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Membrane-mediated control of hepatic β-hydroxy-β-methylglutaryl-coenzyme A reductase

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Previously we [Sabine & James (1976) *Life Sci.* **18**, 1185–1192] proposed that 'the activity of hepatic β-hydroxy-β-methylglutaryl-coenzyme A reductase is critically regulated by the fluidity of its supporting microsomal membrane'. In the present work we examined further this concept of membrane-mediated control, with respect to the specific hypothesis that such control might function as a common mechanism both for the co-ordinated regulation of other enzymes affected by cholesterol feeding and also for the subcellular integration of the several physiological factors known to influence this enzyme's activity. Contrary to earlier expectations, this hypothesis now appears not to hold. We report here that, under those conditions of short-term cholesterol feeding that affected the reductase, a variety of other microsomal enzymes did not display membrane-function interactions, i.e. neither enzymes involved in cholesterol metabolism and also affected by cholesterol feeding (cholesterol 7α-hydroxylase), nor those involved in cholesterol metabolism and not affected by cholesterol feeding (hydroxymethylglutaryl-CoA hydrolase, acyl-CoA:cholesterol acyltransferase), nor those not directly involved in cholesterol metabolism at all (glucose 6-phosphatase). Furthermore, we oberved no evidence for the operation of membrane-mediated control of the reductase in other situations known to influence its activity, i.e. starvation, diurnal rhythm, the very early stages of cholesterol feeding and various manipulations *in vitro*.

The microsomal enzyme HMG-CoA reductase (EC 1.1.1.34) is important for a number of reasons, not the least of which are that under a wide range of physiological and experimental conditions it is the rate-limiting enzyme for mammalian cholesterol biosynthesis (Dempsey, 1974; Rodwell *et al.*, 1976; Sabine, 1977), and that dietary feedback control of its activity, a feature of the liver enzyme in all species examined, is critically defective in all tumorous and pre-tumorous livers (Siperstein, 1970; Sabine, 1975, 1980). Given the importance of cholesterol synthesis in many serious diseases (Sabine, 1977) and given the plethora of conditions under which this synthesis in general, and HMG-CoA reductase activity in particular, can be modified (Rodwell *et al.*, 1976; Sabine, 1977), it is clearly important that further work should be done with the specific objective of clarifying our understanding of the physiological or intracellular mechanism by which the catalytic capacity of the enzyme is regulated.

Currently two hypotheses have been proposed to explain the molecular mechanism controlling the activity of HMG-CoA reductase. Various workers (e.g. Ingebritsen *et al.*, 1978; Nordstrom *et al.*, 1977; and others) have shown that enzyme activity is reversibly modified by a protein-mediated phosphorylation/dephosphorylation reaction. This phenomenon, although examined extensively in recent years, has not provided a satisfactory explanation for any of the various extracellular or 'whole-body' controls known to operate over this enzyme. On the other hand, the second hypothesis for molecular control, namely that 'the activity of HMG-CoA reductase is critically regulated by the fluidity of its supporting microsomal membrane', first proposed from our laboratory (Sabine & James, 1976) and later confirmed by others (Mitropoulos & Venkatesan, 1977), has been implicated as a possible general intracellular mechanism common to several

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

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regulatory situations (Sabine & James, 1976). The present paper concerns our attempts to explore further this possibility.

Thus, if membrane-mediated control of HMG-CoA reductase has important general physiological significance in the regulation of hepatic cholesterol synthesis, then three corollaries might follow: (i) in those situations where such control operates over the reductase, a similar control might operate over other enzymes that are regulated co-ordinately with it; (ii) other microsomal enzymes should be unaffected by the reductase control; and (iii) a variety of different controls over the reductase might operate through the same membrane mechanism.

Materials and methods

Chemicals

Reagents were purchased as follows: CoA (lithium salt), $NADP^+$, NADPH, dithioerythritol, hydroxymethylglutaric acid and mevalonic acid from Sigma Chemical Co., St Louis, MO, U.S.A.; hydroxymethyl[3-^{14}C]glutaric acid from New England Nuclear, Boston, MA, U.S.A.; DL-[2-^{14}C]-mevalonic acid from The Radiochemical Centre, Amersham, Bucks., U.K.; bulk cholesterol from Merck, Darmstadt, Germany; Sephadex G-15 from Pharmacia, Uppsala, Sweden; chromatographic papers from Whatman, Maidstone, Kent, U.K.; charcoal (Norit A) from MCB, Los Angeles, CA, U.S.A. Benzene and acetone (dried over anhydrous $CaSO_4$) were redistilled before use. The charcoal was boiled in 1.0M-HCl, washed thoroughly with distilled water and dried at 60°C.

All other reagents were of analytical grade.

Buffers

Two buffers were used, both at pH 7.6. Buffer A contained 0.05M-triethanolamine, 0.25M-sucrose, 0.01M-Na_2EDTA and 0.01M-mercaptoethanol. Buffer B contained 0.1M-sucrose, 0.05M-KCl, 0.04M-potassium phosphate, 0.03M-Na_2EDTA and 0.01M-dithioerythritol.

Enzyme preparation

The microsomal fraction from rat liver was prepared by standard homogenization and centrifugation techniques, at 4°C, in buffer A. Briefly, the liver was minced in buffer with fine scissors, the buffer decanted (thus removing most of the contaminating plasma and erythrocytes) and replaced with fresh buffer (1:2, wet wt./vol.), and the liver pieces were then homogenized in a glass homogenizer with a loose-fitting Teflon pestle, with four up-and-down strokes (setting 10 on a Caframo RZRI-64 stirrer). The resultant mixture was then centrifuged in the following sequence: MSE Mistral 44, 2500rev./min, 10min (supernatant, S_5); Sorvall SS-3, 10000rev./min, 30min (supernatant, S_{12}); Beckman L5-65 (50 Ti rotor), 40000rev./min, 75min (microsomal fraction, P_{104}, and cytosol, S_{104}). The microsomal fraction was resuspended in buffer B, either immediately or after storage at −15°C.

Preparation of [^{14}C]HMG-CoA substrate

CoA and hydroxymethyl[^{14}C]glutaric anhydride (sp. radioactivity 200Ci/mol, prepared by the method of Goldfarb & Pitot, 1971) were made to react essentially under the conditions described by Louw *et al.* (1969).

Assay for HMG-CoA reductase

This was based on the chloroform-extraction method of Ackerman *et al.* (1977) and did not differ significantly from that procedure. Each enzyme assay was usually done in triplicate, and throughout the paper activity is expressed as nmol of mevalonate formed/30min per mg of protein.

Assay for other enzymes

Other assays were by only minor modifications to standard techniques: acyl-CoA:cholesterol acyltransferase (Goodman *et al.*, 1964); cholesterol 7α-hydroxylase (Van Cantfort *et al.*, 1975); glucose 6-phosphatase (Swanson, 1955). The assay for HMG-CoA hydrolase involved isolation and counting of radioactivity of the hydroxymethyl-[^{14}C]glutarate released in a standard incubation procedure.

Other assay conditions

In our studies of the pH profile of enzyme activity the microsomal pellet was resuspended in buffers in the pH range 6.5–8.0. Where assay temperature was varied, incubations were carried out in a series of water baths set at temperatures in the range 19–40°C, by using Thermomix 1441 water heaters (B. Braun, Melsungen, West Germany). We determined, in preliminary experiments, that in the various physiological states and under the different assay conditions used in these studies enzyme activity was linear with respect to time for the duration of the assay.

Animals and diets

We used mature male rats (Hooded-Wistar strain) as supplied by this University's Central Animal House. They were housed in our laboratory under a 12h:12h lighting regime and fed on a standard diet (Charlick's Mouse Cubes, no. 318107) and water *ad libitum* for at least 2 weeks before any experiment. Unless otherwise stated animals were killed by decapitation in the middle of the dark period. When cholesterol was added to the diet this was done as a 5% (w/w) mixture with crushed food.

Diurnal rhythm

Besides animals kept on a reverse lighting regime (off at 06:00h, on at 18:00h), some animals were maintained under normal seasonal lighting. Thus, for an examination of the diurnal variation in HMG-CoA reductase, two animals kept under the artificial-light conditions and two kept under natural lighting, were killed at noon, thus giving two at mid-light and two at mid-dark. The livers from each pair were pooled.

Time course of the effect of cholesterol feeding

Groups of two animals were placed in individual cages and adapted for 1 week to feed for only 2h each day, i.e. 09:00–11:00h, the lights having gone off at 06:00h. On the day of the experiment one group of meal-fed rats was killed at 09:00h, as the zero-time point, and these were compared with another two that had been fed *ad libitum*. The remainder of the meal-adapted rats were fed on one of the following diets: standard diet; standard diet, including 5% (w/w) cholesterol; standard diet, including 5% (w/w) [^{14}C]cholesterol (6.67μCi/g of cholesterol). One group of animals on each of the above diets was killed at 11:15h, and another at 15:00h (i.e. 2.25h and 6h from the commencement of feeding respectively). Each pair of livers was pooled.

[^{14}C]Cholesterol feeding

The livers of the rats fed on [^{14}C]cholesterol were perfused *in vitro* with ice-cold 0.25M-sucrose, to remove most of the blood, and were then homogenized. Lipids were extracted from the whole homogenate and from the cytosolic and microsomal fractions, with chloroform:methanol (2:1, v/v). The lipid phase, after drying, was counted for ^{14}C, using a toluene-based scintillation mixture. The amounts of dietary cholesterol in each of the liver fractions were estimated from the known specific radioactivity of the cholesterol in the diet.

Results

Arrhenius plots and the effects of cholesterol feeding

The Arrhenius plot of the activity at different temperatures of a membrane-bound enzyme is often used to study the interaction between the enzyme protein and its surrounding lipid environment (Raison, 1973). The presence of any break in such a plot has been attributed to the occurrence of phase changes in the membrane lipids, which in turn induce sufficient structural modification to the enzyme protein that its activity is altered (Raison, 1973).

Fig. 1 shows typical Arrhenius plots for freshly prepared microsomal HMG-CoA reductase from rats that were either with or without supplementary

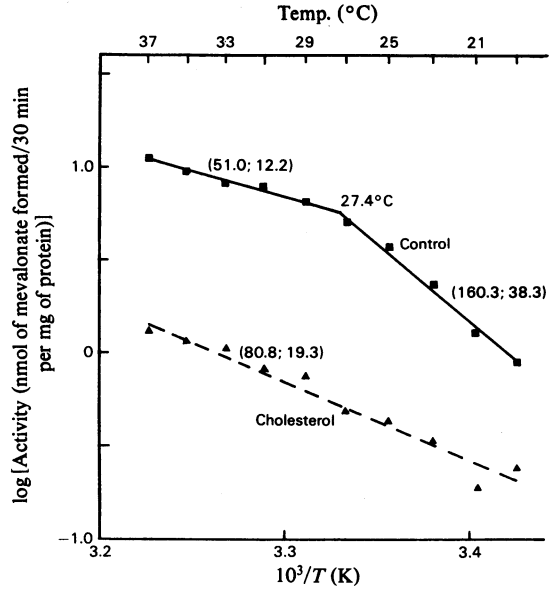

Fig. 1. *Arrhenius plots of activity of freshly prepared microsomal HMG-CoA reductase, derived from livers of control (■; normal fed) and cholesterol-fed (▲) rats and assayed without preincubation*

Each point is the mean of triplicate determinations. The E_a values are shown in parentheses (first as kJ/mol, then as kcal/mol). For details see the text.

dietary cholesterol. From six experiments with control animals the average transition temperature was 27.5±0.58°C, and activation energies (E_a) were 56.9±4.6kJ/mol (13.6±1.1kcal/mol) above the transition temperature and 213.6±36.4kJ/mol (51.1±8.7kcal/mol) below it. In two experiments with cholesterol-fed animals the activation energies calculated were 80.8 and 111.3kJ/mol (19.3 and 26.6kcal/mol), with no phase transition observed. Under our conditions neither the K_m for the reductase (62μM at 37°C) nor its pH profile was affected by cholesterol feeding.

Changes in vitro

We were able to change significantly the activity of the microsomal reductase by storage at −15°C (a decrease) and by preincubation at 37°C of the stored preparation (an increase). In neither case, however, were Arrhenius-plot characteristics changed.

Effect of cholesterol feeding on other hepatic enzymes

In our rats cholesterol feeding resulted in a 2.3-fold increase in the activity of microsomal cholesterol 7α-hydroxylase, but no change in the

activities of the other microsomal enzymes (HMG-CoA hydrolase, acyl-CoA:cholesterol acyltransferase and glucose 6-phosphatase).

Fig. 2 shows Arrhenius plots for cholesterol 7α-hydroxylase from normal and cholesterol-fed rats. From these it appears that there is no critical involvement of the microsomal membrane in the activity of this enzyme, and also that its activation energy is unaffected by dietary cholesterol. Similar results were found with the other microsomal enzymes tested.

Other factors regulating HMG-CoA reductase

During the course of these experiments we were able to confirm both the well-known decrease in reductase activity occasioned by starvation and the equally familiar increase seen at midnight relative to midday. Neither situation, however, significantly altered Arrhenius-plot characteristics.

Time course of effects of cholesterol feeding on reductase activity

Fig. 3 shows reductase activity at various times after the commencement of cholesterol feeding. Despite the significant depression in activity seen at 2.25 h, there were no changes in Arrhenius-plot characteristics at this time. By 6 h after the onset of cholesterol feeding there was a slight shift in transition temperature, to 28.6°C.

The time course of uptake of dietary [^{14}C]-cholesterol in the various fractions of liver at 2.25 and 6 h after feeding is shown in Table 1. Only some 19–24% of the total homogenate [^{14}C]cholesterol was associated with the microsomal fraction, with the rest probably located in the plasma and mitochondrial membranes. By our calculations the

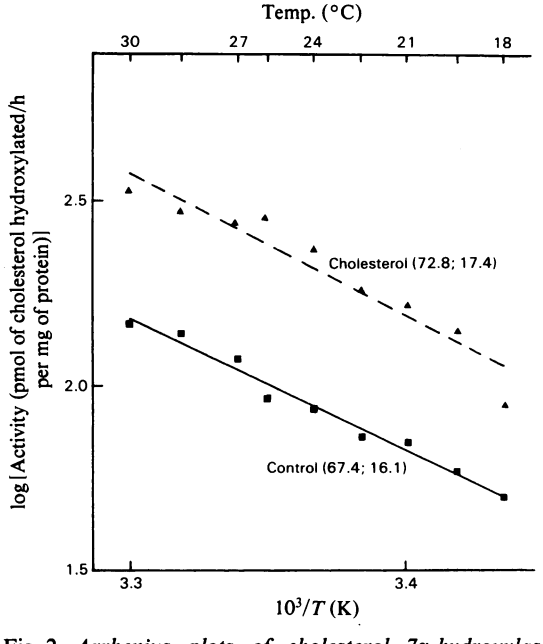

Fig. 2. *Arrhenius plots of cholesterol 7α-hydroxylase from cholesterol-fed (▲) and control (■) rats*
Microsomal cholesterol 7α-hydroxylase from rats fed on either a standard diet or one supplemented with cholesterol (5%, w/w; 2.5 days) was assayed at different temperatures, as described in the text. E_a values (kJ/mol; kcal/mol) are shown in parentheses.

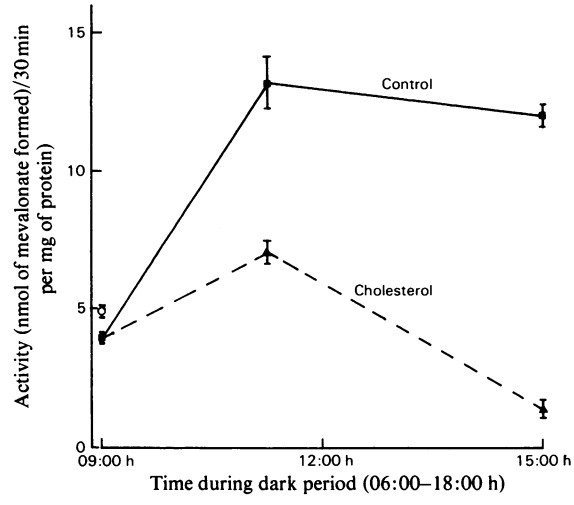

Fig. 3. *Hepatic HMG-CoA reductase activity at various times after the commencement of cholesterol feeding*
Rats that had been meal-fed (2 h/day) on a control diet for at least 14 days were offered either the control diet (■) or one supplemented with cholesterol (5%, w/w) (▲) 3 h after the commencement of the dark period in a 12 h:12 h lighting schedule. ○, Enzyme from rats fed on the control diet *ad libitum*. Each point is the mean ± S.E.M. for three determinations on pooled microsomal fractions from two rats.

Table 1. *Time course of uptake of dietary [^{14}C]cholesterol into the whole liver, the microsomal and the cytosolic fractions*
Experimental details are given in the Materials and methods section. Abbreviation: n.d., not detectable.

	Cholesterol uptake after feeding (μg/g wet wt. of liver)	
Liver fraction	At 2.25 h	At 6 h
Homogenate	70	708
Microsomal	13	166
Cytosolic	n.d.	n.d.

quantity of dietary cholesterol that accumulated in the liver microsomal fraction within 2.25h of cholesterol feeding was only small (less than a 3% increase). By 6h, however, the increase was of the order of 20–30%.

Discussion

The phase-transition characteristics shown for HMG-CoA reductase in the present paper, namely a definite break in the Arrhenius plot (of hepatic microsomal reductase activity from rats fed on a standard diet) at 27.5°C, with an activation energy of 57.1kJ/mol (13.6kcal/mol) above and 214.6kJ/mol (51.1kcal/mol) below this transition temperature, are similar to those reported for other membrane-bound enzymes (Fourcans & Jains, 1974). In view, however, of the several possible interpretations of a temperature discontinuity in an Arrhenius plot of enzyme activity (Dixon & Webb, 1964; Wynn-Williams, 1976; Woolley & Eibl, 1977; Silvius *et al.*, 1978), it would be most helpful if independent supporting evidence could be obtained that a lipid phase change in rat liver microsomal membranes does indeed occur at the particular transition temperature of the reductase plot. It should be noted, however, that any physicochemical probes (cf. Oseroff *et al.*, 1973) that might be used to detect such a change would undoubtedly measure the general or overall characteristics of the total membranes, whereas the reductase might be responding to changes solely in the lipids of its own micro-environment, which might well differ from those of the bulk membrane (Kimelberg & Papahadjopoulos, 1974).

The changes shown here in membrane-related characteristics of the reductase after cholesterol feeding confirm previous results from our laboratory (Sabine & James, 1976) and elsewhere (Mitropoulos and Venkatesan, 1977), with different assay techniques. Our present results have now significantly extended these earlier observations, in that membrane-mediated characteristics are not displayed by other microsomal enzymes, even when (as with cholesterol 7α-hydroxylase) gross activity is altered by dietary cholesterol, nor are they apparent in the physiological control of reductase activity by means other than cholesterol feeding (i.e. by starvation and by diurnal rhythm). Although these obervations do not fulfill our earlier expectations (Sabine & James, 1976) that membrane-mediated regulation might provide a common intracellular basis for a number of different controls over hepatic cholesterol biosynthesis, they do highlight the critical significance of this mechanism in our understanding of the defective control of HMG-CoA reductase observed in cancer, e.g. dietary feedback control is missing (Sabine, 1980), whereas diurnal rhythm is retained (Goldfarb & Pitot, 1971; Sabine *et al.*, 1972).

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