of dietary lipid concentrations.

TABLE II
INCORPORATION OF [1-^{14}C]ACETATE INTO STEROL AND FATTY ACIDS BY RAT LIVER SLICES[a]

Fat Content of Diet (%)	μmoles of Acetate Incorporated	
	Sterol	Fatty Acids
0	0.1457 ± 0.0087	0.1025 ± 0.0322
5	0.1352 ± 0.0137	0.0327 ± 0.0087
10	0.1747 ± 0.0158	0.0356 ± 0.0080
20	0.3251 ± 0.0451	0.0169 ± 0.0007

[a] Conditions of incubation and isolation are described in Experimental Procedures. Data listed are mean values with standard error of each mean.

The distribution of radioactivity from [2-^{14}C]-mevalonate in the intermediates of the sterol biosynthetic pathway and sterol, as well as the prenoic acids, is presented in Table III. Although the amount of label in squalene was greater than in its precursors, i.e., the isoprenols, the incorporation of ^{14}C into squalene paralleled that found in the latter. Prenol and squalene biosynthesis was at a minimum in the liver slices from the 5% and 10% fat-supplemented groups. However, the incorporation of [2-^{14}C]mevalonate into isoprenols and squalene was increased (P = <0.05 and <0.2, respectively) with the addition of 20% fat to the diet. The labeling of the prenols and squalene in the liver slices from the fat-free groups was higher (P = <0.05 and <0.1, respectively) than in the 5% and 10% fat-fed rats.

The incorporation of radioactivity into the isoprenoic acids was somewhat different from that found for squalene and the isoprenols. The label in the prenoic acids decreased with the addition of lipid to the fat-free diet, with a minimum incorporation at 20% dietary lipid (P = <0.05). Thus, an inverse relationship existed between the lipid content of the diet and the radioactivity in the prenoic acids. In liver slices prepared from the same rats, a comparable effect on the incorporation of [1-^{14}C]acetate into fatty acids was observed.

TABLE III
INCORPORATION OF [2-^{14}C]MEVALONATE INTO PRENOLS, PRENOIC ACIDS, SQUALENE, AND STEROL BY RAT LIVER SLICES

Fat Content of Diet (%)	μmoles of Mevalonate Incorporated				
				Sterol	
	Isoprenols	Prenoic Acids	Squalene	Column	Digitonin
0	0.0289 ± 0.0015	0.0753 ± 0.0053	1.2163 ± 0.1670	0.6118 ± 0.0359	0.7617 ± 0.0461
5	0.0224 ± 0.0024	0.0519 ± 0.0067	0.7963 ± 0.1635	0.6166 ± 0.1043	0.7496 ± 0.0685
10	0.0246 ± 0.0011	0.0497 ± 0.0019	0.8648 ± 0.0583	0.7565 ± 0.0555	0.8580 ± 0.0495
20	0.0353 ± 0.0019	0.0409 ± 0.0023	0.9577 ± 0.0201	1.3335 ± 0.0406	1.2251 ± 0.0600

[a] Conditions for incubation and isolation procedures are presented in Experimental Procedures. Data listed are mean values with the standard error of each mean.

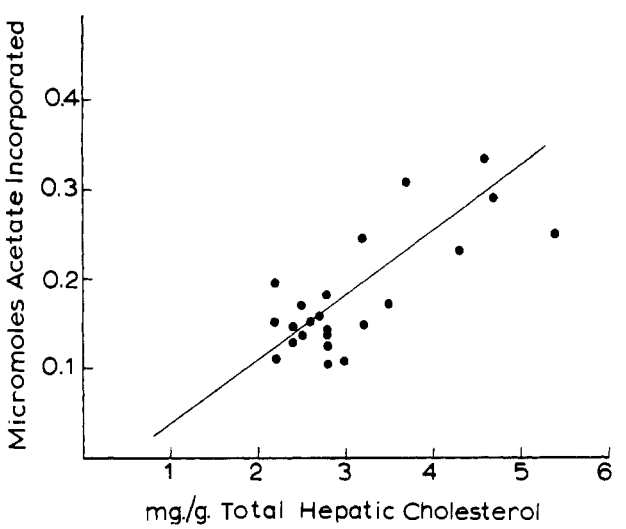

FIG. 1.—The relationship between μmoles of [1-^{14}C]-acetate incorporated into sterol and total hepatic cholesterol concentration. Correlation coefficient = 0.718, "t" value = 4.84 with P = <0.001.

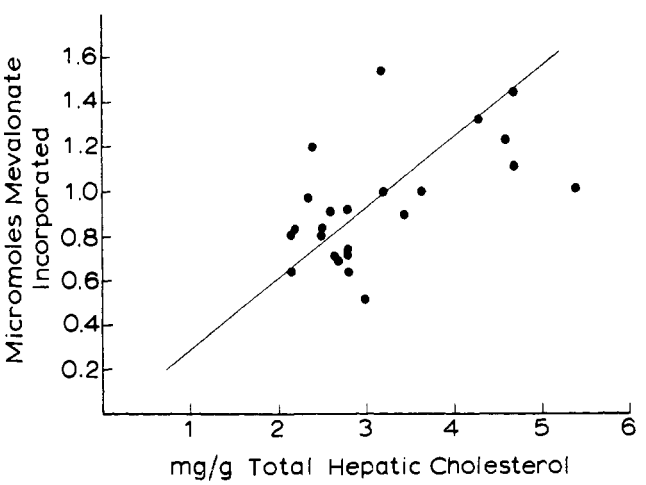

FIG. 2.—The relationship between μmoles of [2-^{14}C]-mevalonate incorporated into sterol and total hepatic cholesterol concentration. Correlation coefficient = 0.618, "t" value = 3.69 with P = <0.001.

Alumina chromatography and digitonin precipitation were used to isolate the sterols in the [2-^{14}C]mevalonate studies. The data obtained by these methods are in excellent agreement. Sterol biosynthesis was at a minimum in liver slices from the fat-free and 5% fat supplemented groups. When the diet was supplemented with 10% and 20% fat, an increased incorporation of label into sterol was found at each increment of dietary lipid with a maximum at 20% fat ($P = <0.05$). Whereas the radioactivity from [2-^{14}C]mevalonate recovered in the prenols and squalene of the fat-free groups was higher than in the 5% and 10% fat-supplemented groups, such a relationship was not evident in the sterols. However, the incorporation of ^{14}C in the prenol, squalene, and sterol fractions was related the lipid content of the diets.

A positive correlation exists between the incorporation of the labeled precursors into sterol and the total hepatic sterol concentration in all groups (Fig. 1 and Fig. 2, respectively). Such a correlation does not exist between the respective substrates and the free hepatic cholesterol.

Discussion

In the present report, the incorporation of acetate into sterol was increased as the lipid content of the diet was elevated, whereas the incorporation of label into fatty acids was depressed. *In vitro* experiments with rat liver slices and subcellular fractions have shown that citrate can effect a similar reciprocity in fatty acid and cholesterol biosynthesis (Foster and Bloom, 1963). The significance of these observations remains to be elucidated. However, it may be postulated that hepatic sterol biosynthesis may represent an alternate pathway for acetyl-CoA when lipogenesis is impaired under certain conditions.

The enhancement of sterol biosynthesis was in striking contrast to the depression of lipogenesis. This effect on sterol biosynthesis was confirmed by the use of [1-^{14}C]acetate and [2-^{14}C]mevalonate as precursors. The results of short-term studies undertaken to elucidate the quantitative relationships of dietary fat to sterol biosynthesis have been equivocal. Linazasoro *et al.* (1958) reported that the *in vitro* incorporation by liver slices of [1-^{14}C]acetate into sterol was directly related to the fat content of the diet. Conversely, Wilson and Siperstein (1959) did not observe such an effect. Although the present data were obtained in rats with prolonged fat feeding, short-term experiments in fat feeding have also resulted in a reduction of fatty acid bisosynthesis (Hill *et al.*, 1958).

Inasmuch as the purified diets were cholesterol free, the elevated cholesterol levels observed in this investigation were of endogenous origin. The elevated plasma and hepatic sterol concentration is indicative of a greater rate of cholesterol biosynthesis than of elimination under these particular experimental conditions. Indeed, the data corroborate the hypothesis of an increased cholesterol biosynthesis; however, they do not exclude the possibility of a lower rate of cholesterol catabolism.

It has been amply demonstrated that elevated hepatic cholesterol concentrations induced by dietary cholesterol supplements depress hepatic cholesterol biosynthesis (Tomkins *et al.*, 1953; Gould *et al.*, 1953; Frantz *et al.*, 1954). A depressed cholesterol biosynthesis has been observed with the accumulation of endogenous hepatic cholesterol as the result of an essential fatty acid deficiency (Mukherjee and Alfin-Slater, 1958). The data of this report demonstrate that the accumulation of endogenous cholesterol does not of itself suppress cholesterol biosynthesis. Rather, an increased cholesterol biosynthesis and hepatic cholesterol concentration is found with high dietary fat levels. *In vitro* experiments have shown that the inhibition of sterol biosynthesis by exogenous cholesterol operates via a negative feedback mechanism, which inhibits the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid (Siperstein, 1960). The nature of the observed stimulatory effect of high levels of dietary lipid on the sterol biosynthetic pathway is under investigation.

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