Relationship between Activity of Hepatic 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase and Secretion of Very-Low-Density-Lipoprotein Cholesterol by the Isolated Perfused Liver and in the Intact Rat

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The hepatic output of triacylglycerol and cholesterol from very-low-density lipoprotein (VLD lipoprotein), and the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase were compared in the isolated perfused rat-liver preparation and in the intact rat. The output of triacylglycerol and cholesterol from VLD lipoprotein by the perfused liver was stimulated by oleate concomitant with stimulation of hepatic microsomal hydroxymethylglutaryl-coenzyme A reductase activity. In the intact animal treated with Triton WR-1339, the magnitude of secretion of triacylglycerol and cholesterol from VLD lipoprotein coincided with the diurnal rhythm of hepatic hydroxymethylglutaryl-coenzyme A reductase activity, which was maximal at 24:00h and minimal at 12:00h. These observations suggest that the stimulation of the reductase and of the secretion of cholesterol from VLD lipoprotein by non-esterified fatty acids, as observed with the isolated perfused rat liver preparation *in vitro*, may also be an important physiological mechanism *in vivo*. Hepatic cholesterogenesis may be stimulated under conditions conducive to the secretion of the VLD lipoprotein, the primary transport form for triacylglycerol in the postabsorptive state.

We have reported previously that oleate stimulated the secretion of total triacylglycerol and total cholesterol by the isolated perfused rat liver *in vitro*. The increase in the secretion of triacylglycerol and cholesterol by the perfused liver was associated with an increase in the synthesis of cholesterol, estimated by the incorporation of $^3\text{H}_2\text{O}$ into cholesterol (Goh & Heimberg, 1973). The stimulation by oleate of hepatic cholesterogenesis was reflected also by an increase in the activity of microsomal HMG-CoA reductase, the rate-limiting enzyme of cholesterogenesis (Goh & Heimberg, 1976, 1977a). These observations led us to suggest that the secretion of cholesterol, presumably as a moiety of the VLD lipoprotein, which is required for the secretion of triacylglycerol, is related to the synthesis of cholesterol by the liver (Goh & Heimberg, 1973, 1976, 1977a). In the present study, the VLD lipoprotein was specifically isolated and the relationship between the secretion of VLD-lipoprotein cholesterol and the

activity of microsomal HMG-CoA reductase was examined in rat livers perfused with or without oleate.

In addition, the secretion of VLD-lipoprotein cholesterol by the rat liver *in vivo* was also measured; determinations were made at 12:00h, when the diurnal activity of hepatic microsomal HMG-CoA reductase was at its nadir, and at 24:00h, when the activity was at its peak (Edwards & Gould, 1974).

The activity of hepatic microsomal HMG-CoA reductase was associated with the secretion of cholesterol and triacylglycerol from VLD lipoprotein, and the increase in the secretion of VLD-lipoprotein cholesterol was accompanied by a simultaneous increase in the activity of hepatic microsomal HMG-CoA reductase in the perfused liver *in vitro* and in the intact animal. Preliminary reports of this work have been published previously (Goh & Heimberg, 1977b; Heimberg *et al.*, 1978).

Experimental

Male Sprague-Dawley rats, obtained from the Charles River Breeding Laboratories, Wilmington, MA, U.S.A., were housed with lighting from 05:00h to 17:00h. The animals were allowed free access to Purina rat chow and water, and were maintained

Abbreviations used: 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34), HMG-CoA reductase; very-low-density lipoprotein, VLD lipoprotein.

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under these conditions for at least 10 days before use in studies either *in vitro* (perfusion) or *in vivo*. The average body weight of the animals from which livers were removed for perfusion experiments was 243 ± 9 g (mean $\pm$ s.e.m.). For perfusion, rats were anaesthetized with diethyl ether and livers were removed surgically between 09:00h and 10:30h. Perfusion equipment and procedures were those described previously (Goh & Heimberg, 1977a). The perfusion medium (starting volume 65 ml) consisted of Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit *et al.*, 1949) containing 3 g of purified bovine serum albumin/dl (Wilcox *et al.*, 1975) and 100 mg of glucose/dl. After insertion of the liver into the perfusion system and equilibration for 20 min, 5 ml of an oleate (70 μ mol)-bovine serum albumin complex [fatty acid (1418 μ mol)/albumin (10 g)/0.9% NaCl (100 ml)], prepared as described previously (Van Harken *et al.*, 1969), was added as a pulse dose. At the same time, infusion of the same complex at a rate of 11.6 ml/h (166 μ mol/h) was begun. Bovine serum albumin alone was infused in experiments without oleate. The livers were then perfused for 4 h. The average perfusion experiment was terminated by 14:30h.

Rats (300–325 g) were removed from the animal-care facilities for experiments *in vivo* 2.5 h before being killed at 12:00h or 24:00h. The animals were anaesthetized with urethane (50% solution; 100 mg/100 g body wt.) injected intraperitoneally and were maintained in the anaesthetized state for the duration of the 2.5 h experiment. Triton WR-1339 (20 g/100 ml of 0.9% NaCl; 40 mg/100 g body wt.) or 0.9% NaCl alone was given intravenously via the femoral vein (Otway & Robinson, 1967). The animals were killed 2 h after receiving either Triton or 0.9% NaCl by exsanguination via aortic puncture. Residual blood in the liver was removed by infusion of 20 ml of 0.9% NaCl through the portal vein. EDTA (1.0 mg/ml of blood) was added as an anticoagulant and Triton WR-1339 (10 mg/ml of blood) was added to blood obtained from the animals treated with 0.9% NaCl to inhibit the activity of phosphatidylcholine-cholesterol acyl-transferase (Soler-Argilaga *et al.*, 1977). Portions (3 ml) of plasma from individual animals were layered under 6 ml of 0.9% NaCl in polycarbonate centrifuge tubes and centrifuged in a model L5-65 Spinco ultracentrifuge in a 65 rotor at 192000g and 10°C for 20 h. The plasma fraction with $d < 1.006$ was recovered by quantitative removal of the top 4 ml of the 0.9% NaCl. Agarose-column chromatography was used for the separation of plasma and perfusate lipoproteins. The procedures were similar to those described by others (Rudel *et al.*, 1974; Sheperd *et al.*, 1975; Fainaru *et al.*, 1977), except that ascending elution was used, which resulted in less trailing, narrower peaks and a more stable gel bed volume.

Lipoproteins were eluted with a solution of 0.9% NaCl, 0.01% EDTA and 0.02% NaN₃ at 16°C. For the isolation of the VLD lipoprotein from the perfusate, a 10 ml portion of perfusate was applied directly to an A-5m agarose column (2.6 cm $\times$ 95 cm). For the isolation of the plasma VLD lipoprotein free of chylomicra, portions of the plasma fraction with $d < 1.006$ (2–4 ml) from the individual fed rats treated with 0.9% NaCl or Triton WR-1339 were applied directly to an A-150m agarose column (2.6 cm $\times$ 92 cm). Fractions containing the VLD lipoprotein were pooled and freeze-dried. The perfusate VLD lipoprotein, when chromatographed on a A-150m column, had an elution profile similar to that observed with plasma VLD lipoprotein.

The hepatic microsomal fraction was isolated at 100000g for 60 min by using procedures and buffer described by Shapiro *et al.* (1974). The activity of HMG-CoA reductase was determined without using [³H]mevalonate as an internal standard (Goh & Heimberg, 1977a). Microsomal protein was determined by the method of Lowry *et al.* (1951) after precipitation with 5% trichloroacetic acid. Lipids were extracted with chloroform/methanol (2:1, v/v) and the lipid classes were separated by t.l.c. (Heimberg *et al.*, 1965). Triacylglycerol (Van Handel & Zilver-smith, 1957; Newman *et al.*, 1961), non-esterified and esterified cholesterol (Rudel & Morris, 1973) and phospholipid (King, 1932) of VLD lipoprotein were analysed. Significance of the differences between groups was determined by Student's *t* test (Steel & Torrie, 1960). Curve fitting (Fig. 1) was done on an HP-97 calculator by using program SD-03A.

Triton WR-1339 was obtained from the Ruger Chemical Co., New York, NY, U.S.A. Agarose gels were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Hydroxymethyl[3-¹⁴C]glutaryl-coenzyme A, obtained from New England Nuclear, Boston, MA, U.S.A., was adjusted to 3000 d.p.m./nmol with non-radioactive coenzyme purchased from P.-L. Biochemicals Inc., Milwaukee, WI, U.S.A. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP⁺ and mevalonic acid lactone were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Thin-layer plates of silica gel G for separation of lipid classes were purchased from Analtech Inc., Newark, DL, U.S.A. LQD thin-layer plates for the separation of mevalonic acid lactone were obtained from Quantum Inc., Fairfield, NJ, U.S.A. Oleate was obtained from Nu-Chek Prep., Elysian, MN, U.S.A. All other chemicals were of analytical grade.

Results

The output of VLD-lipoprotein lipids by the perfused liver is shown in Table 1. Oleate stimulated the output of VLD-lipoprotein triacylglycerol, free

Table 1. Relationship between output of VLD-lipoprotein lipids by the perfused rat liver and activity of hepatic microsomal HMG-CoA reductase

The output of VLD-lipoprotein lipids and activity of HMG-CoA reductase were measured after 4 h of perfusion. Output of lipids by the perfused liver was linear during the period of perfusion (Goh & Heimberg, 1973). Results presented are means $\pm$ S.E.M. Values in parentheses indicate the number of observations. Abbreviations used: TG, triacylglycerol; PL, phospholipid; C, cholesterol; CE, cholesteryl esters.

Experimental group	Output of VLD-lipoprotein lipids (μ mol/g of liver per h)				Activity of HMG-CoA reductase (nmol/min per mg of protein)
	TG	PL	C	CE	
Control (oleate omitted) (4)	0.52 $\pm$ 0.04	0.19 $\pm$ 0.02	0.16 $\pm$ 0.01	0.05 $\pm$ 0.01	0.93 $\pm$ 0.12
Experimental (oleate added) (6)	1.37 $\pm$ 0.09	0.24 $\pm$ 0.02	0.22 $\pm$ 0.01	0.07 $\pm$ 0.01	2.24 $\pm$ 0.22
P values	<0.001	<0.20	<0.02	<0.05	<0.005

Table 2. Diurnal relationship between output of VLD-lipoprotein lipids and activity of hepatic microsomal HMG-CoA reductase *in vivo*

The output of VLD-lipoprotein lipids *in vivo* was calculated from the difference between control animals and rats treated with Triton WR-1339 as described in the text (Soler-Argilaga *et al.*, 1976). The activity of HMG-CoA reductase was measured 2 h after treatment with Triton or 0.9% NaCl. All data are means $\pm$ S.E.M. Values in parentheses indicate the numbers of observations. For explanation of the abbreviations, see the legend to Table 1.

Collection period	Output of VLD-lipoprotein lipids (μ mol/g of liver per h)				Activity of HMG-CoA reductase (nmol/min per mg of protein)	
	TG	PL	C	CE	-Triton	+Triton
12:00h (4)	0.98 $\pm$ 0.06	0.52 $\pm$ 0.03	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01	0.14 $\pm$ 0.01	0.45 $\pm$ 0.01
24:00h (4)	1.20 $\pm$ 0.09	0.57 $\pm$ 0.07	0.11 $\pm$ 0.01	0.10 $\pm$ 0.01	0.79 $\pm$ 0.07	1.16 $\pm$ 0.05
P values	<0.02	<0.30	<0.01	<0.01	<0.001	<0.001

cholesterol and cholesteryl esters. The molar ratios of VLD-lipoprotein lipids relative to triacylglycerol were 0.36 ± 0.02 , 0.31 ± 0.04 and 0.09 ± 0.01 (mean $\pm$ S.E.M.) for phospholipid, free cholesterol and cholesteryl esters respectively, for experiments without oleate. Corresponding values for experiments with oleate were 0.18 ± 0.02 , 0.17 ± 0.01 and 0.06 ± 0.01 (mean $\pm$ S.E.M.) respectively. These ratios are similar to those observed previously in VLD lipoprotein isolated by zonal ultracentrifugation from the perfusate (Wilcox *et al.*, 1975). The activity of microsomal HMG-CoA reductase was increased in the experiments with added oleate in agreement with previous observations (Goh & Heimberg, 1976, 1977a).

The output of VLD-lipoprotein lipids *in vivo* is shown in Table 2. Data were calculated by differences between animals treated with Triton WR-1339 and controls, considering plasma volume to be 4% of body weight (Soler-Argilaga *et al.*, 1976). More triacylglycerol, free cholesterol and cholesteryl esters from VLD lipoproteins were secreted at 24:00h than at 12:00h. In agreement with reports by others (Kandutsch & Saucier, 1969; White & Rudney, 1970), treatment of rats with Triton WR-1339 increased hepatic activity of microsomal

HMG-CoA reductase. Activities of the enzyme at 12:00h and 24:00h from control animals are in good agreement with data reported by others (Edwards & Gould, 1974; Hamprecht *et al.*, 1969). Activity of HMG-CoA reductase increased concomitantly with output of VLD lipoproteins *in vivo*.

Discussion

It was observed in these experiments that the hepatic secretion of cholesterol, cholesteryl esters and triacylglycerol from VLD lipoproteins was stimulated by oleate in the perfusion experiments, and that the secretion of the VLD-lipoprotein lipids in the intact animal was greater at 24:00h than that at 12:00h. The increased output of VLD lipoprotein *in vivo* is coincident with the maximal concentration of plasma non-esterified fatty acid at 24:00h compared with the minimal value at 12:00h (Mlekusch *et al.*, 1975) and presumably is related to the feeding habits of the rat. Activity of hepatic microsomal HMG-CoA reductase was stimulated by oleate in the experiments *in vitro*, and *in vivo* was higher at 24:00h. These observations, considered with the decrease in HMG-CoA reductase activity in rats treated with colchicine (Ottery & Goldfarb,

1976), an inhibitor of VLD-lipoprotein secretion (Stein & Stein, 1973), support the hypothesis that secretion of VLD-lipoprotein cholesterol for transport of triacylglycerol is of physiological importance in the regulation of hepatic cholesterologenesis.

We have taken the liberty of correlating activity of HMG-CoA reductase with output of VLD-lipoprotein cholesterol, without regard to whether the data were obtained with the isolated liver or were measured *in vivo* (Fig. 1). We are aware that the data *in vitro* may only coincidentally be related to the

Fig. 1. Proposed correlation between activity of microsomal HMG-CoA reductase and secretion of the VLD-lipoprotein cholesterol

The secretion of cholesterol in the VLD lipoprotein was calculated as the sum of non-esterified and esterified cholesterol, as reported in Tables 1 and 2. For the study *in vivo*, data were plotted by using the mean activity of HMG-CoA reductase from non-Triton-treated rats. The activity of microsomal HMG-CoA reductase, expressed as nmol of mevalonate formed/min per mg of microsomal protein, was recalculated as rate of synthesis of cholesterol, expressed as μmol of cholesterol/g of liver per h (Edwards *et al.*, 1977), by multiplying the enzyme activity by 0.2. This factor assumes the conversion into cholesterol of all mevalonate synthesized, and a content of 20mg of microsomal protein/g wet wt. of liver (Bergstrand & Dallner, 1969); therefore (nmol of mevalonate synthesized/min per mg of microsomal protein) $\times$ (60 min) $\times$ (20mg/g of liver) $\times$ ($\frac{1}{1000}$ mol of mevalonate/mol of cholesterol) $\times$ (1/1000) = 0.2. By using this conversion factor, the relationship between the rate of secretion of VLD-lipoprotein cholesterol and hepatic cholesterologenesis can be described by the equations $y = 28.82 x^{3.35}$ for the hyperbolic curve and $y = -0.32 + 2.49 x$ for a linear fit, where x = output of VLD-lipoprotein cholesterol and y = cholesterol synthesized. Circles represent results obtained *in vivo* and triangles results obtained *in vitro*. Symbols: $\circ$, 12:00h; $\bullet$, 24:00h; $\blacktriangle$, oleate added; $\triangle$, oleate omitted.

data obtained *in vivo*, but the inferences drawn, if valid, may help us understand regulatory mechanisms affecting synthesis and secretion of cholesterol by the liver. The data correlating activity of HMG-CoA reductase (y) with the secretion of VLD-lipoprotein cholesterol (x) can be fitted to the hyperbolic equation $y = ax^b$ (where $r = 0.98$, $a = 144.12$ and $b = 3.35$). The same data will also fit the linear equation $y = a + bx$ (where $r = 0.96$, $a = -1.62$ and $b = 12.45$). In the first phase of the hyperbolic expression ($y < 0.25$), secretion of VLD-lipoprotein cholesterol is accompanied by small changes in reductase activity. In the next phase ($y = 0.25-2.5$), activity of HMG-CoA reductase is proportional to secretion of VLD-lipoprotein cholesterol. In a third phase ($y > 2.5$), enzyme activity would increase more rapidly than secretion of VLD-lipoprotein cholesterol (Goh & Heimberg, 1977a). Since the highest values reported for hepatic microsomal HMG-CoA reductase from rats treated with cholestyramine were less than 3.0 nmol/min per mg of protein, the last phase probably is of no physiological significance. The presence of a regulatory metabolic pool of cholesterol governing the activity of reductase may be inferred from phase 1. Removal of cholesterol from this pool by the secretion of VLD-lipoprotein cholesterol might gradually increase the activity of HMG-CoA reductase as suggested by the hyperbolic expression, or alternatively, increase enzyme activity after secretion of about $0.13 \mu\text{mol}$ of cholesterol/g of liver per h (x intercept, Fig. 1) according to the linear regression line. These hypotheses undoubtedly oversimplify reality, but do suggest certain intriguing possibilities.

Dissociation between the activity of HMG-CoA reductase and incorporation of a radioactive precursor into cholesterol has been reported (Nervi *et al.*, 1976). In the present studies, cholesterologenesis by the perfused rat liver in the absence or presence of oleate was estimated by measurement of incorporation of ^3H from $^3\text{H}_2\text{O}$ into cholesterol (Goh & Heimberg, 1973) and separately, by the activity of HMG-CoA reductase (Goh & Heimberg, 1976, 1977a). A comparison between these two methods of measurements is shown in Table 3. Similar values for the ratio a/b , with or without added oleate support the concept of a rate-limiting role for microsomal HMG-CoA reductase in hepatic cholesterologenesis. The deviation of the ratio a/b from 1.0 (Table 3) suggests the utilization of mevalonate for pathways other than cholesterologenesis (Righetti *et al.*, 1976), failure to measure the incorporation of $^3\text{H}_2\text{O}$ into other metabolites of cholesterol in estimating the rate of cholesterol synthesis, and/or the apparent increase of microsomal HMG-CoA reductase activity by the homogenization of liver in the absence of 50mM-NaF (Nordstrom *et al.*, 1977). Regardless, the relative changes in hepatic cholesterologenesis by

Table 3. Relationship between cholesterogenesis estimated by incorporation of ^3H of $^3\text{H}_2\text{O}$ into cholesterol and from activity of microsomal HMG-CoA reductase in the perfused rat liver

The rate of synthesis of cholesterol from $^3\text{H}_2\text{O}$, calculated as described by Windmueller & Spaeth (1966) is based on the sum of the relative total activity of free and esterified cholesterol of liver and perfusate, and is from results reported previously (Goh & Heimberg, 1973). The rate of synthesis of cholesterol estimated from the activity of microsomal HMG-CoA reductase was calculated by multiplying the enzyme activity (Table 1) by 0.2, as described by Edwards *et al.* (1977). The perfusion experiments reported previously (Goh & Heimberg, 1973) were similar to those presented in the present paper except that a pulse dose (70 μmol) of oleate was added to the medium at the beginning of the experiments reported in the present paper. Results presented are means $\pm$ S.E.M. Values in parentheses indicate numbers of observations.

Group	Rate of hepatic cholesterogenesis ($\mu\text{mol/g}$ of liver per h) calculated from:		Ratio of a/b
	Activity of HMG-CoA reductase (a)	Incorporation of ^3H (b)	
(A) Control (oleate omitted)	0.18 $\pm$ 0.02 (4)	0.13 $\pm$ 0.02 (7)	1.38
(B) Experimental (oleate added)	0.42 $\pm$ 0.04 (4)	0.32 $\pm$ 0.03 (5)	1.31
Ratio of B/A	2.33	2.46	

perfused livers, with infusion of up to 663 μmol of oleate/4h, were proportional by the two different methods of measurement (B/A, Table 3). Edwards and his co-workers (1972) measured cholesterogenesis *in vivo* in the rat by incorporation of $^3\text{H}_2\text{O}$ into hepatic cholesterol and by measurement of HMG-CoA reductase at 12:00 and 24:00h; the ratios of synthesis at 24:00h to that at 12:00h were 2.6 and 2.7 for $^3\text{H}_2\text{O}$ and enzyme activity respectively. This is comparable with the ratio of B/A shown in Table 3. The proportional changes in hepatic cholesterogenesis estimated by these two methods suggest that the observed activity of microsomal HMG-CoA reductase represents a relative change in hepatic cholesterogenesis as it relates to the secretion of VLD-lipoprotein cholesterol. The efflux of cholesterol from isolated hepatocytes has also been observed by other investigators to be associated with an increase in the activity of microsomal HMG-CoA reductase (Edwards, 1975; Edwards *et al.*, 1976; Breslow *et al.*, 1977).

The simultaneous increase in HMG-CoA reductase activity and hepatic secretion of VLD-lipoprotein cholesterol during the diurnal cycle and in the perfusion experiments *in vitro* suggests an important physiological mechanism for cholesterogenesis related to the quantity and composition of the diet consumed by the animal. Various nutrients that directly or indirectly stimulate the formation and secretion of the VLD lipoprotein by the liver for transport of triacylglycerol may also be expected to stimulate output of VLD-lipoprotein cholesterol and stimulate hepatic cholesterogenesis. Dietary cholesterol and endogenous lipoprotein cholesterol would be expected to decrease sterol synthesis *de novo* by supplying some of the sterol required for the formation and secretion of VLD lipoprotein. Since the composition of dietary neutral fats affects the composition of the plasma non-esterified fatty acid and, subsequently,

the properties and composition of the VLD lipoprotein secreted by the liver (Dunn *et al.*, 1975), the mechanisms by which the amount and kind of fat in the diet affect the rate of hepatic cholesterogenesis may, in part, be regulated through the secretion of VLD-lipoprotein cholesterol. It is also conceivable that the kind and quantity of carbohydrates in the diet, by being substrates for lipogenesis, can give rise to plasma or hepatic fatty acids and subsequently affect the secretion of VLD-lipoprotein cholesterol and hepatic cholesterogenesis, in part, through mechanisms discussed in the present paper.

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