Sterol synthesis and CO_2 production from mevalonate in calves

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Abstract Nonruminating male Holstein calves were fed a reconstituted milk containing 11.7% nonfat-dried-milk solids and 3.5% beef tallow. Calves were slaughtered at 17 weeks of age. Samples of perirenal adipose tissue, liver, muscle, small intestine, kidney cortex, and kidney medulla were assayed in vitro for sterol synthesis and production of $^{14}CO_2$ from [2-^{14}C]mevalonate. Of the tissues tested, adipose tissue and kidney medulla produced nonsaponified lipids at the greatest rates; kidney cortex and liver at half the rate of adipose tissue; muscle and jejunum at one-eighth the rate of adipose tissue; and ileum at a negligible rate. The amount of ^{14}C in squalene, lanosterol, and cholesterol of the nonsaponified lipids of each tissue was determined by thin-layer chromatography. Proportions of ^{14}C in cholesterol to ^{14}C in total nonsaponified lipids ranged from 30% to 59%; squalene, from 5% to 27%; and lanosterol, from 11% to 59% of the total nonsaponified lipids present. The rate of CO_2 production by the "*trans*-methylglutaconate shunt of mevalonate metabolism" was determined. Kidney cortex displayed the greatest shunt activity, producing 15–80-fold more CO_2 than any other tissue tested. Ileum, jejunum, skeletal muscle, and kidney medulla had similar shunt activities; liver sections had less shunt activity, and no shunt activity was detected in adipose tissue. These data reveal a shunt for mevalonate utilization that does not lead to sterols and also show that the kidney is important in the sterol and nonsterol metabolism of mevalonate.

Supplementary key words cholesterol synthesis · *trans*-methylglutaconate shunt pathway

Mevalonate is an established intermediate in cholesterol and isoprenoid biosynthesis. This and other features of mevalonate metabolism recently have been reviewed by Beytia and Porter (1). One novel aspect of mevalonate metabolism is a pathway described by Popjak (2, 3) and Edmond and Popjak (4) termed the "*trans*-methylglutaconate shunt of mevalonate metabolism." Edmond and Popjak (4) showed that radioactive label from [2-^{14}C]mevalonate injected into rats was incorporated into *n*-fatty acids (4). According to their proposal, this was accomplished by conversion of mevalonate to acetyl CoA and acetoacetyl CoA by a nonsterol or shunt mechanism; these molecules then may enter other pathways of intermediary metabolism. This shunt activity also has been assayed independent of measuring fatty acid synthesis. Tissue incubated with [^{14}C]mevalonate will produce $^{14}CO_2$ relative to the shunt activity in tissue. Righetti et al. (5) showed that the shunt pathway accounted for a significant percentage of the mevalonate metabolized in kidney, ileum, spleen, lung, and testes, but was of minor importance or undetectable in liver, brain, skin, and adipose tissue of rats. In the rat, the nonsterol, or shunt, pathway is responsible for 26% of the mevalonate metabolized (6). Kidneys are the most important tissue site for metabolism of circulating mevalonate (6, 7). Observations of Fogelman, Edmond, and Popjak (8) on in vivo conversion of mevalonate to CO_2 in rats and man further supported the existence of a metabolic shunt capable of converting intermediates of sterol biosynthesis to nonsterols.

The purposes of the experiment described here were to determine *a*) if a shunt pathway for mevalonate metabolism exists in a young bovine animal (calf), *b*) the in vitro capacity of this shunt in several tissues of the calf, and *c*) the synthesis rate and the composition of sterols synthesized from mevalonate.

Previous work has shown that the calf is an excellent model animal for study of human atherosclerosis and regulation of blood cholesterol concentration (9–11).

MATERIALS AND METHODS

Materials obtained commercially included: mevalonic acid lactone from Sigma Chemical Co., St. Louis, MO; silica gel G thin-layer chromatography plates, 250 μm, from Analtech, Newark, DE; rhodamine 6G from Applied Science Laboratories, State College, PA; and *RS*-[2-^{14}C]mevalonate, DBED salt, 22.8 mCi/mmol from Amersham/Searle, Arlington Heights, IL. The [2-^{14}C]mevalonate was radiochemically pure when

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chromatographed on a Waters Corp. liquid chromatograph, using a Bondapak C_{18} column eluted with water–methanol 90:10 (v/v). Radioactivity in samples was determined with a Packard model 3002 liquid scintillation counter.

Animals and diet

Intact male Holstein calves, 4 months of age, were fed a reconstituted milk containing 11.7% dried milk solids, 3.5% beef tallow, and a vitamin–antibiotic premix (12). Calves were fed daily an amount of the diet equivalent to 10% of body weight for 16 weeks after birth. Water and trace-mineralized salt were available ad libitum. The average weight of calves at 16 weeks of age was 88 kg.

Tissue isolation and incubation conditions

Within 30 min after death and exsanguination, samples of muscle biceps femoral, liver, small intestine, kidney cortex, kidney medulla, and perirenal adipose tissue were removed and placed in physiological saline at 39°C. Triplicate sections (100–200 mg) of tissues other than the small intestine were sliced with a razor blade. Triplicate intestinal samples of villi plus crypt cells were prepared from ileum (3 m from caudad end) and jejunum (3 m from orad end) by the method of Dietschy and Siperstein (13). The weighed tissue samples were transferred to a flask containing 3 ml of oxygenated Krebs and Henseleit Ringer–bicarbonate buffer (Ca^{2+}-free, pH 7.4) (14), 25 μmol of mevalonate aerated with 95% O_2/5% CO_2, and 2 μCi of *RS*-[2-^{14}C]mevalonate. The flasks were sealed with a septum and incubated at 30°C for 2 hr in a metabolic shaker (100 oscillations min^{-1}). To collect $^{14}CO_2$, 0.1 ml of 25% KOH was injected into a hanging well containing filter paper. Reactions were stopped by adding 0.7 ml of 1 N H_2SO_4 to the incubation medium. These assay conditions were established to be optimal for adipose tissue, liver, and the two sections of small intestine and were assumed to be so for muscle and kidney.

Determination of $^{14}CO_2$

The KOH-impregnated filter paper was transferred from the hanging well to a scintillation vial. The well was washed with two 0.5-ml portions of water. Washes and the filter paper were added to 15 ml of Bray's (15) scintillation fluid for radioactivity determination.

Analytical methods

Tissue samples were removed from the flasks and washed three times with physiological saline. Samples were transferred to test tubes containing 15 ml of chloroform–methanol 2:1 (v/v). After being shaken overnight, the tissue and chloroform–methanol extracts were separated. Extracts were brought to 20 ml with the same solvent and then washed by the method of Folch, Lees, and Sloane Stanley (16). Washed extracts were dried at 60°C under a stream of nitrogen. The resulting residue was saponified in 6 ml of 3% methanolic-KOH (w/v) by refluxing for 2 hr at 85°C. Four milliliters of water were added; then the nonsaponified lipids were extracted with three 5-ml portions of hexanes. More than 90% of the radioactive lipids in tissues were isolated by these extraction procedures. Hexane extracts were combined into a scintillation vial. An aliquot was removed for thin-layer chromatography; the remaining extracts were dried under moving air. The resulting residue was dissolved in a scintillation mixture containing 0.3% PPO and 0.02% POPOP in toluene.

Thin-layer chromatography

Nonsaponified lipid dissolved in hexanes was dried under a stream of nitrogen. The residue was dissolved in chloroform and applied to 18 × 22 cm silica gel G plates. Plates were developed in the solvent system described by Raskin and Siperstein (17). Lipids were visualized by a light spraying of 0.05% rhodamine 6G in water. Spots corresponding to squalene, lanosterol, and cholesterol standards were scraped from the plate and combined with 1 ml of ether in a scintillation vial; 15 ml of Bray's (15) scintillation fluid were added, and the samples were assayed for radioactivity. Nonsaponified lipid not corresponding to standards was handled in the same fashion.

Calculations

It has been shown that only the *R*-isomer of mevalonate is metabolized (8). Accordingly, all calculations were made by assuming that half of the added substrate was utilizable by the tissue. The amount of CO_2 produced by the sterol pathway was calculated by multiplying the nanomoles of *R*-mevalonate converted to cholesterol by 0.2 (5). The CO_2 produced by the shunt mechanism was calculated by subtracting the amount of sterol-derived CO_2 from the total observed CO_2.

Data were analyzed by split-plot analysis; P values < 0.05 were considered significant (18).

RESULTS

Conversion of *R*-[2-^{14}C]mevalonate to $^{14}CO_2$ by sterol and shunt pathways

RS-[2-^{14}C]Mevalonate was incubated in vitro with calf tissues. The conversion rates of *R*-[2-^{14}C]meva-

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TABLE 1. Conversion of R-mevalonate to CO_2 by sterol and shunt pathways

Tissue	Sterol[a] CO_2	Total[b] CO_2	Shunt[c] CO_2	Sterol CO_2	Shunt CO_2
	nmol/(g tissue × 2 hr)			%[d]	
Adipose tissue	19.0 ± 1.6[e]	16.2	0.0	100	0
Liver	9.8 ± 0.2	11.4	1.6 ± 0.4	93	7
Kidney medulla	13.0 ± 3.2	19.0	6.0 ± 0.6	68	32
Jejunum	4.6 ± 1.6	13.2	8.6 ± 2.4	35	65
Muscle	2.6 ± 1.2	9.0	6.4 ± 1.8	29	71
Kidney cortex	9.7 ± 1.4	137.4	127.6 ± 12.0	7	93
Ileum	0.2 ± 0.2	4.6	4.4 ± 1.6	4	96

[a] Sterol CO_2 refers to rate of CO_2 production by the sterol pathway. These data were calculated from nmol of mevalonate converted to cholesterol times 0.2.
[b] Total CO_2 refers to rate of R-mevalonate conversion to CO_2 during incubation.
[c] Shunt CO_2 refers to rate of CO_2 produced by the shunt pathway. These data were obtained by subtracting the rate of sterol CO_2 production from total CO_2 production.
[d] Percentage of sterol CO_2 and shunt CO_2 of the total of the two was calculated.
[e] All rates are expressed as mean ± SEM.

lonate to $^{14}CO_2$ by the sterol and shunt pathways are shown in **Table 1.** The CO_2 produced by the sterol pathway represents the demethylation of lanosterol during its conversion to cholesterol. This activity was greatest in the adipose tissue of the calves, although kidney medulla, kidney cortex, and liver produced substantial amounts of CO_2 by this pathway. Jejunum, muscle, and ileum produced lesser amounts of CO_2 via the sterol pathway than did liver.

The CO_2 produced by the shunt pathway represents the total CO_2 produced from mevalonate minus the CO_2 produced from mevalonate by the sterol pathway. The data show that kidney cortex had the greatest shunt activity, producing 15- to 80-fold more CO_2 from mevalonate than did any other tissue ($P < 0.01$) except adipose tissue, which had no shunt activity. The jejunum had twofold greater shunt activity than did the ileum ($P < 0.05$), which had a rate slightly less ($P > 0.05$) than that of kidney medulla or skeletal muscle. Liver sections produced less CO_2 from mevalonate by the shunt mechanism than did any other tissue ($P < 0.05$) except adipose tissue.

The last two columns of Table 1 summarize the relative capacities of the sterol and shunt pathways to produce CO_2 from mevalonate. Adipose tissue, which demonstrated the greatest capacity to convert mevalonate to sterol, had no apparent shunt mechanism. The reverse situation existed in kidney cortex in which the percentage shunt activity was greater than 90%; thus, the percentage of sterol pathway activity was low. Liver, kidney medulla, jejunum, and skeletal muscle exhibited decreasing (from 86% to 29%) percentages of sterol pathway activity and, thus, increasing percentages of shunt activity. The ileum produced little CO_2 by the sterol pathway in contrast to that by the shunt pathway (4% vs. 96%).

Conversion to R-mevalonate to nonsaponified lipid by the sterol pathway

All tissues studied were capable of converting R-mevalonate to nonsaponified lipid. These data are presented in **Table 2.** Adipose tissue demonstrated a rate greater than those of any other tissues ($P < 0.05$), except kidney medulla for which the difference was not statistically significant ($P > 0.05$). Within the kidney, the medulla seemed to synthesize lipid via the sterol pathway more readily than did the cortex, but these rates were not statistically different ($P > 0.05$). The rate in liver was similar to that in kidney cortex. Muscle and jejunum sections synthesized nonsaponified lipid at one-third the rate of liver. Jejunum synthesized nonsaponified lipid at a rate 27-fold greater than did the ileum ($P < 0.05$). The ileum conducted little synthesis via the sterol pathway, functioning at 0.4% the rate of adipose tissue.

Composition of the nonsaponified lipid fraction

The nonsaponified lipid fraction was composed of sterols and intermediates in the synthesis of sterols. Thin-layer chromatography was used to separate the squalene, lanosterol, and cholesterol in this fraction. The amounts of radioactivity were determined in these specific compounds and are expressed as the percentage of total radioactivity in the nonsaponified lipid from each each tissue (**Table 3**). In all tissues studied, cholesterol was the primary component of the nonsaponified lipid prepared after a 2-hr in vitro incubation of different tissues with mevalonate. The percentage of radioactivity in the cholesterol of nonsaponified lipids was greatest in small intestinal sec-

TABLE 2. Production of nonsaponified lipid by sterol pathway from mevalonate

Tissue	Rate of Synthesis
	nmol/(g tissue × 2 hr)[a]
Adipose tissue	320.4 ± 22.2[b]
Kidney medulla	218.2 ± 39.8
Kidney cortex	139.2 ± 14.2
Liver	133.0 ± 2.2
Jejunum	38.6 ± 11.0
Muscle	35.4 ± 10.4
Ileum	1.4 ± 0.6

[a] Rate of synthesis is expressed as nmoles of mevalonate converted to nonsaponified lipids/(g tissue × 2 hr).
[b] Mean ± SEM.

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tions; that in cholesterol of nonsaponified lipids from other measured tissues was similar. These data indicate that all tissues studied contain an active mechanism for converting mevalonate to squalene and lanosterol and that further conversion of these two intermediates to cholesterol proceeds at a greater rate in the small intestine sections than in other tissues. In no tissue did the radioactivity in squalene, lanosterol, and cholesterol total 100% of the radioactivity in the nonsaponified lipid present. The remaining components were not identified.

DISCUSSION

A shunt pathway for mevalonate utilization, not leading to sterols, has been reported by several researchers (4–8). These studies have provided evidence for nonsterol as well as sterol products of mevalonate metabolism in the rat. Little work has been done, however, with the metabolic fate of mevalonate in tissues of other animals. Because mevalonate is an intermediate of cholesterol synthesis, hypercholesterolemia is associated with atherogenesis, and resistance of different species of animals to atherosclerosis varies considerably; we believe that mevalonate metabolism in animals other than the rat needs investigating. In this communication, we have reported on mevalonate utilization in calves, an animal that has been shown to be an excellent model animal for human atherosclerosis studies.

We estimated the in vitro shunt activity by measuring the production of $^{14}CO_2$ by tissues incubated with [2-^{14}C]mevalonate. This is an indirect assay, relying on the shunt converting [2-^{14}C]mevalonate to radioactive acetyl CoA, or other products, that can be converted to $^{14}CO_2$ by the tricarboxylic acid cycle. Shunt activities determined in this manner represent minimum activities (5) because considerable radioactive label might be trapped in metabolic intermediates and not converted to $^{14}CO_2$ during the test reaction. Furthermore, tricarboxylic acid cycle activities may differ among tissues, which would affect the measured shunt activities. Even acknowledging these problems in assaying shunt activity, we have confirmed the presence of a shunt for mevalonate utilization in the calf. Greatest shunt activity was in the kidney cortex. A high shunt activity in rat kidney cortex also has been observed by in vivo (6, 7) and in vitro (5) experiments. Our data show that, as in the rat (5–7, 19), the kidney of the calf is a major site of mevalonate metabolism not leading to sterols.

TABLE 3. Percentage of radioactivity in squalene, lanosterol, and cholesterol in the nonsaponified lipid fraction

Tissue	Squalene	Lanosterol	Cholesterol[b]
		%[a]	
Adipose tissue	25	22	30
Liver	21	11	37
Kidney medulla	27	21	30
Jejunum	5	23	59
Muscle	15	13	38
Kidney cortex	31	12	35
Ileum	12	25	51

[a] Includes C-27 sterols other than cholesterol.
[b] Reported as percentage of total radioactivity in lipids in nonsaponified lipid fraction.

TABLE 4. Percentage of mevalonate utilized by shunt mechanism

Tissue	%[a]
Adipose tissue	0.0
Liver	1.2
Kidney medulla	2.7
Jejunum	18.2
Muscle	15.3
Kidney cortex	47.8
Ileum	75.8

[a] Percentage of mevalonate utilized by a shunt mechanism of the total mevalonate converted to nonsaponified lipids plus CO_2. Assay was performed in vitro by incubating [2-^{14}C]mevalonate with calf tissues and percentage calculated by this equation:

$$\% = \frac{\text{nmol mevalonate converted to } CO_2 \text{ by shunt mechanism (Table 1)}}{\text{nmol mevalonate converted to } CO_2 \text{ by shunt mechanism plus nonsaponified lipids (Tables 1 and 2).}} \times 100$$

Data in **Table 4** are the percentages of mevalonate utilized by the shunt pathway in relation to that of total mevalonate converted to CO_2 plus nonsaponified lipids. Except for kidney cortex and ileum, tissues utilized low percentages of mevalonate by the shunt pathway. The values in Table 4 probably are minimum values because all ^{14}C-labeled products of the shunt may not have been converted to $^{14}CO_2$. This lack of information makes assessment of true shunt activities and the effect of the shunt on sterol metabolism difficult. It is possible that the level of shunt activity may be involved in the regulation of sterol synthesis because de novo mevalonate synthesis is rate-limiting in cholesterol synthesis (20). This possibility has not been examined.

Calf adipose tissue converted mevalonate to nonsaponified lipids at the greatest rates (Table 2). Adipose tissue of the young goat also has higher rates of sterolgenesis than do other tissues, including liver and small intestine (21). The anatomical site of adipose tissue must also be considered in discussing synthesis rates. Perirenal adipose tissue was used in our studies; we do not know whether adipose tissue from other anatomical sites has similar rates of sterol synthesis from mev-

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alonate. For comparison, rates of lipogenesis in subcutaneous adipose tissue of cattle (22, 23) and pigs (24) range from 2- to 10-fold greater than that in perirenal adipose tissue.

Calf kidney also produced nonsaponified lipids at a high rate. This activity was higher in kidney medulla than in kidney cortex. Other researchers have found cholesterogenesis in rat kidney cortex higher than that in rat kidney medulla (5).

These data have shown that calf tissues, except for adipose tissue, possess the *trans*-methylglutaconate shunt of mevalonate metabolism (4, 8) and that the kidney cortex exhibits the greatest rates of mevalonate flux through this pathway. The relationship of this pathway to control of cholesterol synthesis in the major cholesterogenic tissues of the calf fed typical and highly atherogenic diets needs investigation.

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