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Regulation of cholesterol synthesis in the liver and mammary gland of the lactating rat

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1. The activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase; EC 1.1.1.34) in the lactating mammary gland of rats killed between 10:00 and 14:30h was 2–3 times that in the livers of the same animals. In contrast, after injection of ^3H_2O *in vivo*, the rate of appearance of ^3H in the cholesterol of the gland was much lower than that in the liver. 2. In the mammary gland of virgin and non-lactating animals, the activity of HMG-CoA reductase was less than 10% of that of the lactating gland. 3. The activity of HMG-CoA reductase in the lactating mammary gland was significantly ($P < 0.005$) lower at midnight than at mid-day, and appeared to show an inverse relationship to the activity of the liver enzyme. However, there was no corresponding change in the incorporation of ^3H into the gland cholesterol. 4. Withdrawal of food for 6h had no effect on the activity of HMG-CoA reductase in the lactating mammary gland, but resulted in a significant decrease ($P < 0.005$) in that of the liver. Starvation of lactating rats for 24h produced a significant decrease ($P < 0.005$) in the activity of the enzyme in both organs. There was also a significant decline in the rate at which ^3H_2O was incorporated *in vivo* into the cholesterol of both organs (liver, $P < 0.05$; gland, $P < 0.005$). 5. Giving a high-fat palatable diet together with chow to lactating animals led to a decline in HMG-CoA reductase activity in the mammary gland, but not in liver. This decrease in the gland was not accompanied by a corresponding decline in the apparent rate of cholesterol synthesis.

The hepatic production of lipoproteins and biliary steroids imposes a large demand for cholesterol, which can be met either from dietary sources or by endogenous synthesis within the liver, and the regulation of this process has been intensively studied (see Gibbons *et al.*, 1982). The lactating mammary gland also requires considerable quantities of cholesterol, e.g. the rat secretes approx. 16mg per day in the milk (Clarenburg & Chaikoff, 1966). Unlike the liver, however, the regulation of cholesterol synthesis and uptake in this organ has received relatively little attention. Although the lactating gland is capable of converting acetate into cholesterol *in vitro* (Clarenburg & Chaikoff, 1966; Middleton *et al.*, 1981), the quantity of cholesterol synthesized by the gland compared with the liver is unknown. The activity of HMG-CoA reductase (EC 1.1.1.34) is an important determinant of the rate of cholesterol synthesis in the liver (Rodwell *et al.*, 1976), and a high activity of this enzyme is present in explants of rabbit mammary gland (Middleton *et al.*, 1981). However, it is not known whether mammary-gland HMG-CoA reductase responds to certain physiological changes in the same way as does the liver enzyme. The present investigation was undertaken to provide information about the relationship between the activity of mammary-gland HMG-CoA reductase and the rate of cholesterol synthesis in the gland *in vivo*. A further objective was to determine whether HMG-CoA reductase activity and the rate of cholesterol synthesis in the gland respond to physiological changes such as those that occur over the diurnal cycle, or during starvation, or on consumption of a high-fat diet. Finally, the extent to which these changes occur in the mammary gland has been compared with the

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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changes in the activity of hepatic HMG-CoA reductase and cholesterol synthesis under the same conditions.

Experimental

Animals

Rats of the Wistar strain (200–300g) with between 8 and 12 pups were used after a lactation period of 10–14 days. Virgin rats weighed 200–250g. Rats were fed on PRM diet (E. Dixon and Sons, Ware, Herts., U.K.) and water *ad libitum* except where described and were maintained on a constant lighting schedule (lights on 08:00–20:00h). Starved rats had their food removed at 08:30h and were killed after 6h (14:30h) or 24h. One group of lactating rats were fed from parturition with Crawford's Cheddar-cheese biscuits, which are high in fat (for details of composition see Rolls *et al.*, 1980), in addition to the normal chow. Non-lactating rats refers to rats that had had their pups removed at birth and were then killed 10–12 days later.

Materials

^3H_2O, (3*RS*)-[3-^{14}C]HMG-CoA and [4-^{14}C]cholesterol were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Measurement of the rates of cholesterol synthesis and lipogenesis in vivo

^3H_2O (5mCi in 0.5ml) was injected intraperitoneally into rats to measure the rates of lipogenesis and cholesterol synthesis (Jungas, 1968). Where applicable, rats were left with their litters until the injection of ^3H_2O. The rats were anaesthetized with Nembutal (50mg/kg body wt.; solution in 0.9% NaCl) about 55min after injection of ^3H_2O, and at 60min the inguinal mammary gland and liver were removed. Heparinized blood was collected for the measurement of the specific radioactivity of plasma water. The specific radioactivity of mammary-gland tissue water was similar to that of plasma. The tissues were hydrolysed under alkaline conditions (Stansbie *et al.*, 1976), with the addition of [4-^{14}C]cholesterol (1153 d.p.m.) as internal standard. The non-saponifiable lipids were extracted three times with light petroleum (b.p. 40–60°C; total vol. 20ml) and the organic extract was washed several times with water. After evaporation of the solvent, cholesterol (and accompanying C_{27} sterols) was isolated from the non-saponifiable residue by chromatography on thin-layer plates of silica gel H developed with chloroform/methanol (99:1, v/v). The plate was dried and the cholesterol-containing band was located by spraying with a solution of Rhodamine 6G in acetone (0.1%, w/v). This area of the plate was scraped into a scintillation vial, and the ^3H and ^{14}C contents of the sterol fraction were determined by scintillation counting.

The aqueous phase after extraction of the non-saponifiable lipid was acidified to pH1 with H_2SO_4 and the labelled fatty acid fraction was extracted with light petroleum (Stansbie *et al.*, 1976). After evaporation of the solvent, the ^3H radioactivity of the residue was determined by scintillation counting.

Measurement of HMG-CoA reductase activity

Portions of mammary gland and liver were frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. A sample of each frozen powdered tissue (approx. 1g) was suspended in 3.0ml of 50mM-potassium phosphate buffer, pH7.4 (containing 5mM-dithiothreitol and 1mM-EDTA) and homogenized with a Teflon/glass homogenizer. Samples of the homogenate were removed and diluted with the above buffer to give the required final protein concentration (optimally approx. 2mg/ml; see below). Samples (25μl) of the diluted enzyme preparation were then taken for assay of HMG-CoA reductase by the method described by Kandutsch and co-workers (Saucier & Kandutsch, 1979; Cavenee *et al.*, 1981). Preliminary experiments (at least three) were performed to determine optimum conditions for the assay. In the liver, maximal activity occurred when the diluted homogenate was preincubated at 37°C for 20min in the absence of cofactors before assay of the enzyme. After this period there was an average $298 \pm 46\%$ ($n = 3$) increase in activity compared with the non-incubated controls. Longer preincubation periods resulted in lower measured activities. Preincubation of the diluted homogenate from the mammary gland for periods up to 20min in the absence of cofactors did not result in such a marked increase in the measured activity of HMG-CoA reductase during a subsequent incubation in the presence of substrate and cofactors. After 20min preincubation the activity of the enzyme was $121 \pm 28\%$ ($n = 3$) that of the non-preincubated sample; after 20min preincubation, enzyme activity declined. In liver, enzyme activity was linear with respect to protein concentrations up to 1.0mg/ml in the assay mixture, whereas in mammary gland activity remained linear up to at least 2.5mg of protein/ml of assay mixture. In the homogenates from both organs, there was a linear relationship between enzyme activity and incubation time for at least 40min. In the presence of 3μM-compactin, a competitive inhibitor of HMG-CoA reductase in several types of cell (Brown *et al.*, 1978; Endo *et al.*, 1979), there was no detectable enzymic activity in homogenates of mammary gland irrespective of the length of incubation period, whereas in liver the drug resulted in an average 96.7% ($n = 2$) inhibition after 30 min. On the basis of these experiments, standard conditions for the

assay of HMG-CoA reductase from both organs were set at: preincubation time, 20min; protein concentration in the assay mixture, 0.4–0.9mg/ml; incubation period, 20min. The assay mixture consisted of potassium phosphate buffer (100mM), pH7.4, containing dithiothreitol (2.5mM), EDTA (0.25mM), glucose 6-phosphate (20mM), $NADP^+$ (2.5mM), glucose 6-phosphate dehydrogenase (0.4 unit) and (3*RS*)-[3-^{14}C]HMG-CoA (80μM, containing 100000d.p.m.).

Results

Effects of lactation and dietary manipulation on HMG-CoA reductase activity and the rates of fatty acid and sterol synthesis in liver and mammary gland

The activity of HMG-CoA reductase in the mammary gland of lactating rats was very high compared with that of virgin or non-lactating rats (Table 1). The activity of this enzyme was also higher in the liver of lactating rats than in that of virgin and non-lactating rats (Table 1); this latter finding is in general agreement with the results of Walker & Hahn (1981). The present changes were such that, although the activity of hepatic HMG-CoA reductase was higher than that of the mammary gland in non-lactating or virgin animals, the reverse was true in rats that had been lactating for 10–14 days. However, despite this relatively high activity of HMG-CoA reductase (expressed per mg of protein), the rate of appearance of ^3H in the cholesterol of the lactating mammary gland after injection of ^3H_2O *in vivo* was only about 10% of that in the liver. Even though the lactating mammary gland contains less protein [98.5 ± 2.1 (S.E.M.) mg/g wet wt., $n = 7$] than does liver (151.7 ± 5.3mg/g wet wt., $n = 7$), calculation of HMG-CoA reductase activity per g wet wt. still indicates a much larger activity of enzyme compared with the rate of cholesterol synthesis in the mammary gland than in liver. Removal of food for 6h resulted in a decrease in hepatic HMG-CoA reductase activity, but no significant change occurred in the activity of this enzyme in the lactating gland. This latter group of animals was killed at 14:30h, and comparisons were made with control animals allowed free access to food and killed at the same time (Table 1). Starvation for 24h led to a further decrease in the activity of the hepatic enzyme, and there was also a substantial decrease in the activity of the enzyme in the lactating gland. Similar changes were also observed in the rates of appearance of ^3H in the cholesterol of each organ after injection of ^3H_2O *in vivo*. However, after 6h starvation the changes were not statistically significant. Replacement of the normal diet by one consisting of palatable biscuits with a high vegetable-fat content together with chow

Table 1. *Effects of lactation and dietary manipulation on the rates of cholesterogenesis, lipogenesis and HMG-CoA reductase activity in liver and mammary gland*

Rats were treated as indicated in the Experimental section and ^3H_2O was injected 1h before they were killed. Each group of animals was compared with the fed (control) group killed at 10:00h, except for the 6h-starved group, which was compared with the fed (control) group killed at 14:30h. Numbers of animals in each group are shown in parentheses. Statistical analysis was performed by Student's *t*-test. Values that are significantly different from the corresponding control value are indicated by: $^*P < 0.050$; $^{**}P < 0.005$. Except where stated, the rats were lactating.

State of rats	HMG-CoA reductase activity (pmol of mevalonate/min per mg of protein)		^3H_2O incorporation into cholesterol (μmol/h per g of tissue)		^3H_2O incorporation into fatty acids (μmol/h per g of tissue)	
	Liver	Mammary gland	Liver	Mammary gland	Liver	Mammary gland
Fed (10:00h)	58.3 ± 9.0 (5)	147.6 ± 24.6 (7)	3.03 ± 0.22 (7)	0.35 ± 0.05 (7)	30.6 ± 4.9 (9)	128.4 ± 16.8 (9)
Fed (14:30h)	44.7 ± 3.4 (4)	84.3 ± 5.4 (5)	3.14 ± 0.46 (4)	0.23 ± 0.03 (5)	26.7 ± 13.8 (5)	92.3 ± 29.9 (6)
6h starved	17.6 ± 3.4 (4)**	96.3 ± 24.3 (7)	2.45 ± 0.75 (5)	0.42 ± 0.15 (5)	9.6 ± 1.8 (5)**	15.8 ± 6.8 (5)**
24h starved	8.7 ± 1.4 (5)**	42.1 ± 6.4 (5)**	1.61 ± 0.68 (4)*	0.09 ± 0.04 (4)**	2.7 ± 0.6 (6)**	4.5 ± 1.1 (6)**
Chow–biscuit diet	58.7 ± 9.3 (6)	66.5 ± 11.0 (6)**	3.51 ± 0.32 (6)	0.31 ± 0.03 (6)	34.6 ± 4.6 (6)	62.6 ± 19.4 (6)*
Virgin	22.0 ± 3.7 (7)**	5.8 ± 2.8 (6)**	2.29 ± 0.48 (9)		15.0 ± 2.9 (9)*	
Non-lactating	42.2 ± 12.1 (5)	14.1 ± 1.8 (5)**	2.34 ± 0.26 (9)*		9.5 ± 1.4 (7)**	

(Rolls *et al.*, 1980) did not appear to affect either the activity of HMG-CoA reductase or the rate of incorporation of ^3H_2O into cholesterol in the liver. In contrast, on this diet there was a greater than 50% decrease in the activity of HMG-CoA reductase in the lactating mammary gland. In this case, however, there was no change in the amount of ^3H incorporated into mammary-gland cholesterol. The rate of ^3H_2O incorporation into fatty acids in lactating mammary gland and liver showed greater variations in the situations examined than did the rate of cholesterol synthesis, confirming previous results (Table 1; Agius *et al.*, 1980; Munday & Williamson, 1981).

Diurnal changes in HMG-CoA reductase activity and the rates of sterol synthesis in the liver and lactating mammary gland

The activity of HMG-CoA reductase in the lactating mammary gland of rats killed around mid-day (i.e. at 10:30h) was significantly higher [147 ± 24.6 (7) pmol of mevalonate/min per mg of protein] than that of those killed around midnight (00:30h) [70.9 ± 4.7 (7) pmol/min per mg of protein; $P < 0.005$]. Rats killed between these times (i.e. at 07:00h and 19:00h) showed intermediate values for the activity of mammary gland HMG-CoA reductase (Figs. 1*a* and 1*b*). The activity of this enzyme in the livers of the same rats exhibited a diurnal variation as expected, with a peak around midnight and a nadir around mid-day (Rodwell *et al.*, 1976). The amplitude of this latter variation was greater than that in the mammary gland, and there appeared to be an inverse relationship between the activities of HMG-CoA reductase in each organ. Thus around mid-day, the activity of the liver enzyme was lower than that in the lactating mammary gland, confirming the results obtained previously (Table 1). However, at midnight the activity of the liver enzyme was appreciably higher than that in the mammary gland (Fig. 1*a*). Although the rate of incorporation of ^3H into liver cholesterol *in vivo* showed a similar diurnal pattern to that observed with hepatic HMG-CoA reductase, the contour of incorporation of ^3H into mammary-gland cholesterol was very low over the whole diurnal period and appeared to reach a minimum value at 10:30h (0.35 ± 0.05 μmol of ^3H_2O/h per g, compared with 0.58 ± 0.05 μmol of ^3H_2O/h per g at 00:30h) when the activity of mammary-gland HMG-CoA reductase was maximum.

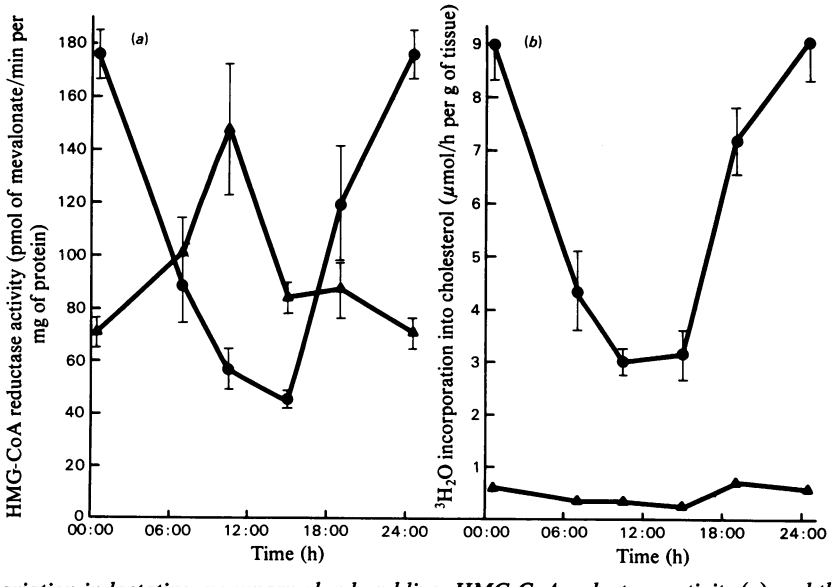

Fig. 1. *Diurnal variation in lactating mammary-gland and liver HMG-CoA reductase activity (a) and the rate of appearance of ^3H in cholesterol after administration of ^3H_2O in vivo (b)*

Rats were injected with ^3H_2O at different times of the day as described in the Experimental section. Rats were killed 1h later and the liver and mammary gland were removed. A portion of each organ was used for determination of ^3H incorporation into cholesterol, and another portion was utilized for assay of HMG-CoA reductase activity. The results are mean values for at least seven rats. The vertical bars represent the S.E.M. for each group; these have been omitted for clarity for mammary-gland cholesterol synthesis. ▲, Values for mammary gland; ●, values for liver.

Discussion

Site of milk cholesterol synthesis

The low rate of incorporation of ^3H into mammary-gland cholesterol compared with that observed in the liver after administration of ^3H_2O *in vivo* suggests that, relative to the liver, the rate of cholesterol synthesis *de novo* in this organ is very low. With some assumptions, it is possible to use the present data to calculate the maximum contribution of cholesterol synthesized endogenously by the gland to the total quantity of cholesterol secreted in the milk. From data obtained in liver cells derived from normal rats fed on a standard chow diet, 7.59 μmol of ^3H_2O are incorporated into each μmol of cholesterol synthesized (C. R. Pullinger & G. F. Gibbons, unpublished work). If this relationship holds in the mammary gland, then from the data shown in Table 1 (fed animals killed at 10:00h) the organ synthesizes a maximum of 0.046 μmol of cholesterol/h per g. On the asumption that this is an average rate over the day, and Fig. 1(*b*) appears to bear this out, then the gland synthesizes 1.11 μmol (0.43 mg) of cholesterol/day per g of tissue. This is equivalent to 5.15–6.45 mg of cholesterol/day for a whole gland weighing 12–15 g (D. H. Williamson, unpublished work) in rats weighing between 250–300 g. Clarenburg & Chaikoff (1966) have calculated that a lactating rat of this size secretes 16.0 mg of cholesterol into the milk per day. In the steady state, therefore, and assuming equilibration of cholesterol derived from exogenous and endogenous sources, then a maximum of 32.2–40.3% originates from endogenous synthesis within the organ. In rats fed on a diet containing a similar quantity of cholesterol to that used in the present work, only 11% of the milk cholesterol was derived from the diet (Clarenburg & Chaikoff, 1966). It is probable therefore that the cholesterol synthesized within the liver is responsible for most of the cholesterol secreted by the lactating gland into the milk. Several other steroid-secreting organs also preferentially utilize cholesterol synthesized by the liver to meet their requirements (Andersen & Dietschy, 1977, 1978; Balasubramaniam *et al.*, 1977*a*; Azhar & Menon, 1981).

Mammary-gland HMG-CoA reductase activity

In view of the relatively small quantity of cholesterol produced by the lactating mammary gland, the high activity of HMG-CoA reductase, particularly around mid-day, is enigmatic and, compared with the liver, would appear to be vastly in excess of that required to meet the demand for cholesterol synthesized *de novo*. The activity of HMG-CoA reductase is also high in cultured explants of rabbit mammary gland (Middleton *et al.*, 1981). It is of course possible that, because of the high demand of the mammary gland for substrate for other biosynthetic pathways, e.g. fatty acid synthesis, a high activity of HMG-CoA reductase is required in order to ensure that even a small quantity of common substrate is diverted into the cholesterol pathway. Evidence for the demands made on the pool of common intermediary metabolites in the gland compared with the liver is the 4-fold higher rate of incorporation of ^3H_2O into fatty acids in the former tissue (Table 1). In addition, changes in the activity of mammary-gland HMG-CoA reductase appear to parallel changes in the demand for substrate for fatty acid synthesis, as reflected by the actual rate of lipogenesis (measured by ^3H_2O incorporation) (Table 1). If competition for substrate is indeed the cause of the high HMG-CoA reductase activity in the mammary gland, then it follows that substrate availability, rather than HMG-CoA reductase activity, is the primary determinant of the rate of cholesterol production in this organ. Whatever the reason for the high activity of HMG-CoA reductase in the lactating mammary gland, it is clear that the activity of this enzyme as measured *in vitro* cannot be relied on as an indicator of the rate of cholesterol synthesis in this organ. In this connection, it is noteworthy that starvation and a high-fat diet (biscuits plus chow) both lower mammary-gland reductase activity, but, whereas starvation decreases ^3H incorporation into cholesterol and fatty acids, the high-fat diet only decreases lipogenesis (Table 1). Although the changes in the activity of HMG-CoA reductase and the rate of ^3H_2O incorporation into cholesterol usually occurred in parallel in liver, this was not the case after short-term (6h) starvation (Table 1).

We cannot completely discount the possibility that in mammary gland the primary determinant of HMG-CoA reductase activity is not sterol, but some other non-sterol product of mevalonate metabolism. However, careful fractionation of the non-saponifiable lipid fraction labelled *in vivo* with ^3H_2O in the mammary gland showed little radioactivity corresponding to non-cholesterol compounds.

Diurnal variations

It is widely recognized that cyclical changes in the activity of hepatic HMG-CoA reductase occur over a 24h period and that this variation is accompanied by corresponding changes in the rate of acetate conversion into cholesterol *in vitro* (Rodwell *et al.*, 1976). A variation in the incorporation of ^3H into hepatic cholesterol and total non-saponifiable material also occurs *in vivo* in rats injected with ^3H_2O at midnight and mid-day (Edwards *et al.*, 1972; Jeske & Dietschy, 1980). The present results indicate that lactation does not alter these diurnal changes in the liver (see also Walker & Hahn, 1981).

No other tissue studied appears to exhibit similar circadian variations, although there is some disagreement as regards the diurnal constancy of the rates of cholesterol synthesis in the intestine (Edwards *et al.*, 1972; Andersen & Dietschy, 1977), and the rhythm in the activity of HMG-CoA reductase in the adrenal gland (Balasubramaniam *et al.*, 1977*b*) is apparently not reflected in similar changes in the conversion of labelled acetate into adrenal-gland cholesterol *in vivo* (Andersen & Dietschy, 1977). Thus the present observations of a circadian rhythm in HMG-CoA reductase activity in the mammary gland is of considerable interest, not only because it appears to be one of the few tissues to exhibit such changes, but also because the variations in activity appear to bear an inverse relationship to the activity of the hepatic enzyme. Another interesting feature is that the diurnal changes in the activity of mammary-gland HMG-CoA reductase do not seem to be related to corresponding changes in the rate of cholesterol synthesis, at least as indicated by the present method involving ^3H incorporation. The reasons for the variations in the activity of the mammary-gland enzyme are at present obscure. However, it is possible that the high activity observed in the lactating mammary gland around mid-day may be due to a decrease in the amount of cholesterol which the gland receives from the liver, since the rate of hepatic cholesterol synthesis is low during this period (Fig. 1*b*). Alternatively, if changes in the rate of cholesterol synthesis in the mammary gland are accurately reflected in variations in the ^3H content of the glandular cholesterol, then the low rate of cholesterol production in the gland during this period may be the stimulus for the high activity of HMG-CoA reductase.

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