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INHIBITION OF HEPATIC *S*-3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE AND *IN VIVO* RATES OF LIPOGENESIS BY A MIXTURE OF PURE CYCLIC MONOTERPENES

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Abstract—A proprietary mixture of pure cyclic monoterpenes (Rowachol) inhibited hepatic HMGCoA reductase by 50–60% when measured 17 hr after the oral administration of a single dose to rats. The extent of this inhibition was independent of the normal activity range of HMGCoA reductase within its diurnal cycle and the same inhibition (65%) was found in 24 hr starved animals where the control reductase activity was less than 20% that of normal fed rats. *De novo* sterol and fatty acid synthesis in intact, fed rats was measured by incorporation of ^3H from injected H_2O. In rats treated with Rowachol the rate of sterol synthesis *in vivo* was inhibited 52% in liver and 44% in testis with no significant effects in other tissues. The synthesis of non sterol (isoprenoid) compounds in testis was unaffected and the inhibition of sterol synthesis in this tissue probably reflects decreased acquisition of newly synthesized material from liver rather than any effect on the endogenous process. In the same animals the rate of fatty acid synthesis was inhibited 55% in liver. These effects were associated with a significant depletion of liver glycogen which may account for the reduction in rate of fatty acid synthesis. We conclude that the reported cholelitholytic action of monoterpenes is associated with the physiological inhibition of hepatic sterol synthesis mediated by decreased HMGCoA reductase activity.

The formation of cholesterol gallstones in man [1] and in laboratory animals [2] is associated with increased activity of hepatic *S*-3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA)† reductase, the rate limiting enzyme for cholesterol synthesis in this tissue [3]. In man, chronic administration of Rowachol, a proprietary choleretic containing the following pure monoterpenes, menthol 32% (w/w), pinene 17% (w/w), menthone 6% (w/w), borneol 5% (w/w), camphene 5% (w/w) and cineole 2% (w/w) in olive oil, can cause gallstone dissolution [4, 5] and causes a 60% inhibition of hepatic HMGCoA reductase [6]. Previously [7] we have shown that a single dose of Rowachol to rats causes the same (60%) inhibition of hepatic HMGCoA reductase when measured at the peak of its diurnal activity and therefore this system might be used as a model to investigate the mechanism of the cholelitholytic action of Rowachol in man. However, there is no evidence that diurnal variations occur in human hepatic HMGCoA reductase and in this paper we have investigated whether the monoterpene inhibition of this enzyme in rats was (a) independent of the normal diurnal variation in activity and (b) the cause of any inhibition in the rate of cholesterol synthesis when measured *in vivo* in liver and other tissues.

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† Abbreviations used: HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A; DBED, dibenzylethylenediamine; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

MATERIALS AND METHODS

^3H_2O, [3-^{14}-C]HMGCoA, [4-^{14}C]cholesterol and [1-^{14}C]oleic acid, were purchased from Amersham International Ltd (Amersham, U.K.). [5-^{3}H]-Mevalonic acid (DBED salt) was purchased from New England Nuclear (Boston, MA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) $NADP^+$ and glucose assay kits were obtained from Boehringer (Mannheim, F.R.G.). Aluminium backed silica gel precoated sheets for thin-layer chromatography were obtained from Merck (Darmstadt, F.R.G.). Fisofluor scintillation fluid was from Fisons Ltd (Loughborough, U.K.). Rowachol was supplied by Rowa Ltd (Bantry, Ireland). The composition of Rowachol was as follows: menthol 32% (w/w), pinene 17% (w/w), menthone 6% (w/w), borneol 5% (w/w), camphene 5% (w/w), cineole 2% (w/w) and olive oil (B.P. and U.S.P.) 33% (w/w).

Male Wistar rats (180–220 g) were subjected to normal lighting (lit from 08.00 to 20.00 hours) or reversed lighting (lit from 15.00 to 03.00 hours) and unless specified were fed 41B pellet diet *ad libitum*. Animals were dosed with Rowachol liquid or olive oil (2 ml, 6 mmole terpene mixture per kg body wt) by stomach tube at 17.00 hours and killed 17–19 hr later.

In vivo rates of lipid synthesis were determined by measurement of ^3H incorporation into the appropriate tissue lipids following injection with ^3H_2O [8, 9]. Approximately 17 hr after dosing with terpene mixture of olive oil, animals were injected intraper-

itoneally with ^3H_2O (50 mCi/kg body wt) in 0.9% (w/v) saline. One hour later the animals were decapitated, blood was collected, allowed to clot at 0° and serum obtained. Livers and other tissues were rapidly removed from the carcass, chilled to 0°, weighed and samples of up to 1 g were saponified for 2–3 hr at 70° with 5 ml of 15% (w/v) KOH in 90% (w/v) ethanol. At this stage recovery standards ([4-^{14}C]cholesterol, 2000 dpm and [1-^{14}C]oleic acid, 4000 dpm) were added to each tube. Non-saponifiable lipids were extracted into petroleum ether (boiling range 40–60°) which was washed extensively with water, dried in a stream of N_2 and finally *in vacuo* over P_2O_5 to remove the last traces of water. This procedure successfully prevented any contamination of lipid fractions with ^3H_2O. β-Hydroxysterols were isolated from the non-saponifiable lipid fraction by digitonin precipitation [10]. The dried lipid fraction was dissolved in 5 ml acetone:ethanol (1:1, v/v) acidified with one drop of 10% (w/v) acetic acid and the digitonide precipitated by 2 ml of 0.5% (w/v) digitonin in 50% (v/v) ethanol. Before isolating the precipitated material the amount of sterol digitonide was estimated from the absorbance due to light scattering of the suspension measured at 800 nm using cholesterol digitonide as a standard. This method gave a linear relationship between mass of cholesterol and light absorbance over the range 10–300 μg of cholesterol. The digitonides were subsequently collected by low speed centrifugation and washed with acetone:diethylether (1:2, v/v) followed by diethylether. The precipitate was then dried under N_2 and finally dissolved in 1 ml of methanol and mixed with 10 ml of Fisofluor for determination of radioactivity due to ^3H and ^{14}C. In some experiments the ^3H incorporation into total non-saponifiable lipid was determined without further purification as digitonide. We found that digitonin precipitated sterol accounted for 80–88% of the non-saponifiable lipid in liver, intestine and adrenal and 60–66% in kidney and testis. Analysis of liver non-saponifiable lipid fraction by thin-layer chromatography on silica gel in a solvent system of cyclohexane:ethyl acetate (6:4, v/v) showed that [^3H]cholesterol accounted for 83% of the total radioactive products, equivalent to 94% of the digitonin precipitable sterol in liver.

^3H-Labelled fatty acids were extracted into petroleum ether from the saponifiable fraction after acidification of the alkaline aqueous medium with 50% (v/v) H_2SO_4. The fatty acid fraction was extensively washed with water, dried with a stream of N_2 and finally dehydrated *in vacuo* over P_2O_5. After drying, samples were taken for determination of radioactivity due to ^3H and ^{14}C as described above. The ^3H radioactivity in non-saponifiable lipid, sterol and fatty acid was corrected for recovery using the ^{14}C counts: recoveries averaged 80, 76 and 70%, respectively. Incorporation of radioactivity from ^3H_2O into lipid fractions was found to be linear with time (from 15 min) in liver and extrahepatic tissues. In order to convert this ^3H radioactivity into μg atom of H incorporated, samples of serum water were counted to obtain a measure of the body water specific radioactivity. Jeske and Dietschy [9] have shown that equilibration between injected ^3H_2O and body water occurs rapidly and that serum water specific radioactivity accurately represents that in body tissues from 5 min after injection. Rates of lipid synthesis were expressed as μg atom of H incorporated into the appropriate lipid fraction/hr per g wet wt of tissue (or per ml of serum in that case).

For determination of hepatic glycogen content, liver portions (approx 1 g) were rapidly frozen in liquid N_2, weighed and powdered while frozen and finally blended with 6 ml 5% (w/v) $HClO_4$. Glycogen was isolated from this homogenate by the method of Good *et al.* [11]. Glycogen was hydrolysed to glucose by heating with 1 M H_2SO_4 at 100° for 1 hr. After neutralizing, glucose content was determined using the glucose oxidase/peroxidase method [12].

Hepatic microsomal fraction was prepared from livers homogenized in 4 vol. of 0.3 M sucrose, 25 mM mercaptoethanol, 100 mM EDTA, pH 7. The supernatant fraction obtained after spinning at 12,000 *g* for 15 min was centrifuged at 100,000 *g* for 1 hr to obtain a microsomal pellet. This fraction was washed by suspension in the homogenization buffer and recentrifugation at 100,000 *g* for 1 hr before being finally suspended in 100 mM phosphate buffer, pH 7.5, containing 10 mM EDTA and 5 mM DTT to a final concentration of 20 mg protein/ml.

HMGCoA reductase was assayed in microsomal suspensions at 37° essentially as described in [13] using a preincubation of 10 min and starting the reaction with [3-^{14}C]HMGCoA. The 0.15 ml incubation system contained RS-[3-^{14}C]HMGCoA (3000 dpm/nmole), 100 μM; $NADP^+$, 2.5 mM; glucose-6-phosphate, 20 mM; glucose-6-phosphate dehydrogenase, 3 U/ml; DTT, 5 mM; EDTA, 10 mM; 50–750 μg of microsomal protein and potassium phosphate, pH 7.5, 100 mM. The reaction was terminated after 15–30 min with 10 μl 10 M HCl and [5-^{3}H]mevalonate (40,000 dpm) was added as recovery standard. After lactonization the mevalonolactone was separated by thin-layer chromatography as described by Shapiro *et al.* [14] and counted for ^{14}C and ^3H radioactivity in Fisofluor. Formation of [^{14}C]mevalonate under these conditions was linear with time and protein and product recovery averaged 90%. The enzyme activity was expressed as pmole of mevalonate formed/min per mg of microsomal protein. Yield of microsomal protein was unaffected by terpene pretreatment of animals.

The protein content of microsomal suspensions was determined by the microbiuret method [15] after initial precipitation by 10% (w/v) trichloroacetic acid and subsequent dissolution in 1 M NaOH.

RESULTS

In the rat hepatic HMGCoA reductase shows considerable diurnal variation in activity [16] being maximal around the mid-point of the dark period and minimal at the middle of the light period. This variation is reflected in the results of Table 1 where animals were killed at the 7th hour of the dark period (D7) or the 2nd hour of the light period (L2). The ratio of reductase activity at D7 to that at L2 was 2.8 but at both times a single pretreatment with Rowachol resulted in 50–60% inhibition ($P < 0.01$) of the enzyme activity. Thus, the extent of the Rowachol mediated inhibition was not affected by

Table 1. Effect of diurnal variation or starvation on terpene inhibition of hepatic HMGCoA reductase

Diet	Animals killed at	HMGCoA reductase activity (pmoles/min/mg) Controls	Rowachol	Inhibition (%)
Normal	D7	1080 ± 300 (10)	400 ± 120* (6)	63
Normal	L2	380 ± 20 (3)	190 ± 40* (3)	50
Starved (24 hr)	L2	74 ± 33 (6)	33 ± 10† (6)	65

Rats were maintained on a 12 hr light and dark schedule and killed at the 7th hour of dark (D7) or the 2nd hour of light (L2) 17 hr after being incubated with olive oil (controls) or terpene mixture (Rowachol). HMGCoA reductase was assayed as described in Materials and Methods. Results are means ± S.D. with the number of animals in parentheses.

* $P < 0.01$.

† $P < 0.05$ with respect to controls.

the normal diurnal variation of HMGCoA reductase, neither did Rowachol pretreatment affect the diurnal rhythm itself since the ratio of activity at D7 to that at L2 after treatment was 2.1, not significantly altered from controls. To determine that this inhibition was not due to decreased food intake, the weights of stomachs plus contents were determined post mortem at L2 in six terpene treated and control rats. Rowachol significantly increased ($P < 0.05$) the weights of stomachs and contents by 90% (11.4 ± 3.5 g) compared to control values of 6.0 ± 2.7 g. The possibility remained that the monoterpene mixture might prevent digestion and absorption of food resulting in a decreased hepatic reductase activity. To test this a group of rats were starved for 24 hr before Rowachol administration. In control animals (Table 1) starvation resulted in an 80% decrease ($P < 0.01$) in HMGCoA reductase activity but Rowachol treatment of starved rats gave a further inhibition of 65% ($P < 0.05$). In summary the effect of monoterpene (Rowachol) pretreatment of rats was to give between 50 and 65% inhibition of HMGCoA reductase independent of the initial control enzyme activity over a 14.6-fold range.

To determine whether these changes in hepatic HMGCoA reductase activity were reflected in the rate of sterol synthesis *in vivo* this process was measured by incorporation of ^3H from ^3H_2O injected into control and terpene treated rats. This method is well established for measurement of fatty acid synthesis [8] but has been little used for sterol synthesis until recently [9, 17]. In order to establish whether the activity variation observed in HMGCoA reductase activity in animals at D7 and L2 (Table 1) was reflected in the rates of *in vivo* sterol synthesis, control rats were killed at these times, 1 hr after injection with ^3H_2O, and tissue lipid fractions isolated and ^3H incorporation measured (Table 2). Significant diurnal variation in the rate of non-saponifiable lipid synthesis was observed in liver and adrenal but not in other tissues (data not shown). The ratio of rates at D7 to rates at L2 was 2.0 in liver and 1.8 in adrenals. The former value compares well with the ratio of HMGCoA reductase activities in liver at the same times (Table 1) while the ratio of rates in the adrenals is in agreement with the published activity ratio of HMGCoA reductase in adrenals of rats at D7 and L2 [18]. The results imply that, measured at these times, the activity of HMGCoA reductase in liver and adrenal appears to closely reflect the flux through sterol synthesis. Table 2 also showed that the rate of fatty acid synthesis in rat liver (but not other tissues) was elevated in the dark period in accord with similar findings for the mouse [19].

Seventeen hours after rats were dosed with Rowachol the rate of synthesis of digitonin precipitable sterol *in vivo* (Table 3) was inhibited 57% ($P < 0.01$) in the liver and 44% in testis ($P < 0.05$).

Table 2. *In vivo* rates of synthesis of non-saponifiable lipid and fatty acid measured in rats at different stages of the lighting cycle

Tissue	Rate of lipid synthesis *in vivo* (μg atom H/hr/g) Non-saponifiable lipid D7	L2	Fatty acid D7	L2
Liver	11.8 ± 1.6 (5)	5.9 ± 1.7* (6)	64.1 (2)	21.2 ± 4.4 (5)
Adrenal	13.4 ± 3.0 (4)	7.5 ± 3.5† (4)	13.7 (2)	15.1 ± 6.8 (3)

Normal fed rats were maintained on a 12 hr light and dark schedule and killed at the 7th hour of dark (D7) or 2nd hour of light (L2). *In vivo* rates of synthesis of non-saponifiable lipid and fatty acid were determined as described in Materials and Methods and are expressed as μg atom H incorporated/hr per g wet wt ± S.D. with the number of animals in parentheses.

* $P < 0.01$.

† $P < 0.05$ with respect to rate at D7.

Table 3. The effect of Rowachol on sterol synthesis *in vivo*

Tissue	Controls (μg atom H/hr/g or ml)	Rowachol (μg atom H/hr/g or ml)
Liver	5.22 ± 1.44 (6)	2.52 ± 0.69* (6)
Adrenal	6.51 ± 3.07 (4)	5.15 ± 2.95 (4)
Kidney	0.82 ± 0.36 (6)	0.71 ± 0.24 (6)
Testis	0.32 ± 0.09 (6)	0.19 ± 0.05† (6)
Intestine	4.42 ± 2.47 (4)	2.73 ± 1.42 (4)
Serum	0.73 ± 0.24 (3)	1.06 ± 0.46 (3)

Rats were incubated with olive oil (controls) or the terpene mixture in olive oil (Rowachol) and 16–17 hr later injected with ^3H_2O. They were killed 1 hr later at L2 (see Table 1) and the rate of sterol synthesis (as digitonin precipitable sterol) determined in various tissues as described in Materials and Methods. Rates are expressed as μg atom H incorporated/hr per g wet wt of tissue or per ml of serum ± S.D. with the number of animals in parentheses.

* $P < 0.01$.

† $P < 0.05$ with respect to controls.

Table 5. The effect of Rowachol treatment on fatty acid synthesis *in vivo*

Tissue	Controls (μg atom H/h/g or ml)	Rowachol (μg atom H/h/g or ml)
Liver	21.2 ± 4.4 (5)	9.6 ± 2.6* (5)
Adrenal	15.1 ± 6.8 (3)	12.0 ± 6.1 (3)
Kidney	4.6 ± 1.9 (3)	2.7 ± 1.6 (3)
Testis	3.6 ± 1.9 (5)	3.1 ± 1.7 (5)
Intestine	7.5 (2)	5.5 (2)
Serum	2.1 ± 1.3 (3)	5.7 ± 4.5 (3)

Rats were incubated with olive oil (controls) or the terpene mixture in olive oil (Rowachol) and 16–17 hr later injected with ^3H_2O. They were killed 1 hr later at L2 (see Table 1) and the rate of fatty acid synthesis determined in various tissues as described in Materials and Methods. Rates are expressed as μg atom H incorporated/hr per g wet wt of tissue or per ml of serum ± S.D. with the number of animals in parentheses.

* $P < 0.01$ with respect to controls.

Inhibition did not reach statistical significance in other tissues and did not occur in serum. In no tissue did monoterpene treatment alter the total sterol content. There was evidence that Rowachol treatment altered the balance of the overall pathway in some tissues. This was indicated by the ratio of the rate of synthesis of digitonin precipitatable sterol to that of total non-saponifiable lipid (Table 4). While this ratio was unaffected in liver and intestine, it was increased by 25% ($P < 0.001$) in kidney and decreased by 35% ($P < 0.001$) in testis. This difference was due to a 65% inhibition of non sterol (isoprenoid) synthesis in kidney where terpene treatment reduced the rate from 0.43 ± 0.17 (6) to 0.15 ± 0.05 (5) μg atom H/hr per g ($P < 0.01$); but in testis non sterol (isoprenoid) synthesis was unaffected, terpene treatment causing a specific inhibition of sterol (digitonin precipitable) only.

In addition to the effect on the rate of sterol synthesis, treatment with Rowachol significantly inhibited the synthesis of fatty acids *in vivo* in liver (Table 5). Effects on other tissues were not significant.

During these investigations it became apparent that a single dose of Rowachol significantly lowered liver weights when measured in normally fed animals at L2. The drop in liver weight (Table 6) was accompanied by a 66% ($P < 0.05$) decrease in liver glycogen glucose content in fed animals. In 24 hr starved animals the very low level of glycogen glucose found in control livers was not significantly altered when they were treated with Rowachol.

Table 4. Effect of Rowachol treatment on the relative rate of sterol synthesis to total non-saponifiable lipid synthesis

Tissue	Rate of sterol synthesis / Rate of non-saponifiable lipid synthesis: Controls	Rowachol
Liver	0.88 ± 0.09 (6)	0.86 ± 0.11 (6)
Kidney	0.66 ± 0.002 (6)	0.83 ± 0.003* (5)
Testis	0.60 ± 0.007 (6)	0.39 ± 0.004* (5)
Intestine	0.79 ± 0.07 (3)	0.89 ± 0.10 (3)

Rates of sterol synthesis and total non-saponifiable lipid synthesis were determined by ^3H_2O injection after pretreating animals with olive oil (controls) or Rowachol. Rats were killed at L2 (see Table 1). Results are expressed as ratios of rates of sterol synthesis to rates of total non-saponifiable lipid synthesis in each tissue ± S.D. with the number of animals in parentheses.

* $P < 0.001$ with respect to controls.

DISCUSSION

The inhibition of hepatic HMGCoA reductase activity following a single treatment with Rowachol (Table 1) is shown by this study to cause an identical percentage inhibition in the rate of sterol and isoprenoid synthesis in liver (Tables 3 and 4) when measured *in vivo* by the ^3H_2O method. The only other tissue to show significant inhibition of sterol synthesis *in vivo* was testis, although non sterol (isoprenoid) synthesis in kidney was significantly reduced by the monoterpene mixture. However, two pieces of evidence indicate that the newly synthesized sterol found in testis was probably derived from liver via transport on plasma lipoproteins. Firstly, the ratio of the rate of sterol synthesis in testis to the rate of sterol accumulation in serum (Table 3) was <1 in both control and Rowachol treated animals; secondly the rate of non sterol (isoprenoid) synthesis in testis was quite unaffected by Rowachol treatment, unlike that in kidney. Potter *et al.* [20] have shown that synthesis of dolichol (a non sterol isoprenoid) is particularly elevated in relation to cholesterol synthesis in normal adult mouse testes and this high rate of isoprenoid synthesis is required for spermatogenesis. It is therefore probable that this process would be independent of hepatic influence and would be maintained in spite of reduction of sterol synthesis in liver.

Although the processes of hepatic sterol and fatty acid synthesis are linked via their subsequent assem-

Table 6. The effect of Rowachol treatment on liver weight and liver glycogen content

Diet	Liver weight (g/100 g body wt)		Liver glycogen glucose (μmoles/g wet wt)	
	Controls	Rowachol	Controls	Rowachol
Normal	4.4 ± 0.3 (6)	3.7 ± 0.4* (6)	287.6 ± 69.7 (3)	96.8 ± 27.4† (3)
Starved (24 hr)	3.2 ± 0.2 (6)	3.2 ± 0.2 (6)	1.6 ± 0.7 (3)	0.4 ± 0.2 (3)

Rats were allowed food *ad libitum* or were starved 24 hr before incubation with olive oil (controls) or the terpene mixture in olive oil (Rowachol). They were killed at L2 (see Table 1) and their livers frozen in liquid N_2 for glycogen determination. See Materials and Methods for further details. Results are means ± S.D. with the number of animals in parentheses.

* $P < 0.01$.

† $P < 0.05$.

bly into very low density lipoprotein [21], the effect of Rowachol on fatty acid synthesis in liver (Table 5) is unlikely to be due to inhibition of lipoprotein assembly or release since accumulation of newly synthesized sterol or fatty acid in serum (Tables 3 and 5) was not affected by Rowachol. The massive depletion of liver glycogen (Table 6) following Rowachol treatment of fed rats (amounting to about 1.9 mmoles of glucose/rat) could account for the inhibition of fatty acid synthesis *in vivo* because liver glycogen has been shown [22] to be the major precursor for *de novo* fatty acid synthesis in the postprandial state. The mechanism for the glycogen depletion is not clear but it is known that menthol (the major constituent of Rowachol) is rapidly excreted as the glucuronide [23], the glucose component of which could be derived from liver glycogen.

The monoterpene induced decrease in hepatic HMGCoA reductase activity was not mediated by the effect on liver glycogen since Rowachol still caused inhibition after 24 hr of starvation during which period the hepatic glycogen stores in control animals were depleted by more than 99% (Table 6). This study has demonstrated that the mechanism of Rowachol inhibition of hepatic HMGCoA reductase is independent of those factors that mediate the changes of this enzyme's activity either during the diurnal cycle or after food deprivation. This implies that the mechanism of action is unlikely to involve hormones such as insulin, glucocorticoids, triiodothyronine, and glucagon. In this respect the inhibitory action of monoterpenes resembles that caused by feeding cholesterol [24] or its oxygenated derivatives [25] and it is possible that the cyclic monoterpenes in Rowachol could act as simple analogues of these inhibitory sterols.

In conclusion, Rowachol, an effective agent for cholesterol gallstone dissolution in man, can inhibit sterol synthesis *in vivo* by specifically decreasing hepatic HMGCoA reductase activity in a manner independent of the normal hormonal control of this enzyme. The cholelitholytic action of this monoterpene mixture could thus be explained by its inhibition *in vivo* of hepatic lipogenesis, especially cholesterol synthesis.

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