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Hepatic 3-Hydroxy-3-Methylglutaryl CoA Reductase Activity in Germfree Rats (39662)

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Previous investigations have shown that germfree rats have a reduced fecal excretion of bile acids and neutral steroids and higher levels of serum and liver cholesterol compared with conventional rats (1-4). In accordance with this finding the 7α-hydroxylation of cholesterol, which is the rate-limiting step in the conversion of cholesterol to bile acids, tends to be decreased in germfree rats (5). The rate-determining step in the biosynthesis of cholesterol is the formation of mevalonic acid from HMG CoA (3-hydroxy-3-methylgutaryl CoA) (6). Recently techniques have been described for determining the activity of the HMG CoA reductase (7). In order to further clarify the mechanisms behind the differences in cholesterol metabolism between germfree and conventional rats, it was considered of importance to investigate the cholesterol synthesis. The present paper describes the activity of the HMG CoA reductase in liver microsomes from germfree and conventional rats.

Materials. [Glutaryl-3-^{14}C]HMG CoA (specific radioactivity, 20 μCi/mg) and DL-[mevalonic-5-^{3}H(N)]mevalonic acid (dibenzylethylenediamine salt) (specific radioactivity, 25 μCi/mg) were obtained from New England Nuclear Corp., Boston, Mass. The radioactively labeled HMG CoA was diluted with unlabeled material, synthesized by P-L Biochemicals, Inc., Milwaukee, Wis., to yield a specific radioactivity of 1.45 μCi/mg. Unlabeled DL-mevalonic acid lactone, NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo.

Methods. Animals and preparation of microsomes. Three germfree and three conventional male rats of the AGUS[1] strain about 100 days of age and weighing about 250-300 g were used. The germfree animals were reared according to the technique of Gustafsson (8, 9) and fed a standard diet *ad libitum* (9). This diet is semisynthetic with 10% (w/w) arachis oil as source of fat and contains 0.004% (w/w) cholesterol. Conventional animals of the same strain were reared outside the germfree isolators on the same sterilized diet. The germfree animals were killed between 9 and 10 AM by a blow on the head immediately after they had been taken out of the germfree isolators; the conventional animals were killed in the same way at the same time. The livers were removed immediately and chilled on ice. Liver homogenates (10%, w/v) were prepared with a Potter-Elvehjem homogenizer equipped with a loosely fitting Teflon pestle. The homogenizing medium contained: sucrose, 0.3 *M*; nicotinamide, 0.075 *M*; EDTA, 0.002 *M*; and mercaptoethanol, 0.02 *M*. The homogenate was centrifuged at 20,000*g* for 15 min. The microsomal fraction was obtained by centrifuging the 20,000*g* supernatant fluid at 100,000*g* for 60 min. The microsomes were washed with the homogenizing medium and centrifuged again at 100,000*g* for 30 min. The microsomal fraction was then suspended in the homogenizing medium in a volume corresponding to that of the 20,000*g* supernatant fluid. The protein concentration was determined by the method of Lowry *et al.* (10).

Assay of HMG CoA reductase activity. The assay system used was similar to that described by Shefer *et al.* (7). The complete system contained in a volume of 0.85 ml: 0.2 ml of microsomal fraction; phosphate buffer, pH 7.2, 100 m*M*; NADP, 3 m*M*; glucose 6-phosphate, 10 m*M*; glucose 6-phosphate dehydrogenase, 5 enzyme units;

[1] This rat strain of Long Evans origin has been reared under germfree conditions at the Department since 1956. It has later been established at Laboratory Animal Center, Carshalton, England and labeled AGUS.

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[3-^{14}C]HMG CoA, 0.2 m*M*. The incubation was run for 15 min at 37°C. At the end of this period 0.01 μCi of tritium-labeled mevalonic acid was added as an internal standard together with 3 mg of unlabeled mevalonic acid lactone and the reaction was stopped by the addition of 0.1 ml of 5 *M* HCL. The incubation mixture was further shaken for 30 min at 37°C. After cooling to room temperature, 0.5 ml of absolute ethanol was added and the mixture was dried over anhydrous Na_2SO_4. The solution was extracted three times with 3 ml of ethyl ether. The ethyl ether layers were combined, dried over anhydrous Na_2SO_4 and evaporated. The residue, dissolved in acetone, was subjected to thin-layer chromatography (precoated silica-gel plates; 250 μm; Merck, Darmstadt, Germany) with benzene–acetone (1:1, v/v) as solvent. The chromatoplate was sprayed with 0.2% (w/v) solution of 2,7-dichlorofluorescein in methanol and the mevalonic acid lactone zone was located under uv-light. The mevalonic acid lactone band was scraped off into a counting vial. The radioactivity was determined in a Packard Liquid scintillation spectrometer, Model 3003, using Instagel as a scintillator liquid. Corrections were made for quenching by using suitable ^{3}H- and ^{14}C-labeled standards. Corrections were also made for losses during extraction and thin-layer chromatography procedures by the internal standard (tritium-labeled mevalonic acid lactone).

Results. The amount of formed [3-^{14}C]mevalonic acid lactone was expressed as picomoles per milligram of protein per minute. As seen in Table I the labeled mevalonic acid lactone formed in conventional rats amounted to 50.5 ± 2.9 pmole/mg of protein/min. In germfree animals the formation of mevalonic acid lactone was 21.7 ± 3.8 pmole/mg of protein/min which is about 40% of the reaction rate found in conventional rats.

Discussion. It is a well-known fact that germfree rats have higher levels of serum and liver cholesterol compared with conventional rats (3, 4, 11). Further, Kellogg and Wostmann (12) have shown that germfree rats fed with only moderate amounts of cholesterol accumulate 2–3 times as much cholesterol in the liver as conventional rats.

TABLE I. The Activity of HMG CoA Reductase in Liver Microsomes from Germfree and Conventional Rats.[a]

Rat	Mevalonic acid lactone formed (pmole/mg of protein/min)	
	Germfree	Conventional
1	25.8	52.5
2	20.9	51.8
3	18.3	47.2
Means ± SEM	21.7 ± 3.8	50.5 ± 2.9

[a] The values are the means of two determinations in each rat.

Several factors are of importance in the regulation of cholesterol metabolism in rats, i.e., (A) intestinal cholesterol absorption, (B) cholesterol synthesis, and (C) fecal excretion of cholesterol as neutral steroids and bile acids. Recently, Wostmann (13) reported that germfree rats have a 25% higher absorption of dietary cholesterol compared with conventional rats. The more efficient absorption of cholesterol in the germfree animals can probably be ascribed to the higher concentration of bile acids in the small intestine (14). The fecal loss of the bile acids in germfree rats is about half of that recorded in conventional rats (1, 2). Mainly due to the more efficient reabsorption of cholesterol, the fecal excretion of cholesterol and other neutral steroids is also reduced by about 50% in germfree rats compared with conventional.

Another factor of importance for the larger pool of cholesterol in germfree animals could be the endogenous formation of this compound. The present results demonstrate, however, that the synthesis of cholesterol is depressed in germfree rats compared with conventional rats. According to current concepts the formation of cholesterol in rat liver is homeostatically regulated by cholesterol as well as by bile acids reaching the liver (15, 16). As mentioned above germfree rats indeed have an increased reabsorption of cholesterol as well as of bile acids. Even though germfree rats have a lower rate of formation of cholesterol compared with conventional rats, they accumulate cholesterol probably due to a more efficient reabsorption from the intestine, a decreased loss via feces and a decreased degradation of cholesterol to bile acids.

Summary. The cholesterol synthesis was

studied in germfree and conventional rats by assaying the HMG CoA (3-hydroxy-3-methylglutaryl CoA) reductase activity in the liver microsomal fraction. Germfree rats had a decreased activity (21.7 ± 3.8 pmole/mg of protein/min) compared with conventional rats (50.5 ± 2.9). The results are discussed in relation to present knowledge of cholesterol metabolism in the two types of animals.

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