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IN VIVO INHIBITION OF HEPATIC LIPOGENESIS IN THE RAT BY CYCLANDELATE (3,3′,5-TRIMETHYLCYCLOHEXANYLMANDELATE)

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Abstract—Rates of hepatic lipogenesis were measured *in vivo* in rats by incorporation into lipids of [^{3}H] from injected [^{3}H]H_2O 17 hr after a single oral dose of cyclandelate (3,3′,5-trimethylcyclohexanylmandelate, a vasoactive substance). Cyclandelate administration resulted in a significant inhibition (40–60%) of both sterol and fatty acid synthesis in the livers which was independent of the 3.2-fold diurnal variation in the rates of hepatic sterol and fatty acid synthesis. The inhibition of accumulation of newly synthesized fatty acid in intestine also reached statistical significance. The accumulation of newly synthesized sterol was significantly depressed in serum but did not result in any change in the concentration of serum total cholesterol. These results are interpreted in terms of the inhibitory effect of cyclandelate on hepatic 3-hydroxy-3-methylglutaryl-CoA reductase previously reported by us (*Biochem. Pharmac.* **32**, 649, 1983).

In a previous investigation [1] we have shown that cyclandelate (3,3′5-trimethylcyclohexanylmandelate, a vasoactive substance) causes the specific inhibition of hepatic 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase 17 hr after a single oral dose given to rats. Although HMG-CoA reductase is generally accepted as the rate limiting enzyme of sterol synthesis, its activity does not always parallel rates of sterol synthesis in liver [2, 3] and its inhibition may not necessarily imply any impact on sterol synthesis. To investigate the possible inhibitory effects of cyclandelate on sterol synthesis *in vivo*, we measured [^{3}H] incorporation into lipid following injection of [^{3}H]H_2O. The technique relies on the incorporation of [^{3}H] into sterols, isoprenoid compounds and fatty acids at a number of specific enzymic steps in the synthesis of these compounds [4–6]. The use of [^{3}H]H_2O has many advantages over other radioactive lipid precursors: it rapidly equilibrates throughout the body, crosses biological membranes easily, is not significantly diluted by metabolically generated H_2O, does not disturb the metabolism of the animal on injection and does not have to be extensively metabolized prior to incorporation into lipid [7, 8]. However, in common with other radioactive precursors used to measure *in vivo* rates of lipogenesis, this technique only gives unequivocal information in those tissues where endogenous synthesis is substantially greater than influx of radioactive lipid from the plasma compartment. This paper examines the effect of a single dose of the vasoactive substance cyclandelate (Cyclospasmol®, Gist Brocades N.V., Delft, The Netherlands) on rates of lipogenesis *in vivo* in liver and the accumulation of lipid in some extrahepatic tissues of the rat.

MATERIALS AND METHODS

[^{3}H]H_2O, [4-^{14}C]cholesterol and [1-^{14}C]oleic acid were purchased from Amersham International Ltd., Amersham, Bucks, U.K. Cholesterol assay kit (C system) was obtained from Boehringer, Mannheim, West Germany. Fisofluor scintillation fluid was from Fisons Ltd., U.K. Cyclandelate (3,3′,5-trimethylcyclo-hexanylmandelate) was supplied by Brocades (GB) Ltd., West Byfleet, Surrey, U.K.

Male Wistar rats (220–250 g) were subjected to normal lighting (from 08.00 to 20.00) or reversed lighting (from 15.00 to 03.00) and were fed 41B pellet diet *ad libitum*. Animals were acclimatized to their lighting schedules for 14 days before use at the weight stated. They were given cyclandelate in olive oil (4 ml/kg body wt) by gavage at a dose of 3 mmole/kg body wt and controls were given the appropriate volume of olive oil. Rats were dosed at 17.00 and killed 17–19 hr later. Administration of the drug did not prevent normal feeding and weights of stomachs plus contents *post mortem* were not lower in experimental groups than in controls.

In vivo rates of hepatic lipid synthesis and extrahepatic lipid accumulation were determined by measurement of [^{3}H] incorporation into the appropriate tissue lipids following injection with [^{3}H]H_2O. This technique has been shown to measure true rates of lipogenesis *in vivo* in liver and intestine [6, 9–11]. The appearance of [^{3}H]lipid in other tissues represents the sum of endogenous synthesis and uptake from the plasma. Plasma [^{3}H]lipid is derived from liver and intestine [9]. Approximately 17 hr after dosing with cyclandelate or olive oil, animals were

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injected i.p. with $[^3H]H_2O$ (50 mCi/kg body wt) in 0.9% (w/v) saline. One hour later the rats were decapitated, blood was collected, allowed to clot at 0° and serum obtained. A 1 ml portion of the serum was further fractionated by precipitation of apo-B containing lipoproteins with heparin and $MnCl_2$ [12] (final concentrations 33.3 units/ml and 92 mM, respectively). The precipitate and supernatant were collected after centrifugation at 15,000 *g* for 5 min. Liver and other tissues were rapidly removed from the carcass and weighed and chilled to 0°. Portions of tissues (approx 1 g maximum), samples of serum and of serum fractions were then saponified for 2–3 hr at 70° with 4 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. This procedure successfully prevented any contamination of lipid fractions with $[^3H]H_2O$. β-Hydroxysterols (of which > 90% was cholesterol) were isolated from the non-saponifiable lipid fraction by digitonin precipitation [13]. The dried lipid fraction was dissolved in 5 ml acetone:ethanol (1:1 v/v) acidified with 1 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. 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 $[^{14}C]$.

$[^3H]$ 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 $[^{14}C]$ in Fisofluor as described above. The $[^3H]$ radioactivity in sterol and fatty acid was corrected for recovery using the $[^{14}C]$ standards: recoveries averaged 75 and 65%, respectively. In previous work, we [14] and others [8, 10] have found that incorporation of radioactivity from $[^3H]H_2O$ into lipid fractions was 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 [8] have shown that equilibration between injected $[^3H]H_2O$ and body water occurs rapidly and that serum water specific radioactivity accurately represents that in body tissues from 5 min after injection. Furthermore, Turley *et al.* [9] have shown that the small amount of blood trapped in body tissues does not contribute significantly to the total intracellular $[^3H]$lipid of the tissues. Rates of lipogenesis and lipid accumulation were expressed as μg atom of H incorporated into the appropriate lipid fraction/hr per g wet weight of tissue (or per ml of serum).

The total (free plus esterified) cholesterol content of serum or serum fraction after precipitation with heparin-Mn^{2+} was determined enzymatically (Boehringer C system kit).

Statistical significance testing used Student's *t*-test. Lines were fitted to graphical data by least-squares linear regression analysis.

RESULTS AND DISCUSSION

Sterol synthesis and $[^3H]$*sterol accumulation* in vivo

The *in vivo* rates of sterol synthesis determined in rats at the seventh hour of the dark period (D7) or the second hour of the light period (L2) are given in Table 1. Rats were dosed with cyclandelate (in olive oil) or the olive oil vehicle only (controls) 17 hr before $[^3H]H_2O$ injection. In the controls there was a significant difference ($P < 0.05$) in rates of $[^3H]$ appearance in the sterol fraction at the two times studied in liver, serum, kidney and adrenals, the values at D7 being on average 2.7 times those at L2. In liver the ratio of rates at D7 compared with that at L2 was 2.5 while the ratio of hepatic microsomal HMG-CoA reductase activities determined in similarly treated rats killed at the same times in the diurnal cycle was 2.4–3.8 [1]. In finding a significant diurnal variation in hepatic sterol synthesis rate *in*

Table 1. The effect of cyclandelate on sterol synthesis and accumulation rates *in vivo*

Tissue	Rates measured at D7		Rates measured at L2	
	Controls	Cyclandelate	Controls	Cyclandelate
	(μg atom H/hr/g or ml)			
Liver	11.55 ± 0.65(8)	7.53 ± 1.004*(6)	4.56 ± 0.48(6)	2.44 ± 0.33*(6)
Serum	1.61 ± 0.13(4)	1.13 ± 0.13†(4)	0.80 ± 0.07(4)	0.58 ± 0.06(4)
Intestine	1.81 ± 0.35(4)	2.18 ± 0.33(4)	1.84 ± 0.42(4)	1.49 ± 0.22(4)
Kidney	0.69 ± 0.27(4)	0.25 ± 0.05(4)	0.15 ± 0.09(4)	0.14 ± 0.01(4)
Adrenal	5.82 ± 0.66(4)	3.90 ± 1.12(4)	3.19 ± 0.77(4)	1.70 ± 0.17(4)

*$P < 0.01$, †$P < 0.05$ with respect to controls.

Rats were maintained on a 12 hr light and dark schedule and injected with $[^3H]H_2O$ at the 7th hour of dark (D7) or 2nd hour of light (L2), 17 hr after oral dosing with olive oil (controls) or cyclandelate (3 mmole/kg) in olive oil. The animals were killed 1 hr after $[^3H]H_2O$ administration and *in vivo* rates of sterol synthesis in liver and intestine or rates of $[^3H]$sterol accumulation in the other tissues were determined as described in Materials and Methods and are expressed as μg atom H incorporated/hr/g wet wt of tissue or per ml serum ± S.E.M. with the number of animals in parentheses.

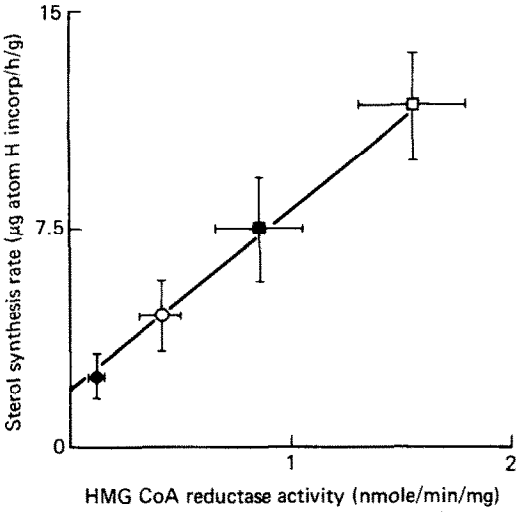

Fig. 1. Linear correlation between *in vivo* rate of hepatic sterol synthesis and HMG-CoA reductase activity in rat controls and cyclandelate treated rats killed at different times in the lighting cycle. Controls at D7 and L2: □, ○ respectively. Cyclandelate at D7 and L2: ■, ● respectively. Data points are means ± S.D. for at least 4 animals. HMG-CoA reductase activities are from ref. [1].

vivo we are in disagreement with the results of Fears and Morgan [3] but in accord with the earlier studies of Edwards *et al.*[15] and with the thorough investigation of Jeske and Dietschy [8]. These latter two studies observed a diurnal variation of 2.2–4.2-fold in hepatic sterol synthesis determined *in vivo* by [^{3}H] incorporation.

Table 1 shows the effect of a single dose of cyclandelate on sterol synthesis in liver and its accumulation in extrahepatic tissues. In liver this treatment resulted in significant inhibition (compared to controls) of 35% at D7 and 45% at L2. Thus, the inhibitory effect of cyclandelate was independent of the diurnal variation in control rates and administration of the drug did not alter this diurnal variation, implying no disturbance of those factors such as food intake and insulin concentration [16] known to influence daily patterns of sterol synthesis in rats. We found an excellent linear correlation ($r = 0.99$, $P < 0.001$) between rates of *in vivo* sterol synthesis in liver (Table 1) and hepatic HMG-CoA reductase activity (data from [1]) in all groups of rats (Fig. 1). This correlation suggests that the cause of the inhibition of hepatic sterol synthesis following cyclandelate administration was the specific decrease in activity of HMG-CoA reductase reported previously [1]. The vertical intercept of the regression line was significantly greater than zero ($P < 0.05$) indicating that a portion of newly synthesized sterol found in liver was not of hepatic origin. This labelled sterol presumably was synthesized in the intestine and transferred to the liver via chylomicron remnants. Uptake of remnants by the liver is known [17] to achieve high velocity even at very low concentrations in the plasma such as would be found at L2, more than 8 hr after the period of maximum food intake in the rat [18]. This intestinal contribution was 1.9 μg atom H/hr per g liver which was about 16% of the maximum (D7) rate for controls. This is similar to the value of 0.6 μg atom H/hr/g liver for the same parameter obtained by Turley *et al.* [9]. Their value was 13% of their maximum hepatic rate.

Cyclandelate did not significantly affect the sterol accumulation rate in any of the extraheptic tissues examined: the rate in intestine was not altered and while the rate in adrenal was lowered by 40% this did not reach statistical significance (Table 1).

The accumulation of [^{3}H]sterol in serum was inhibited by 30% after cyclandelate treatment (Table 1) and the effect reached statistical significance at D7. The newly synthesized sterol of serum is largely derived from liver [9] as a component of lipoprotein particles and in support of this we found a good correlation ($r = 0.88$; $P < 0.001$) between hepatic sterol synthesis rates and [^{3}H]sterol accumulation in serum in control and cyclandelate-treated rats at both time points. Despite the effect on newly synthesized cholesterol cyclandelate treatment did not alter serum total cholesterol concentration (Table 2). Whole serum was also fractionated into heparin-Mn^{2+} precipitable (VLDL + LDL) and non-precipitable (HDL) fractions which were ana-

Table 2. The effect of cyclandelate on the concentration of cholesterol and its accumulation rate in serum and serum fractions

Serum parameter	Measured at D7		Measured at L2	
	Controls	Cyclandelate	Controls	Cyclandelate
Total cholesterol (mM)	1.95 ± 0.15(8)	2.17 ± 0.18(8)	2.26 ± 0.24(6)	2.21 ± 0.11(6)
HDL cholesterol (mM)	0.95 ± 0.03(4)	0.77 ± 0.03*(4)	1.02 ± 0.08(4)	0.87 ± 0.06(4)
[^{3}H]sterol entry into HDL (μg atom H/hr/ml)	0.92 ± 0.12(4)	0.47 ± 0.03†(4)	0.40 ± 0.06(3)	0.23 ± 0.02*(4)
[^{3}H]sterol entry into VLDL + LDL (μg atom H/hr/ml)	0.69 ± 0.05(4)	0.66 ± 0.12(4)	0.48 ± 0.12(4)	0.35 ± 0.05(4)

*$P < 0.05$, †$P < 0.01$ with respect to controls.

Rats were maintained on a 12 hr light and dark schedule and injected with [^3H_2O] at D7 or L2 (see Table 1), 17 hr after oral dosing with olive oil (controls) or cyclandelate (3 mmole/kg) in olive oil. The animals were killed 1 hr after [^{3}H]H_2O administration and *in vivo* rates of sterol accumulation and cholesterol concentrations were determined in whole serum or in serum treated with heparin and Mn^{2+} as described in Materials and Methods. Rates are expressed as μg atom H incorporated/hr/ml serum and concentrations are mM. Values are given ± S.E.M. with the number of animals in parentheses.

Table 3. The effect of cyclandelate on fatty acid synthesis and accumulation rates *in vivo*

Tissue	Rates measured at D7		Rates measured at L2	
	Controls	Cyclandelate	Controls	Cyclandelate
	(μg atom H/hr/g or ml)			
Liver	60.82 ± 6.82(8)	27.01 ± 2.68*(8)	17.79 ± 2.7(6)	10.84 ± 1.2†(6)
Serum	5.03 ± 0.72(4)	2.99 ± 0.60(4)	1.81 ± 0.43(4)	1.68 ± 0.27(4)
Intestine	11.22 ± 1.48(4)	6.74 ± 0.32†(4)	5.35 ± 0.47(4)	5.96 ± 0.32(4)
Kidney	6.02 ± 0.76(4)	4.11 ± 0.39(4)	4.45 ± 0.22(4)	4.87 ± 0.44(4)
Adrenal	19.85 ± 4.0(4)	15.05 ± 2.3(4)	16.22 ± 2.8(4)	17.16 ± 5.55(4)

*$P < 0.001$, †$P < 0.05$ with respect to controls.

Rats were maintained on a 12 hr light and dark schedule and injected with [^{3}H]H_2O at D7 or L2 (see Table 1), 17 hr after oral dosing with olive oil (controls) or cyclandelate (3 mmole/kg) in olive oil. The animals were killed 1 hr after [^{3}H]H_2O administration and *in vivo* rates of fatty acid synthesis in liver and intestine or accumulation in other tissues were determined as described in Materials and Methods and are expressed as μg atom H incorporated/hr/g of tissue or ml of serum ± S.E.M. with the number of animals in parentheses.

lysed for [^{3}H]sterol and total cholesterol concentration (Table 2). Cyclandelate treatment caused a 47% ($P < 0.05$) decrease in accumulation of [^{3}H]sterol in the HDL fraction which was accompanied by a smaller (19%) but significant decrease in the cholesterol concentration. However, rat serum HDL, unlike that of humans, contains a high proportion of a subgroup (HDL_1) which has the 'LDL-like' function of donating cholesterol to extra hepatic tissues [19, 20] and we did not determine which subgroup of HDL particles was affected by the treatment.

Fatty acid synthesis and [^{3}H]*fatty acid accumulation* in vivo

In accord with the observations of others [10, 15] there were marked diurnal variations in rates of fatty acid synthesis in liver and intestine of control animals (Table 3). Liver (the most active of the tissues investigated) showed a D7:L2 rate ratio of 3.4 while for intestine this value was 2.1. Thus, fatty acid synthesis (like sterol synthesis) was maximal during the dark period when food intake and lipoprotein assembly are maximal. Surprisingly, cyclandelate treatment also caused the inhibition of *in vivo* rates of fatty acid synthesis in liver and intestine (Table 3). In the liver the effect was significant at both times, inhibiting by 66% at D7 and 40% at L2. Inhibition of fatty acid synthesis in intestine reached statistical significance (60%, $P < 0.05$) only at D7. As with sterol synthesis, the effect of cyclandelate on hepatic fatty acid formation was independent of the diurnal variation in rate. Thus, in cyclandelate treated animals, as in controls, the difference between rates of fatty acid synthesis at D7 and L2 was maintained, the rate ratio being 2.5. These results indicate that the effects of cyclandelate are unlikely to be mediated by those hormones (insulin, glucocorticoids) whose diurnal concentration changes are associated with cyclical variations in fatty acid and sterol biosynthesis [10, 16].

The observed inhibition of fatty acid synthesis in liver could be a direct effect on the enzymes involved in this pathway and this possibility is now under investigation. However, it could also be explained by the close links existing between fatty acid and sterol biosynthesis via their incorporation into VLDL particles [21]. Cyclandelate-induced inhibition of fatty acid synthesis could thus be secondary to its effects on sterol synthesis exerted at the level of HMG-CoA reductase [1]. If this were so one would expect a correlation between the rates of the two processes (fatty acid and sterol biosynthesis) in livers of control and cyclandelate treated animals. A significant correlation was indeed observed ($r = 0.52$, $P < 0.01$), the ratio of rates of hepatic fatty acid synthesis to sterol synthesis being maintained at an average value of 4.2 ± 0.5 (Tables 1 and 3) for all the groups of animals in this study. It is of interest to note that the 3,3',5-trimethylcyclohexanol component of cyclandelate structurally resembles the cyclic monoterpene menthol, the administration of which to rats inhibits hepatic HMG-CoA reductase [22] as well as decreasing the rates of sterol and fatty acid synthesis *in vivo* [14].

In summary, the administration to rats of a single oral dose of cyclandelate, a widely used vasoactive drug, results in significant inhibition of hepatic lipogenesis *in vivo*. The inhibitory effects of cyclandelate are exerted on both fatty acid and sterol biosynthesis and are probably the result of the specific decrease in activity of hepatic HMG-CoA reductase reported earlier [1].

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