The effect of dietary fat supplements on cholesterol metabolism in ruminants

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Abstract The serum cholesterol of ruminant animals rises when supplemental fat is fed in a form that ensures the absorption of long-chain fatty acids. The effects of these fat supplements on cholesterol metabolism have been studied in sheep and goats. The proximal part of the small intestine was the major site of sterol synthesis in sheep. Supplementing the diet with fat significantly enhanced sterolgenesis in the small intestine both in vivo and in vitro, whereas in vitro sterolgenesis appeared to be suppressed in the liver. Increased intestinal sterolgenesis was seen with several varieties of fat, but was greatest when palm oil was fed. The reciprocal findings in the intestine and liver may reflect the increased requirement for cholesterol for the transport of triglyceride in chylomicrons and the secondary inhibiting effect of this cholesterol on sterol synthesis in the liver. Dietary fat supplementation did not alter the excretion of neutral steroids in the feces of goats but did cause a marked reduction in the excretion of acidic steroids which may have been due to the decreased formation of sterols in the liver. In two lactating goats in which an injection of [14C]cholesterol was followed by daily intraruminal administration of labeled cholesterol, fat supplementation lowered the specific radioactivity of cholesterol in alimentary particles and in milk, being consistent with an increase in intestinally synthesized cholesterol. The hypercholesterolemia that develops in fat-fed ruminants appears to be primarily due to an increased intestinal biosynthesis of cholesterol but may also be partly due to a decreased fecal excretion of bile acids.

Supplementary key words fat feeding · bile acid excretion

A number of studies have shown that the feeding of fat supplements to ruminants raises the cholesterol concentration in the serum but not in the tissues or milk (1–3). In nonruminants, including primates and man, hypercholesterolemia may be induced by dietary manipulations such as feeding excessive cholesterol or fats with a high saturated fatty acid content. The serum cholesterol concentration does not rise uniformly and hyper-responsiveness has been variously attributed to excessive absorption of cholesterol, e.g., in some species of monkey (4), to diminished re-excretion of cholesterol or bile acids (5, 6), or to the failure

of absorbed cholesterol to exert appropriate feedback inhibition on cholesterol synthesis (7, 8). As ruminants normally derive all of their cholesterol from endogenous biosynthesis, it is reasonable to suppose that the fat-induced hypercholesterolemia in ruminants is due to either an increased de novo synthesis of cholesterol and/or a decreased fecal excretion of cholesterol or bile acids. These studies were conducted to examine these two possibilities.

METHODS

Animals and diets

Sheep were used for all in vitro and in vivo studies of sterol synthesis from radiolabeled precursors. Lactating goats were used for all studies on fecal steroid excretion and for all studies where radiolabeled cholesterol was used.

The basal or control diets contained chopped alfalfa and crushed oats. Lipid supplements prepared as previously described (9, 10) were generally fed in amounts sufficient to supply approximately 10% fat by weight of the total diet. Specific details of animals and diets are described together with the respective results.

Measurement of sterol synthesis in vivo

Lipid supplements were fed for periods of 4–8 weeks before radioactive precursors were injected

into the sheep for measurements of sterol synthesis in vivo, or for up to 4 months before tissues were obtained for similar in vitro studies (see Results and Tables). All animals were weighed prior to injection of the radiolabeled sterol precursors.

Sheep were injected with 200–500 μCi of either sodium [1-^{14}C]acetate (sp act 40–60 mCi/mmol) and/or DL[2-^{3}H]mevalonic acid lactone (sp act 100–500 mCi/mmol). Both of these radioactive sterol precursors were supplied by the Radiochemical Centre, Amersham, U.K. and were dissolved in approximately 10 ml of 0.9% NaCl; this solution was injected intravenously. This injection of radioactivity was followed by two further injections of saline (5 ml each) to rinse the syringe and needle.

For studies on the rate of incorporation of label into plasma sterols, blood was withdrawn by jugular puncture at defined intervals after injection, and was immediately mixed with anticoagulant (EDTA); the plasma was frozen.

For studies on the incorporation of radioactivity into tissue sterols, the sheep were exsanguinated about 30 min after injection, blood was collected and the tissues and organs were quickly removed. Gastrointestinal tissues were emptied of their contents and washed with saline. Other samples, e.g., liver, were rinsed with saline and blotted to remove blood. The small intestine was divided into three segments of approximately equal length (proximal, middle, and distal portions). The weights of the blood, liver, and intestinal segments were recorded and samples (1–2 g) of various tissues were accurately weighed and immediately saponified by heating at 80°C for 2 hr with a mixture of 5 N NaOH (5 ml) and 95% ethanol (5 ml). For some studies tissue samples were collected in a mixture of chloroform–methanol 2:1 (v/v).

Separation of serum lipoproteins

Serum lipoproteins were isolated by ultracentrifugation of fresh serum using the general procedure of Havel, Eder, and Bragdon (11). The serum was stabilized by adding 0.02% EDTA and 0.01% merthiolate and the centrifugation was conducted using polycarbonate centrifuge tubes and a 60 Ti rotor in a Beckman L265 B centrifuge. In general, the serum was separated into four classes of lipoproteins plus the infranatant. Serum (25 ml per tube) was initially centrifuged for 9×10^7 g-min (33,000 rpm for 20 hr) at 5°C. The upper phase (fraction I), comprising chylomicrons and very low density lipoprotein, was removed and the density of the lower phase was adjusted to 1.04 g/ml using a solution of NaCl/KBr and recentrifuged to obtain fraction II (d 1.006–1.040 g/ml).

The density of the remaining lower phase was adjusted to 1.06 g/ml and recentrifuged to obtain fraction III (d 1.040–1.060 g/ml or low density lipoprotein). The density of the remaining lower phase was then adjusted to 1.21 g/ml and centrifuged for 3.9×10^8 g-min (33,000 rpm for 88 hr) to obtain fraction IV (high density lipoprotein). The lower remaining phase was termed fraction V. The volume of each fraction was measured and an aliquot was extracted with chloroform–methanol.

Extraction of sterols from serum and tissues

The lipid extracts were separated by thin-layer chromatography (TLC) using Adsorbosil-1 (Applied Science Labs., State College, PA) and a solvent system of petroleum ether–diethyl ether–acetic acid 85:15:1 (v/v/v). The separated free sterol and esterified sterol fractions were eluted. The sterol esters were saponified and the radioactivity of all sterol extracts was determined in a liquid scintillation spectrometer (Packard Instrument Co.) as described by Downes et al. (12).

Lipid extracts were saponified, the nonsaponifiable tissue lipids were extracted with petroleum ether, and the radioactivity was measured either in total sterols or in the free and esterified cholesterol fractions after separation by TLC.

Characterization of radiolabeled nonsaponifiable lipids

The labeled nonsaponifiable lipids were characterized by using three different TLC systems and also by digitonin precipitation. The three TLC systems were a) silica gel (Adsorbosil-1) with a solvent system of cyclohexane–ethyl acetate 60:40 (v/v); b) silica gel (Adsorbosil-1) mixed with 5% AgNO$_3$ and using a solvent system of chloroform–acetone 98:2 (v/v); and c) aluminum oxide G with a solvent system of benzene–diethyl ether 70:30 (v/v). The bands corresponding to cholesterol were removed and counted, and the radioactivity in the remaining constituents present on the TLC plates was also measured. The digitonin precipitation procedure was similar to that described by Crawford (13). The radioactivity of the digitonin-precipitated sterols was measured by liquid scintillation counting.

The digitonin-precipitated sterols and the cholesterol bands isolated by the three TLC systems contained 80–90% of the radioactivity present in the nonsaponifiable fraction derived from the small intestine, liver, and serum of all animals injected with [1-^{14}C]acetate. These results strongly suggest that the major radioactive sterol generated from acetate in vivo was cholesterol.

Measurement of sterol synthesis in vitro

The tissue sampling and incubation procedures were the same as described by Hood, Thompson, and Allen (14). The proximal segment of the small intestine and the liver were the only two tissues that were studied. The small intestine was washed with saline as previously described and the mucosa was removed by scraping with a glass slide. Samples (approximately 150 mg) of intestinal mucosa or of liver slices were incubated with 10 μmol of sodium [1-^{14}C]acetate (sp act 0.1 mCi/mmol) in 25-ml Erlenmyer flasks containing 0.3 units of insulin, 10 μmol of glucose, and Ca^{2+}-free Krebs-Ringer bicarbonate buffer at pH 7.4 in a volume of 3 ml. Each flask was flushed with oxygen, sealed with a rubber serum cap, and incubated with agitation at 37°C for 2 hr. The reaction was terminated by injecting 0.25 ml of 0.5 M H_2SO_4 through the serum cap into the incubation medium.

The contents of the incubation flasks were rinsed into a glass vial with 5 ml of methanol and homogenized. After homogenization, 5 ml of chloroform and 5 ml of water were added and the mixture was allowed to stand for 24 hr to allow separation of the phases. The lower chloroform layer containing the lipid was removed, washed, and evaporated to dryness. Lipids from the intestinal mucosa incubations were interesterified with methanol (15) and the cholesterol was separated from fatty acid methyl esters by TLC on fiber glass sheets impregnated with silica gel (Gelman Instrument Co.) developed in hexane–diethyl ether–acetic acid 90:8:2 (v/v/v). The identified cholesterol spot was cut out, placed in a scintillation counting vial containing 10 ml of Brydet scintillation solution (16), and assayed for radioactivity by liquid scintillation spectrometry. The background sterol radioactivity attributable to the in vivo incorporation of labeled acetate was subtracted where necessary and the results were expressed as nmol of [1-^{14}C]acetate converted to sterol per 2 hr per mg of soluble protein.

For one in vitro study the liver slices were prepared and incubated as described previously; the lipid extracts were not interesterified with methanol but were separated by TLC into the major component lipids (triglycerides, phospholipids, cholesteryl esters, and free cholesterol). The radioactivity in the lipids as well as in the liberated CO_2 (14) was then measured.

Sterol balance studies

Fecal steroid excretion was measured in seven lactating goats during periods when either supplemental fat (supplemented diet) or chopped alfalfa and oats (control diet) were fed.

Five goats (no. 1–5) were each tested twice during the fat-supplemented period. The other two goats (no. 6 and 7) were tested once during fat supplementation. The supplements were generally formaldehyde-treated (protected) oilseed or oil–casein supplements, but goats no. 1, 2, and 3 were also fed supplements of unprotected oilseeds. The supplemented periods were flanked by control periods. Goats were allowed to consume their respective rations for at least 2 weeks before fecal collection was commenced. Feces were collected for 7 days and the pooled feces were analyzed for neutral steroids only (goats no. 1, 2, 3) or for both neutral and acidic steroids (goats no. 4, 5, 6, and 7). Serum cholesterol concentrations were also measured during the control and supplemented periods.

Analysis of fecal steroids

Neutral steroids were quantified chemically by the method of Miettinen, Ahrens, and Grundy (17). Aliquots of fecal homogenates representing 7-day pooled samples were saponified and the nonsaponifiable fraction was separated into three classes: cholesterol, coprostanol, and coprostanone together with the corresponding plant sterols. Recoveries, estimated from the inclusion of radioactive standards, averaged between 80–90% in different goats.

The acidic steroids in the saponifiable fraction were methylated and isolated by thin-layer chromatography as described by Grundy, Ahrens, and Miettinen (18). Losses through the procedure were less than 15%. The separated neutral and acidic steroids were converted to trimethylsilyl derivatives and quantified by gas–liquid chromatography using 5α-cholestane as an internal standard. The liquid phase was 1% DC 560 (Applied Science Laboratory); the column and detector temperatures were 240°C and 260°C, respectively. Standards of cholesterol and cholic acid were analyzed at frequent intervals and gave a 1:1 response against 5α-cholestane over a wide mass range. The major acidic steroid, which accounted for about 80% of acidic steroids, was identified as deoxycholic acid on the basis of retention time and mass spectra.

Serum cholesterol specific activity–time curves

Radiolabeled cholesterol was infused into four goats: [4-^{14}C]cholesterol was dissolved in ethanol and 49–195 μCi was infused with an excess volume of NaCl solution. This was followed in two goats by the daily intraluminal administration of 1.17 μCi of [4-^{14}C]-cholesterol which had been incorporated into small amounts of "protected" lipid, to prevent ruminal hydrogenation. The specific radioactivity of serum cholesterol was then measured at intervals as shown in the results.
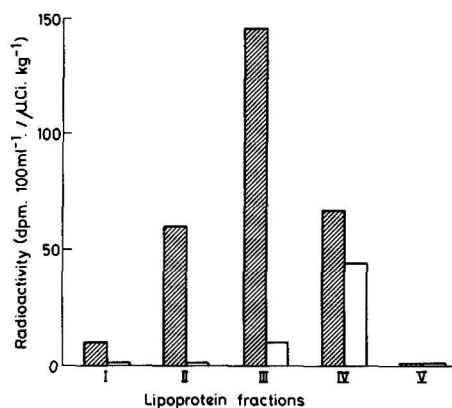
Fig. 1. Incorporation of [1-^{14}C]acetate into the sterols and sterol esters of serum lipoproteins. Two 5-yr-old sheep were each injected with 500 μCi of [1-^{14}C]acetate. The animals were slaughtered 30 min after injection, the blood was collected, the sera were pooled, and serum lipoproteins were isolated by ultracentrifugation as described in the text. The free sterols (◨) and sterol esters (□) were separated by TLC and the radioactivity was determined as described in the text. The diet was alfalfa and crushed oat grain (1/1, 1500 g/animal/day). dpm · 1 ml^{-1}/μCi · kg^{-1} represents dpm in 1 dl of serum normalized to 1 μCi of administered radioactivity per kg body weight.

RESULTS

In vivo sterol synthesis

Incorporation of labeled precursors into plasma sterols. The incorporation of [1-^{14}C]acetate and [2-^{3}H]mevalonic acid into the plasma sterols occurred rapidly during the first 30 min after injection; most of the radioactive sterol was present in the unesterified form.

Fig. 1 shows the distribution of newly synthesized esterified and unesterified cholesterol among the serum lipoproteins of sheep that had been injected with [1-^{14}C]acetate 30 min prior to exsanguination. Most of the label in the low density lipoproteins (II:d 1.006–1.040 g/ml, and III: d 1.040–1.060 g/ml) and in the very low density lipoproteins (I or d < 1.006 g/ml) was in the form of unesterified sterol whereas the high density lipoprotein fraction (IV: d 1.060–1.21 g/ml) contained approximately equal proportions of radiolabel in the esterified and unesterified forms. Fraction V, (d > 1.21) contained very little radioactive sterol.

Effects of fat supplementation on the incorporation of labeled precursors into tissue and plasma sterols. **Fig. 2** shows the incorporation of [1-^{14}C]acetate into the tissue sterols of sheep fed either control diets or diets supplemented with tallow, palm oil, or safflower oil. Animals were slaughtered 30 min after injecting the labeled acetate.

The wall of the small intestine showed the greatest incorporation of radioacetate into sterols and, except for the safflower oil-fed sheep, this incorporation was greater in the proximal and middle than in the

distal part of the small intestine. The feeding of lipid supplements enhanced the incorporation of label into sterols of the intestine, especially when palm oil was fed. The feeding of fat did not alter the amount of radiolabel in the hepatic, cecal, or adipose tissue sterols. (In these and other studies we have found that sterols in adipose tissue, rumen wall, cecum, kidney, heart, and skeletal muscle were only minimally labeled after radioacetate injections.)

The data in **Fig. 3** are derived from the same study as described in Fig. 2 and show the average incorporation of radioacetate into sterols throughout the small intestine as well as the incorporation of radioactivity into serum sterols and the serum cholesterol concentrations for all groups of sheep. The feeding of fats increased the amount of label present in serum sterols 30 min after injection of radioacetate and the pattern of this increased labeling resembled the increased amounts of cholesterol in the serum. Sheep fed the protected palm oil showed the greatest degree of sterol labeling in the intestinal wall and in the serum and these sheep also showed the highest serum cholesterol concentration.

Thin-layer chromatographic separation of the free and esterified sterol in the serum of all sheep from
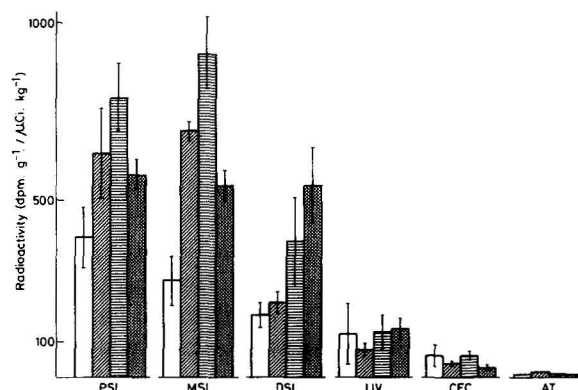

Fig. 2. Effect of dietary fat supplements on the in vivo incorporation of [1-^{14}C]acetate into tissue sterols. Values are means and standard errors for tissue sterol radioactivity for four 16-week-old sheep, each of which was injected with 300 μCi of sodium [1-^{14}C]-acetate. The animals were slaughtered 30 min after injection. Control diets contained chopped alfalfa (400 g) and crushed oats (400 g). Fat-supplemented diets contained chopped alfalfa (350 g), crushed oats (140 g), and formaldehyde-treated, spray-dried, oil–casein supplements (210 g). The oil–casein supplements were prepared as described previously (5) and the ratio of the oil:casein was 2:1 (w/w). Animals were fed their respective rations for 6 weeks prior to slaughter. □ Control, ◨ tallow, ▤ palm oil, ▥ safflower oil. PSI, MSI, and DSI are proximal, middle, and distal segments of the small intestine, respectively. Liv., liver; Cec., cecum wall; AT, subcutaneous adipose tissue. Statistical analysis: differences for radioactivity in small intestinal sterols between control vs. tallow or palm oil or safflower oil treatments respectively, were significant at the 5%, 1%, and 2% levels for proximal segments; 1%, 1%, and 1% for middle segments; and nonsignificant, 2%, and 1% for distal segments.

each treatment showed that 80–90% of the labeled sterol was present in the unesterified form and that the amount of label in this form was markedly increased by dietary fat supplementation. The effect of fat supplementation on the radioactivity of sterol esters was of a lower magnitude (**Table 1**).

Table 1 also shows the total cholesterol content of lipoprotein fractions from these sheep. The cholesterol content of combined very low density and low density lipoproteins was approximately the same as that of high density lipoprotein, and the effect of fat supplementation was to cause an approximately two-fold increase in the cholesterol content of both.

The data from Figs. 2 and 3 were used in conjunction with organ weights and plasma volumes to calculate the total organ and serum sterol radioactivities. When the data were expressed in this manner (**Table 2**) the effect of fat supplementation on sterol radioactivity was essentially the same as when the data were expressed per unit weight of tissue or per unit volume of serum. Table 2 shows clearly the importance of the small intestine as a source of newly labeled sterol.

Fat supplementation did not increase the sterol content of the liver or of the small intestine. Since, in these and other studies, cholesterol mass was measured colorimetrically using an Autoanalyzer, it is possible that sterols other than cholesterol may have been included.

Table 3 shows the incorporation of [³H]mevalonic acid into the tissue and serum sterols of sheep fed control diets or diets supplemented with sunflower oil supplements. In contrast to the results obtained with [¹⁴C]acetate, the liver contained considerably more ³H-labeled sterols than the intestinal wall and there was no effect of fat supplementation on liver or intestinal sterol labeling. Furthermore, fat supple-
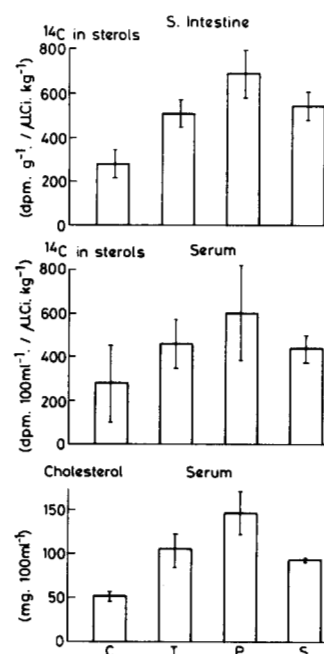

Fig. 3. Effect of dietary fat supplements on the in vivo incorporation of [1-¹⁴C]acetate into sterols of intestinal wall and serum. These data are derived from the same animals as in Fig. 2 and show the means and SE for four sheep. The values for radioactivity of sterols in the small intestine are average values for all intestinal segments. The serum collected at slaughter was also analyzed for cholesterol concentration. C, control; T, tallow; P, palm oil; S, safflower oil. Statistical analysis: differences between control vs. tallow or palm oil or safflower oil, respectively, were significant for intestinal sterol radioactivity at the 1%, 1%, and 1% levels, but not significant for serum sterol radioactivity.

mentation did not influence the ³H content of serum sterols.

In vitro sterol synthesis

The in vitro incorporation of [1-¹⁴C]acetate into sterols of liver and small intestinal mucosa was stud-

TABLE 1. Effect of dietary fat supplements on the content and radioactivity of sterols in serum and serum lipoproteins

	Control		Tallow		Palm Oil		Safflower Oil	
Whole serum sterol radioactivity (dpm/dl/μCi kg⁻¹)[a]								
Free sterol[b]	151 ± 39		310 ± 39		426 ± 97		366 ± 35	
Esterified sterol[b]	26 ± 13		33 ± 6		43 ± 6		54 ± 11	
Lipoprotein sterol concentration (mg/dl serum)[d]								
VLDL + LDL	17.8	23.4	50.2	39.9	62.7	48.8	44.0	49.0
HDL	21.4	19.6	51.0	40.6	57.9	56.6	41.5	45.8
Lipoprotein-deficient serum[c]	5.0	3.7	4.6	3.7	2.7	3.8	3.6	3.5

The sheep, diets, and injection procedures were previously described (see Fig. 2). Lipoproteins were separated as described in the text.

[a] dpm in 1 dl of serum normalized to a dose of 1 μCi of administered radioactivity per kg body weight.

[b] Results are means and standard errors for four sheep in each group.

[c] Lipoprotein deficient serum is infranate of serum spun at d = 1.21 g/ml.

[d] Each value is the mean for two animals.

Statistical analysis: differences for free sterol radioactivity between control versus tallow or palm oil or safflower oil treatments respectively were each significant at the 1% level (unpaired *t* test).

TABLE 2. Effect of dietary fat supplements on total organ and serum sterol radioactivity

| | Control | Fat Supplement | | |
		Tallow	Palm Oil	Safflower Oil
Live weight (kg)	29.0 ± 0.9	30.5 ± 0.6	30.6 ± 0.6	29.1 ± 0.5
Liver (dpm × 10^{-3})	454 ± 302	333 ± 91	577 ± 241	617 ± 117
Small intestine (dpm × 10^{-3})	1387 ± 422	3344 ± 244	4568 ± 522	2939 ± 275
Serum (dpm × 10^{-3})	44 ± 19	60 ± 9	79 ± 17	59 ± 4

Refer to Figure 2 for details of sheep, diets, and injection procedures. Serum was assumed to be 55% of the volume of blood.

Statistical analysis: differences for small intestine sterol radioactivity between control vs. tallow or palm oil or safflower oil treatments, respectively, were significant at the 5%, 1%, and 1% levels. Hepatic radioactivities were not significantly different between treatments.

ied using tissues from sheep fed either control or lipid-supplemented diets and the results are presented in **Fig. 4** and **Table 4.**

The small intestine showed a greater in vitro incorporation of radioacetate into sterols than did the liver (Fig. 4). Furthermore, the in vitro incorporation into intestinal sterols tended to be enhanced by fat feeding, with the palm oil supplement inducing the largest response. Labeling of hepatic sterols on the other hand, was markedly reduced by fat feeding (Fig. 4). The effect of fat supplementation on intestinal sterol radioactivity was not as great as was observed in vivo (Figs. 2 and 3).

The inhibitory effect of fat supplementation on in vivo hepatic sterolgenesis was also observed in another study with tissues from adult sheep (Table 4). There was a lesser reduction in the in vitro incorporation of [^{14}C]acetate to CO_2 which was not significantly affected by fat supplementation. The possible dilution of label in an expanded acetate pool should be considered in interpreting the data from the liver studies.

TABLE 3. Effect of dietary sunflower oil supplements on the in vivo incorporation of [^{3}H]mevalonate into tissue and serum sterols

	Control	Sunflower Oil
Tissue sterol radioactivity (dpm · g^{-1}/μCi · kg^{-1})		
Liver	3068 ± 1494	2308 ± 1644
Small intestine, middle segment	46 ± 13	43 ± 18
Perirenal adipose tissue	36 ± 13	7 ± 7
Serum sterol radioactivity (dpm · 100 ml^{-1}/μCi · kg^{-1})	6646 ± 1244	7112 ± 888
Serum cholesterol concentration (mg/dl)	73 ± 7	94 ± 7

Each value is the mean and standard error for five sheep injected with 220 μCi of DL [2-^{3}H]mevalonic acid 15 min before slaughter. The control diet contained chopped alfalfa (400 g) and crushed oats (800 g); the sunflower oil-supplemented diet contained chopped alfalfa (400 g), crushed oats (400 g), and formaldehyde-treated sunflower oil/casein (2/1; 200 g). The respective diets were fed for 6 weeks prior to slaughter.

Fecal steroid excretion

Fig. 5 shows the effect of feeding fats on the plasma cholesterol concentration and the fecal excretion of neutral and acidic steroids. These results were obtained using lactating goats and either formaldehyde-
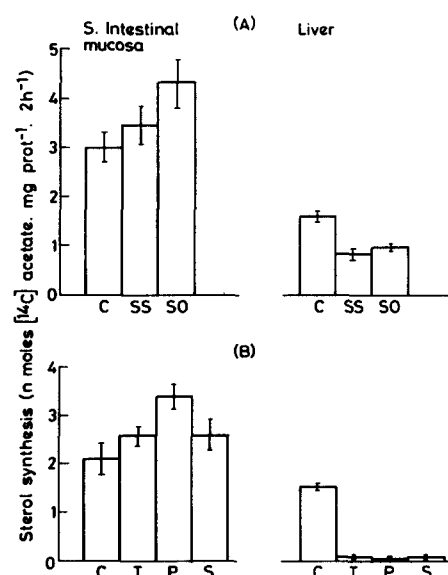

Fig. 4. Effect of dietary fat supplements on the in vitro incorporation of [^{14}C]acetate into tissue sterols at 2 hr. A. Means and SE for four sheep. Each sheep was about 5 years of age and weighed approximately 65 kg. Control animals (C) were fed chopped alfalfa (375 g) and crushed oats (750 g) and supplemented animals were fed either sunflower seed supplements (SS) or sunflower oil/casein (SO) supplements prepared as previously described (10). The SS supplement (36% oil, 375 g/day) and the SO supplement (66% oil, 250 g/day) were each fed with chopped alfalfa (250 g) and crushed oats (500 g). Respective diets were fed for 12 weeks prior to slaughter. Tissues were incubated and radioactive sterols measured as described in the text. B. Means and standard errors for four sheep. Refer to Fig. 2 for details of sheep and diets. Tissues were incubated and radioactive sterols measured as described in the text. C, control; T, tallow; P, palm oil; S, safflower oil. Statistical Analysis. A. Differences for sterol synthesis between control vs. sunflower seed or sunflower oil supplements were, respectively, nonsignificant and significant at the 1% level in intestine and significant at the 1% levels in liver. B. Differences for sterol synthesis between control vs. treatments were all nonsignificant in the intestine and significant at the 2% levels in liver.

TABLE 4. Effect of sunflower seed supplement on the in vitro incorporation of [1-^{14}C]acetate into hepatic lipids

	Control	Sunflower Seed Supplement
	nmol [1-^{14}C]acetate incorporated/2 hr/mg soluble protein[a]	
Total lipid	14.22 ± 1.62[b]	5.79 ± 1.40
Triglyceride	2.02 ± 0.27	0.80 ± 0.29
Phospholipid	4.77 ± 0.77	2.94 ± 0.72
Sterol esters	0.61 ± 0.23	0.46 ± 0.30
Free sterol	6.82 ± 1.40	1.59 ± 0.81
Carbon dioxide	3.44 ± 0.22	2.92 ± 0.26

[a] Incubation procedures are described in the text. Statistical analysis: differences between control vs. sunflower seed supplement for triglyceride, phospholipid, sterol esters, free sterol, and CO_2 were significant at the 1%, 2%, 2%, nonsignificant, 1%, and 5% levels, respectively.
[b] Each value is a mean and standard error for four animals. The animals (5 yr old Merino wethers) were fed their respective diets for 4 months prior to slaughter. At slaughter the animals weighed 50–60 kg. The control diet was chopped alfalfa (400 g) and crushed oats (1100 g); the fat supplemented diet was chopped alfalfa (700 g), crushed oats (250 g) and sunflower seed supplement (36% fat) (500 g) (5).

treated or untreated safflower oil or formaldehyde-treated sunflower oil as the fat supplements.

The feeding of either treated or untreated fat produced a clear increase in the plasma cholesterol concentration in all seven goats used for these studies.

The excretion of neutral steroids was enhanced when fat supplements were fed to goats 1, 2, and 6, but there were no corresponding clear-cut effects on the other five goats (Fig. 5).

Acidic steroid excretion was measured in four goats and, in contrast to the results for neutral steroids, the feeding of fat caused a consistent and substantial decrease in the excretion of acidic steroids in all four goats (Fig. 5) that was sufficient to cause a net decrease in fecal excretion of total steroid in three.

Metabolism of administered radioactive cholesterol

Figs. 6 and 7 show the effects of dietary fat supplementation on the plasma cholesterol concentration and the specific radioactivities of cholesterol in milk and plasma of goats injected with [4-^{14}C]cholesterol. Goats 4 and 5 (Fig. 6 shows data for no. 5) were given an intravenous priming dose of [^{14}C]cholesterol followed by daily intraruminal administration of [^{14}C]-cholesterol; goats 6 and 7 (Fig. 7 shows data for no. 7) were given only a single intravenous injection of labeled cholesterol at the commencement of the experiment.

Feeding the fat supplements caused a marked increase in the plasma cholesterol concentration of all four goats and removal of fat supplements caused a decline in plasma cholesterol concentration. With
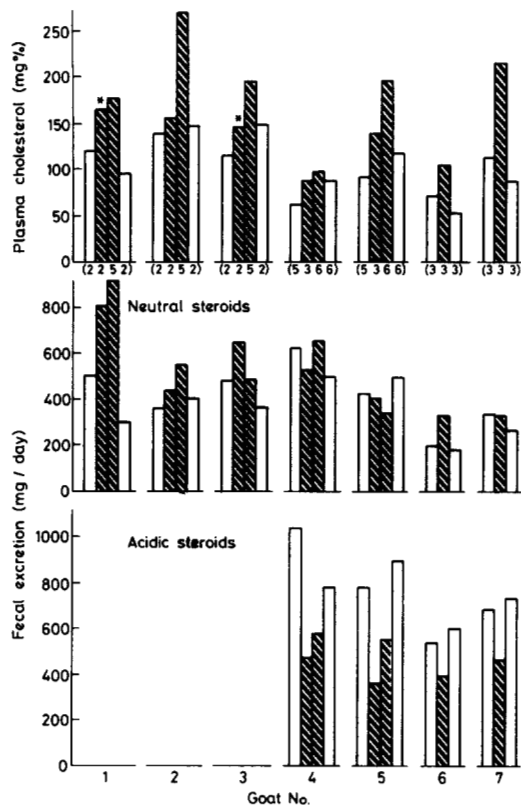

Fig. 5. The effect of dietary fat supplements on serum cholesterol concentration and fecal steroid excretion. Lactating goats were fed control diets of chopped alfalfa and crushed oats (1:1) (1–2 kg/day) or similar diets supplemented with either safflower oil (goats 1–5) or sunflower oil (goats 6 and 7). All supplements were treated with formaldehyde except for some of the safflower supplements fed to goats 1, 2, and 3—the asterisk denotes the nonformaldehyde-treated safflower supplement. The serum cholesterol concentrations shown in this figure were measured on the first day of each 7-day fecal collection period. There were two fecal collection periods for goats 1–5 during the fat supplementation periods. The values in parentheses show the number of weeks that each diet had been fed prior to the start of fecal collections. Acidic steroid excretion was not measured in goats 1–3. □ control rations, ▨ fat supplemented rations.

goats 4 and 5 (Fig. 6) the feeding of fat did not markedly affect the decline in the specific radioactivity of the plasma cholesterol but did appear to cause a decline in the milk cholesterol specific radioactivity; removal of fat from the diet of these goats was followed by an increase in the specific activity of the milk cholesterol. There appeared to be a time lag of approximately 10–15 days between the introduction or removal of fat and the effect on milk cholesterol specific radioactivity.

With goats 6 and 7 (Fig. 7) the feeding of fat caused a transient increase in the specific activity of cholesterol in both milk and plasma but no other clearly defined effect could be attributed to fat supplementation in these two goats. There was a minimal time lag between fat supplementation and the observed tran-
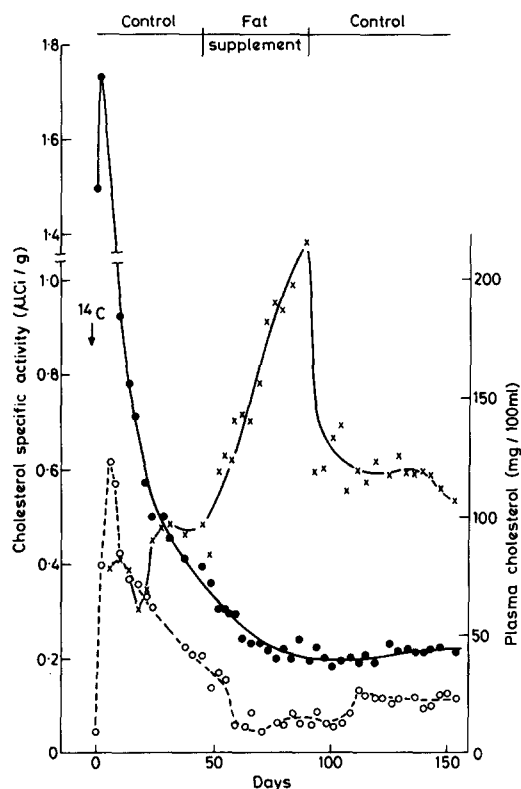
Fig. 6. Effect of dietary fat supplement on the metabolism of plasma and milk cholesterol. Goat no. 5 (see Fig. 5) was injected with a priming dose of [4-^{14}C]cholesterol (sp act 44 mCi/mmol) (42 μCi) at day 0 and this was followed by the daily intraruminal administration of 1.17 μCi of [4-^{14}C]cholesterol. Plasma and milk samples were extracted and the concentration and radioactivity of the cholesterol fraction were determined as described in the text. The basal diet consisted of chopped alfalfa and oats (1:1, w:w, 2000 g/day) and the supplemented diet consisted of 1500 g of chopped alfalfa and oats (1:1, w:w) and 330 g of formaldehyde-treated safflower oil:casein (2:1, w:w). Supplement prepared by procedures previously described (9). ●, Plasma cholesterol specific radioactivity; ○, milk cholesterol specific radioactivity; ×, plasma cholesterol concentration.

sient increase in milk and plasma cholesterol specific activities in these goats. The different effects of fat supplementation on cholesterol specific activities in goats 4 and 5 vis a vis goats 6 and 7 may be related to the different means of administering [^{14}C]cholesterol. Goats 4 and 5 were given an initial intravenous injection of [^{14}C]cholesterol whereas goats 6 and 7 were given only the single injection. The possible implications will be considered later.

The rapid decrease in the plasma cholesterol concentration when fat supplementation was stopped was not associated with a clear change in the slope of the plasma specific radioactivity curves (Figs. 6 and 7).

Table 5 shows the comparison of cholesterol specific radioactivities in the serum, in the combined chylomicrons and very low density lipoproteins, and in the milk of goats 4 and 5 during the fat-supplemented
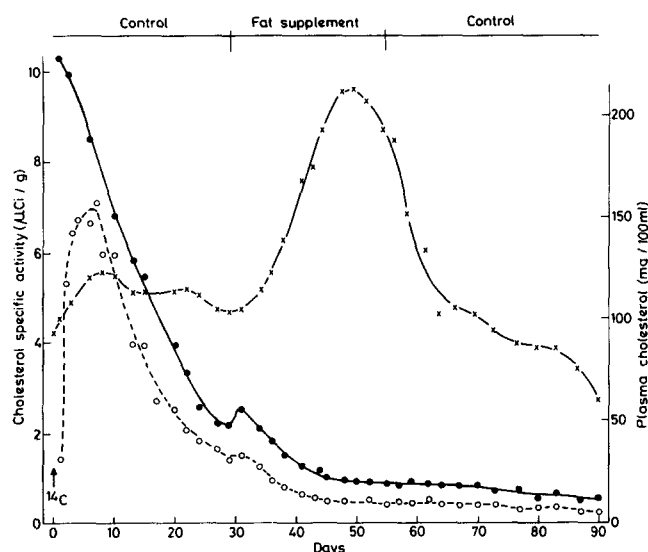

Fig. 7. Effect of dietary fat supplement on the metabolism of plasma and milk cholesterol. Goat no. 7 (see Fig. 5) was given a single intravenous infusion of [4-^{14}C]cholesterol at day 0. Plasma and milk samples were extracted and the concentration and radioactivity of the cholesterol fraction were determined as described in the text. The basal diet consisted of chopped alfalfa and oats (1:1, w:w, 1500 g/day) and the supplemented diet consisted of 1000 g of chopped alfalfa and oats (1:1, w:w) and 400 g of sunflower seed supplement prepared by procedures previously described (9). Legends as for Fig. 6.

period and during the second control period. These data show clearly that fat supplementation led to lower cholesterol specific radioactivities in the triglyceride-rich lipoproteins than in whole serum and this was the case for both free and esterified cholesterol; during the control period the specific radioactivities of cholesterol were similar. The specific radioactivity of cholesterol in milk was always less than in the lipoproteins or whole serum.

TABLE 5. Specific radioactivities of cholesterol in serum lipoprotein fraction I (chylomicrons and very low density lipoprotein) and in milk of goats[a]

	Serum		Chylomicrons and VLDL		Milk
	FC[b]	EC[c]	FC	EC	FC[d]
	μCi/g cholesterol				
Goat supplemented	0.16	0.15	0.13	0.13	0.04
#4 Second control period	0.12	0.14	0.14	0.14	0.07
Goat supplemented	0.19	0.23	0.15	0.15	0.05
#5 Second control period	0.23	0.21	0.26	0.22	0.13

[a] Serum and milk samples were taken at day 96 (supplemented) and day 142 (second control period) (see Fig. 7).
[b] FC, free cholesterol.
[c] EC, esterified cholesterol.
[d] Approximately 98% of the labeled sterol in milk was present in the unesterified form.

DISCUSSION

The purpose of this study was to establish the basis for the dietary fat-induced hypercholesterolemia in ruminants. This was approached in three ways: first, by the measurement of the incorporation of radiolabeled precursors into sterols with in vivo and in vitro techniques; second, by estimating the sterol balance from the excretion rates of neutral and acidic steroids; and third, by studying the metabolism of injected radiolabeled cholesterol.

The in vivo incorporation of radioacetate into sterols after injecting the label was clearly and consistently greater in the small intestines of lipid-supplemented than of control sheep (Figs. 2 and 3, Table 2). This was true for all the varieties of fed fat but the effect was perhaps greatest with palm oil supplementation. The labeling of serum sterols at 30 min after injecting the radioacetate was also greatest in the lipid-supplemented sheep and the pattern of radiolabeling of serum sterols resembled the pattern of intestinal sterol labeling (Fig. 3). In addition, the magnitude of the fat-induced increase in sterol labeling in the intestine and serum resembled the increase in the fat-induced serum cholesterol values (Fig. 3).

Since the small intestine, and especially its more proximal part, was the only organ to show a fat-induced enhancement of sterolgenesis, (the liver, kidney, heart, skeletal muscle, and other sections of the gastrointestinal tract did not show any change), it seems highly probable that increased cholesterolgenesis in the small intestine is a major factor in the development of the hypercholesterolemia. It is likely that this is related to the increased demand for cholesterol in the biosynthesis of chylomicrons or very low density lipoproteins. Additional data showed that the cholesterol mass in the intestinal wall was not changed by fat feeding. Not only was the small intestine the only organ to show a fat-induced increase in the radiolabeling of sterols but it was also the organ that showed the greatest absolute incorporation of labeled acetate into sterols both on the basis of unit weight and total organ content (Figs. 3 and 4, Table 2).

Entirely different results were obtained when [³H]-mevalonate was injected into sheep. The tissue sterol radiolabeling was almost exclusively confined to the liver as it is in rats (19) and there was no effect of fat feeding on either the tissue or serum ³H sterol labeling (Table 3). It is clear that in sheep the biosynthesis of sterols from mevalonate is not under the same biochemical control as is the sterol synthesis from acetate.

In vitro incubation of tissue preparations with [¹⁴C]-acetate confirmed the predominance of the small intestine in the sheep's total potential for cholesterol synthesis, both in normally fed and lipid-supplemented animals (Fig. 4). These in vitro studies also revealed greater radiolabeling of intestinal sterols using tissue from fat supplemented sheep. The in vitro studies also demonstrated apparent suppression with lipid supplementation of sterol synthesis in the liver, (bearing in mind possible dilution of radioacetate by an expanded acetate pool due to fat feeding), which stood in marked contrast to the findings in the small intestine (Fig. 4, Table 4). Since in the control sheep the liver was the only other organ tested to show substantial in vivo sterol synthesis, the reduced labeling of hepatic sterols in vitro strongly supports the view that the fat-induced hypercholesterolemia reflects increased intestinal cholesterolgenesis. The suppression of hepatic cholesterolgenesis may represent the inhibitory effect of cholesterol-rich intestinal particles which, at least in the rat, appear to be important in the regulation of cholesterol production in the liver (20, 21). The failure of the in vivo studies to show similar reduction in hepatic sterol synthesis may have been due to the rapid isotopic equilibration of radiocholesterol since newly synthesized labeled cholesterol would rapidly reach the liver from the intestine.

The gut has increasingly become recognized as a major site of cholesterolgenesis in animals such as the rat (22), monkey (23), and pig (24), as well as in man (25). In fact, the incorporation of radioacetate into sterols of rat small intestine suggests that cholesterolgenesis may be more active there than in the liver (26). The relative importance of the two organs will depend on dietary factors since cholesterolgenesis is more readily suppressible in the liver (23).

In contrast to the present findings in sheep, cholesterol synthesis in other nonruminant species studied is higher in the ileum than in the upper parts of the intestine (23, 25, 27). Since the absorption of bile acids also occurs mainly in the ileum, it has been postulated that the concentration of bile acids may regulate the rate of sterol synthesis (27). Equally, however, cholesterol synthesis may be regulated by the rate of absorption of cholesterol, which, being least in the ileum, would minimally inhibit synthesis in this region (28). Direct measurement of HMG CoA reductase activity in intestinal crypt cells of rats has confirmed the interrelated influence of bile acid flux and the cellular concentration of cholesterol (29).

The sterol balance data in goats tend to complement the findings with radioacetate in sheep. All seven goats showed a dietary fat-induced hypercholesterolemia and four of these goats also showed a fat-induced increase in fecal neutral steroid excretion (Fig. 5). On the other hand, the feeding of fat caused

a consistent reduction in the fecal excretion of acidic steroids in all four goats in which this was measured. In three of these four goats the total sterol excretion during fat supplementation was also reduced. These observations are consistent with an initial dietary fat-induced increase in sterol synthesis in the gut followed by compensating mechanisms, presumably in the liver, that counteracted the continuing heightened cholesterolgenesis in the small intestine. The marked reduction in the in vitro incorporation of radioacetate into hepatic sterols in fat-supplemented sheep and the consistent and substantial fall in bile acid excretion in the fat-supplemented goats were consistent with suppression of sterol synthesis in the liver. The reduced production of bile acids suggests that this is linked in ruminants to cholesterol metabolism in the liver as in other species (5). It was certainly clear that the expanded cholesterol pool in plasma did not stimulate the re-excretion of cholesterol as bile acid.

In the two goats (no. 4 and 5) receiving daily intraruminal doses of radiocholesterol (Fig. 6) the amount of excreted radioactivity equalled the amount being fed after 70 days, i.e., during the latter half of the supplemented period and the whole of the second control period. This finding established that each 7-day pool represented a 7-day period of sterol metabolism, and that neutral sterol losses were minimal.

The interpretation of sterol balance studies depends on the presence of steady-state conditions for cholesterol metabolism. Only in the steady state will the cholesterol balance equal cholesterol synthesis (in the absence of dietary cholesterol). Although the serum cholesterol had not clearly reached steady levels in the fat-supplemented goats 4, 5, 6, and 7 (see Figs. 6 and 7), it is reasonable to conclude that the unchanged or reduced sterol balance during fat supplementation probably reflected unchanged or reduced synthesis and/or excretion. It is consistent with little overall change in total cholesterol turnover, which in the presence of increased intestinal cholesterolgenesis points to an equivalent reduction in synthesis in other tissues, such as liver.

This metabolic "balance" was also indicated by the results of studies where radiolabeled cholesterol was administered to lactating goats. The feeding of fat did not markedly alter the decline in the plasma cholesterol specific radioactivity decay curves despite very marked alterations in the serum cholesterol concentrations (Figs. 6 and 7). Notwithstanding, there were some effects of fat supplementation on the distribution of the injected [14C]cholesterol that appeared to be related to the method of administering the labeled cholesterol. With goats 4 and 5 (Fig. 6), who received an initial injection of labeled cholesterol followed by

constant daily intraruminal infusions of labeled cholesterol, there was a fat-induced fall in the specific radioactivity of milk cholesterol; this appeared to be related to a reduction in the specific radioactivity of cholesterol in chylomicrons and very low density lipoproteins (Table 5). This suggests that increased intestinal sterolgenesis during fat supplementation lowered the specific radioactivity of the intestinally-derived chylomicron and very low density lipoprotein cholesterol, which in turn reduced the specific activity of cholesterol in milk. The data in these goats showed that approximately 30–60% of milk cholesterol was derived from circulating alimentary particles. The absence of similar changes in goats 6 and 7, who received only a single [14C]cholesterol infusion (Fig. 7), may be explained on the basis that increased intestinal cholesterolgenesis would affect the specific radioactivity of chylomicron cholesterol much more in goats 4 and 5, who absorbed radiocholesterol from the gut regularly each day.

The acute rise in the serum cholesterol specific radioactivity in goats 6 and 7 (Fig. 7) might reflect influx into plasma of tissue cholesterol with a higher specific radioactivity. This might not have been observed in goats 4 and 5 because of the continuing influx of freshly labeled cholesterol from the gut.

In summary it appears that the feeding of fat to ruminants caused an increase in intestinal sterolgenesis which in turn may have inhibited hepatic sterolgenesis and thereby the hepatic synthesis of bile acids. The net effect of these reactions was an increase in circulating blood cholesterol but little overall change in cholesterol turnover. The reason for the increase in intestinal cholesterolgenesis is not clear, though it seems highly likely that it was stimulated by the need to transport the large load of fat. Increased reabsorption of endogenous cholesterol, facilitated by the simultaneous absorption of fat, might also have contributed to the hypercholesterolemia.▮▮

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