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Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in rat liver and Morris hepatomas 5123C, 9618A and 5123t.c.

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Characteristics of 3-hydroxy-3-methylglutaryl-CoA reductase from normal liver, Morris hepatomas 5123C, 5123t.c. and 9618A, and host liver were studied. Animals were fed on control and 5%-cholesterol diets. Microsomal membranes from all tissues were found to accumulate cholesterol after 3 days on the 5%-cholesterol diet. The enzyme of the tumours showed no feedback inhibition by dietary cholesterol, and that of host liver gave a variable response, whereas that of control liver was constantly inhibited by 90% or more. Arrhenius-plot analysis was conducted on the microsomal enzyme isolated from the various tissues. Control animals showed that the phase transition present at 27°C was removed when animals were fed on 5%-cholesterol diet for 12h. The hepatomas failed to show this change even after 3 days of 5%-cholesterol diet and a significant increase in microsomal cholesterol. This failure to remove the break in Arrhenius plots also occurred in host liver, even though enzyme inhibition occurred. The reason why hepatomas fail to regulate 3-hydroxy-3-methylglutaryl-CoA reductase activity in response to dietary cholesterol may be a decreased membrane–enzyme interaction.

Regulation of cholesterol biosynthesis in rat liver is controlled predominantly by the enzyme HMG-CoA reductase (EC 1.1.1.34), which catalyses the reduction of HMG-CoA to mevalonic acid. Changes in the rate of cholesterogenesis can be correlated with changes in activity of this enzyme (Rodwell *et al.*, 1976). A high-cholesterol diet results in a very much decreased activity of HMG-CoA reductase, a phenomenon referred to as dietary feedback control. The mechanism by which dietary cholesterol inhibits the enzyme's activity is still unknown, but several suggestions have been made, including activity modulation by a reversible phosphorylation of the enzyme protein (Gibson & Ingebritsen, 1978) and inhibition by various cholesterol derivatives (Gibbons *et al.*, 1980).

In addition, Sabine & James (1976) proposed that the changed fluidity of the microsomal membrane that results from an increased cholesterol content after cholesterol feeding may act as a regulator of enzyme activity. The work of Mitropoulos and co-workers (Mitropoulos & Venkatesan, 1977; Mitropoulos *et al.*, 1978*a*,*b*; 1980) has supported this idea of viscotropic control. They used digitonin precipitation of cholesterol from microsomal membranes, injection of mevalonic acid, injection of Triton WR 1339 and alteration of the saturation of membrane phospholipids by dietary lipid intake to alter the activity of HMG-CoA reductase, and linked this change in enzyme activity with modified temperature-induced kinetics of the enzyme.

With the possible exception of spontaneous hepatomas of C3H-A^{VY} mice, dietary feedback control of cholesterogenesis is absent from all hepatomas and leukaemic cells (Chen *et al.*, 1978). It has been suggested that this loss of regulation of cholesterol synthesis may play an important role in malignancy (Siperstein, 1970). In the light of the well-documented changes in the characteristics of membranes associated with the malignant state, the possibility of defective viscotropic control in the hepatomas was investigated. We have studied the effect of high-cholesterol diets on the cholesterol content and HMG-CoA reductase activity of three Morris transplantable hepatomas and the host livers. The hepatomas selected (5123C, 9618A and

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

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5123t.c.) demonstrate a range of growth rates and minimal deviation.

Materials and methods

Chemicals

[2-^{14}C]Mevalonic acid lactone (18 μCi/μmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. DL-[*Me*-^{3}H]HMG-CoA was obtained from New England Nuclear, Boston, MA, U.S.A. HMG-CoA was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., or synthesized by the method of Goldfarb & Pitot (1971), as modified by Sipat (1978). Cholesterol was obtained from Sigma. All other chemicals were analytical grade.

Animals and tumours

The animals used were mature male Buffalo-strain rats (200–300 g) supplied by the Central Animal Breeding House, University of Queensland, and the Waite Agricultural Research Institute, Adelaide. Morris hepatoma 5123t.c. was obtained from Professor H. Schrieber, University of Melbourne, and hepatomas 5123C and 9618A were from stocks maintained in the Waite Agricultural Research Institute. All tumours were maintained by intramuscular transplantation into both hind limbs.

Routinely, animals were fed on rat pellets or diet supplemented with 5% (w/w) cholesterol, plus water *ad libitum*, for 3 days before they were killed. All animals were maintained on a 12h-light/12h-dark regime for at least 2 weeks before experiments and were killed at the middle of the dark period, by a sharp blow to the head followed by decapitation.

Preparation of microsomal fraction

Livers and tumours were removed and placed in cold 0.01 M-potassium phosphate buffer (pH 7.6) containing 0.1 M-sucrose and 0.01 M-2-mercaptoethanol. The weighed tissues were cut into small pieces and washed to remove blood. The supernatant was decanted, and the tissues were homogenized with a Teflon/glass Potter–Elvehjem homogenizer and buffer 1 (10 mM-potassium phosphate, pH 7.6, 0.1 M-sucrose, 30 mM-EDTA, 50 mM-KCl and 5 mM-dithiothreitol; 2 ml/g of tissue). The homogenate was centrifuged at 15000 *g* for 20 min and the supernatant centrifuged at 105000 *g* for 75 min. The microsomal fraction was resuspended in buffer 1 at a protein concentration of approx. 5 mg/ml and kept on ice until assay.

HMG-CoA reductase assay

HMG-CoA reductase was assayed as described by Ackerman *et al.* (1977). The reaction mixture contained: microsomal protein (<300 μg); 3 mM-$NADP^+$; 30 mM-glucose 6-phosphate; 0.2 unit of glucose 6-phosphate dehydrogenase and 100 μM-DL-[*Me*-^{3}H]HMG-CoA (4.5 mCi/mmol) in 0.15 ml of buffer 1. Except where indicated, the reaction was started without preincubation by addition of HMG-CoA. After appropriate times, usually 30 min, the reaction was stopped with 0.1 ml of 4 M-HCl. To monitor extraction efficiency and act as a carrier, 0.01 ml of 0.05 mM-[2-^{14}C]mevalonic acid lactone (18 mCi/mmol) was added. After a further 30 min incubation, to allow lactonization, mevalonic acid lactone was isolated, and radioactivity was determined in a Packard liquid-scintillation spectrometer, by using a toluene-based scintillant.

Assays were linear over the time and protein concentrations used.

Other assays

Total cholesterol was determined by the method of Solow & Freeman (1970). Fluorescence was measured with an Aminco Spectrofluorimeter, with an excitation wavelength of 530 nm and an emission wavelength of 565 nm. Protein was measured by the method of Lowry *et al.* (1951), after precipitation with 5% trichloroacetic acid to remove dithiothreitol.

Results

A failure of uptake or storage of cholesterol has been suggested as an explanation for the defect in dietary feedback regulation of HMG-CoA reductase in hepatomas (Sabine, 1965; Harry *et al.*, 1971; Horton & Sabine, 1973). However, other workers have found uptake to be normal, or at least of a magnitude sufficient to have decreased the enzyme activity of normal liver (Horton *et al.*, 1973*a*,*b*; Calandra *et al.*, 1973). To ensure that the Morris hepatomas used in this study were taking up cholesterol, a 5%-cholesterol diet for 72 h was used and the microsomal cholesterol content was measured.

Table 1 gives the cholesterol content of the microsomal fraction from control livers, hepatoma 5123t.c. and host liver, all taken from animals fed on either a control or 5%-cholesterol diet. Individual animals were analysed and the data pooled. These data show that the cholesterol content of the microsomal fraction from all tissues (control liver, host liver and tumour) was significantly higher in cholesterol-fed animals ($P < 0.05$, <0.001 and <0.05 respectively). The cholesterol content in host liver was higher than in the controls ($P < 0.05$). This is possibly due to the cholesterol synthesized by the tumour accumulating in the liver.

Arrhenius-plot analysis of microsomal HMG-CoA reductase from animals fed on a control or 5%-cholesterol diet gave results similar to those obtained by other workers (Sabine & James, 1976;

Mitropoulos & Venkatesan, 1977; Sipat & Sabine, 1981). A normal diet resulted in a discontinuous Arrhenius plot over the region 19–37°C with a temperature transition (T_t) at 27°C and activation energies (E_a) of 72 and 134.3 kJ/mol, above and below the T_t respectively. A 5%-cholesterol diet decreased enzyme activity, and a linear plot was evident, with E_a 67.4 kJ/mol.

Fig. 1 shows the Arrhenius plots for microsomal enzyme for three Morris hepatomas (5123C,

Table 1. *Cholesterol contents of microsomal membranes from control liver, hepatoma 5123t.c. and host liver on normal and 5%-cholesterol diets*

Animals, diets, tissue preparation and cholesterol assays were as described in the Materials and methods section. The data represent means ± S.D. for assays of individual animals; the numbers of animals per group are shown in parentheses. The two tumours carried by each animal were pooled to give a single determination. Where specified, 5% cholesterol was fed *ad libitum* for 3 days before animals were killed.

Tissue	Diet	Cholesterol (μg/mg of protein)
Control liver (4)	Normal	15.6 ± 1.45
Control liver (4)	Cholesterol	21.8 ± 2.1
Host liver (6)	Normal	21.3 ± 4.2
Host liver (4)	Cholesterol	32.5 ± 7.15
Hepatoma 5123t.c. (6)	Normal	27.3 ± 3.8
Hepatoma 5123t.c. (4)	Cholesterol	35.9 ± 4.9

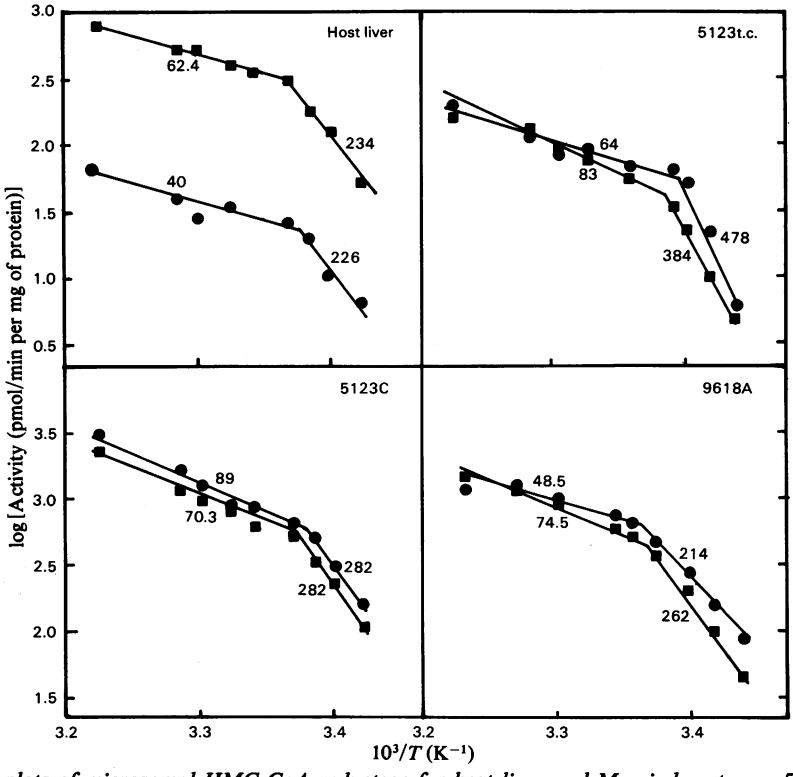

Fig. 1. *Arrhenius plots of microsomal HMG-CoA reductase for host liver and Morris hepatomas 5123C, 5123t.c. and 9618A from animals fed on control* (■) *and 5%-cholesterol diets* (●)

Enzyme and cholesterol assays are as described in the Materials and methods section. Groups of three animals were maintained on diets for 3 days before being killed, and the tumours and livers were pooled. Host livers from all tumour-bearing rats gave similar results; the data presented for host liver are for animals carrying hepatoma 5123C. The cholesterol contents (μg/mg of protein) for the microsomal fractions are as follows for control and cholesterol diets respectively: host liver, 27.0, 42.2; 5123C, 30.7, 47.8; 5123t.c., 15.7, 35.7; 9618A, 21.0, 42.5. The activation energies (kJ/mol) are indicated next to each line.

5123t.c. and 9618A) and host liver from animals carrying hepatoma 5123C fed on control or 5%-cholesterol diet for 3 days. Only one host liver is shown. The livers from animals carrying the other tumours gave similar results. All cholesterol-fed groups had increased microsomal cholesterol content.

Table 2 shows the activation energies and transition temperatures from a series of separate experiments. Where sufficient data were available, statistical analysis showed no differences in activation energies or transition temperatures for enzyme from the tumours and host livers isolated from animals fed on a normal or high-cholesterol diet. The T_t was lower than that observed in control livers. Considerable variation in the activation energies below the T_t occurred. This variation may be due to changes in membrane characteristics with tumour growth and with the amount of necrotic tissue present. Host-liver enzyme, although being inhibited by dietary cholesterol, failed to show the change from a broken to a linear Arrhenius plot observed in control animals. The transition temperature of 22–24°C is also much lower than has been reported for control animals and that we have found.

The duration of cholesterol feeding may change many membrane characteristics. Arrhenius plots of the enzyme from hepatoma 5123C and host liver after cholesterol feeding for 0 and 12h are compared in Fig. 2. Enzyme activity in the host liver was very low (0.187nmol/min per mg of protein, compared with control values of >0.35nmol/min per mg). However, dietary cholesterol was able to inhibit enzyme activity even further. The decreased activity in host liver again indicates the effects that the presence of a tumour has on cholesterol synthesis in that tissue. The Arrhenius-plot parameters of neither host-liver nor tumour enzyme were altered by cholesterol feeding for 12h.

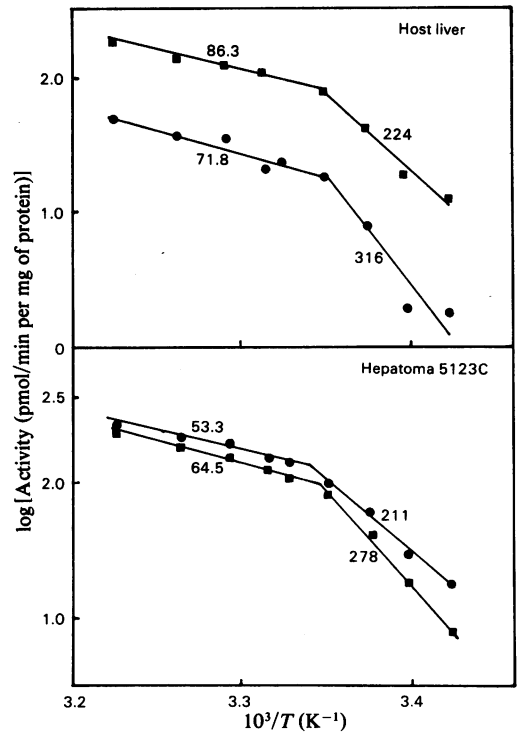

Fig. 2. *Arrhenius plots of microsomal HMG-CoA reductase from Morris hepatoma 5123C and host liver from animals fed on control (■) and 5%-cholesterol (●) diets for 12h*

Enzyme and cholesterol assays are as described in the Materials and methods section, except that cholesterol feeding was for 12h. Cholesterol contents (μg/mg of protein) are 17.2 and 21.3 for liver and 27.1 and 26.8 for hepatoma, for control and cholesterol diets respectively. Transition temperature for host-liver enzyme was 25.6°C on either diet and for the tumour enzyme 25.9 and 21.1°C for control and cholesterol diets respectively.

Discussion

The failure of hepatomas and precancerous liver to regulate cholesterol biosynthesis in response to dietary cholesterol is well documented and may be important in malignancy (Siperstein, 1970; Sabine, 1979). This defect is reflected in the failure of dietary cholesterol to inhibit the rate-controlling enzyme of cholesterol synthesis, namely HMG-CoA reductase. Hepatomas do not lack all control over cholesterol synthesis, since some show a diurnal rhythm, a feeding/starvation response and a response to an injection of Triton WR 1339 (Chen *et al.*, 1978). Further, George & Goldfarb (1980) noted that HMG-CoA reductase of Morris hepatoma 7800 retained an inhibitory response to intravenously administered mevalonic acid.

The suggestion that defective uptake of cholesterol from the blood may explain the lack of dietary feedback in hepatomas has stimulated considerable work. However, results have been inconsistent. Most workers have found that cholesterol ester content of the microsomal fraction is decreased in hepatomas as compared with normal liver (Sabine, 1965; Harry *et al.*, 1971; Horton & Sabine, 1973; Calandra *et al.*, 1973). However, the cholesteryl ester content of liver and hepatomas is a small fraction of the total cholesterol, and Mitropoulos *et al.* (1978*a*) have produced convincing evidence to show that it is the free cholesterol, rather then esterified cholesterol, that is responsible for feedback inhibition. The work of Bell *et al.* (1976), who found that in HTC cells cholesterol ester formation was not required for

Table 2. *Effect on dietary cholesterol on activation energy and transition temperatures of HMG-CoA reductase from control livers, hepatomas and host liver*

HMG-CoA reductase assays are as described in the Materials and methods section. For each experiment, three rats on each diet [normal (N) and 5%-cholesterol (C)] for 3 days were killed, and livers and tumours were pooled to give the microsomal suspension used for assays. The data for hepatoma 9618A was determined from duplicate assays from one group of rats. Other data represent means, with ranges, for two experiments or means ± S.D. for a minimum of three separate experiments.

Tissue	Diet	Activation energy (kJ/mol) Above T_t	Activation energy (kJ/mol) Below T_t	Transition temp. (T_t) (°C)
Control liver	N	70.5 (72,69)	149 (134.3, 163.2)	27.5
	C	91.6 (67.4, 115.9)		
Host liver	N	75.7 ± 8.2	250.2 ± 64.4	24.1 ± 0.29
	C	78.2 ± 15.1	272.4 ± 35.6	23.5 ± 0.44
Hepatoma 5123t.c.	N	79.9 ± 1.7	373.6 ± 30.1	23.6 ± 0.49
	C	72.4 ± 4.8	447.7 ± 58.2	23.0 ± 0.79
Hepatoma 5123C	N	70.9 ± 12.75	262.3 ± 19.9	23.9 ± 1.7
	C	84.9 (88.7, 81.2)	273.6 (281.6, 265.6)	24.4 (24.0, 24.8)
Hepatoma 9618A	N	74.5	262	24.0
	C	48.5	214	24.3

enzyme inhibition by a range of sterols, would support this concept. In the present study we measured the total cholesterol of the microsomal fraction and found that each of the three tumours tested accumulated cholesterol after the animals had been 72h on a high-cholesterol diet, but that the HMG-CoA reductase activity was not decreased.

The uptake of cholesterol by these tumours and its failure to decrease enzyme activity after 72h feeding indicates that dietary cholesterol itself does not inhibit enzyme activity or, more likely, that the cholesterol is unable to bind to the appropriate effector 'site', which, by an unknown mechanism, decreases enzyme activity. The concept of viscotropic control requires this site to be in the microenvironment of the membrane-bound enzyme.

The influence of membrane fluidity on enzyme activity is generally studied by Arrhenius-plot analysis. The abrupt change in the slope of the line in plots of enzyme activity against the reciprocal of temperature is considered to be due to an abrupt phase transition of the lipids of the supporting membrane, and this transition in turn is dependent on a number of factors, in particular the component phospholipids and their acyl chains and on the cholesterol content.

Studies on normal liver, host liver and hepatoma have shown that the change from broken to linear Arrhenius plots is not a concomitant to HMG-CoA reductase inhibition. The host liver enzyme is inhibited, but fails to show altered Arrhenius-plot characteristics. Sipat & Sabine (1981) have also shown that cholesterol feeding over short periods inhibits enzyme activity, but fails to change Arrhenius-plot parameters. This decrease in enzyme activity in the absence of Arrhenius-plot changes may be due to decreased enzyme protein or enzyme inactivation. The failure of hepatoma and host-liver enzyme to show the changes characteristic of normal liver could result from a decreased membrane–enzyme interaction or a failure of cholesterol to be located at the appropriate site in the membrane. Other studies (R. G. Gregg & P. A. Wilce, unpublished work), using fluorescence polarization of diphenylhexatriene, show that the microsomal fraction from hepatoma 5123C has an increased apparent microviscosity compared with those of liver. An effect of increased microviscosity would be increased exposure of the membrane proteins to the aqueous environments, or decreased protein–membrane interaction (Shinitzky & Barenholz, 1978). Thus, although some enzyme–membrane interaction is present in the hepatoma, as evidenced by the fact that Arrhenius plots do show a phase transition, the failure of the hepatoma to respond to dietary cholesterol may be due to a decrease or alteration in this interaction. We have shown here a lower T_t and an increased activation energy below the break for the hepatoma enzyme compared with that of normal liver, and these changes may be due to the well-documented alterations in tumour lipid composition (Ruggieri & Fallani, 1979*a*,*b*).

Host liver also fails to respond to dietary cholesterol as expected, as a phase transition is still

evident after cholesterol feeding. This indicates that the presence of the tumour has effects which modify the response of host liver HMG-CoA reductase to dietary cholesterol. Effects of transplanted hepatomas or other host-liver characteristics have been reported. George & Goldfarb (1980) found that an injection of mevalonate into animals carrying hepatoma 7800 failed to inhibit the host-liver enzyme, although this treatment results in a 90% decrease in enzyme activity in control livers. Further, and with particular relevance to viscotropic control, the lipid composition of host liver has been shown to be altered compared with control liver, in animals carrying hepatoma 5123C and Yoshida hepatoma AH130 (Ruggieri & Fallani, 1979*a*,*b*). These changes may be responsible for the altered response of the host-liver enzyme to dietary cholesterol and intravenous injection of mevalonic acid.

In conclusion, the lack of feedback inhibition of HMG-CoA reductase in hepatomas could be due to a defect in the enzyme–membrane interaction. These changes are also seen to some extent in host liver. This conclusion must remain speculative in the absence of any direct evidence as to the physical state of the lipid microenvironment of the enzyme under various experimental conditions.

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