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INHIBITION OF ACYL COENZYME A: CHOLESTEROL ACYL TRANSFERASE BY TRIMETHYLCYCLOHEXANYLMANDELATE (CYCLANDELATE)

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Abstract—Cyclandelate was an effective inhibitor of rat hepatic acycloenzyme A: cholesterol acyltransferase (ACAT) with a concentration of 80 μM being required for half maximal inhibition. A similar effect was seen with human and rabbit liver microsomal enzymes. The drug did not compete with oleoyl CoA or cholesterol and could be removed from enzyme preparations by washing. It was hydrolysed rapidly by rat liver microsomes to products which were non inhibitory. No hydrolysis of the drug was seen with non hepatic microsomes and the concentration of cyclandelate required to cause half maximal inhibition of ACAT in the transformed mouse macrophage J774 microsomal fraction was less than 30 μM. The possible significance of the differential actions of cyclandelate towards hepatic and extra hepatic ACAT *in vivo* is discussed.

Atherosclerosis is characterized by the accumulation of both calcium and cholesteryl ester in arterial walls. The major intracellular enzyme catalysing the synthesis of cholesteryl ester, acylcoenzyme A: cholesterol acyltransferase (ACAT), is subject to regulation by the supply of cholesterol substrate and also by phosphorylation [1].

Reverse cholesterol transport i.e. efflux from the cell, involves free rather than esterified cholesterol [2] and there is a growing interest in drugs which will inhibit ACAT and lead to an increased intracellular concentration of free cholesterol. Such molecules include derivatives of cholesterol [3, 4] and a range of chemically unrelated molecules (reviewed in Ref. 5) of which the most potent are the *N*-acylamides 58035 and 57118 produced by Sandoz [6, 7]. None of these drugs is currently in use as a therapeutic agent.

Cyclandelate (Cyclospasmol, Gist-Brocades n.v. Delft, Holland), the trimethylcyclohexanyl ester of mandelic acid is non toxic in high doses in man and has been used extensively for many years in the treatment of cerebrovascular disease and peripheral vascular disorders. It inhibits hepatic sterol synthesis *in vivo* in rats probably through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A [8]. A direct action of the drug on arterial tissue was suggested in experiments using rabbits fed a high cholesterol diet [9]. Cyclandelate reduced the extent and the severity of aortic atheroma when the cholesterol fed animals were returned to a regression diet. More recently cyclandelate was shown to inhibit the esterification of low density lipoprotein (LDL)-borne cholesterol in cultured human skin fibroblasts [10] and also the incorporation of [^{3}H]oleate into cholesterol ester but not triglyceride in the same cells. Some characteristics of the inhibition of ACAT by cyclandelate are described in the present paper.

MATERIALS AND METHODS

[1α,2α-^{3}H]Cholesterol (1.59 TBq/mmol), cholesteryl [1-^{14}C]oleate (2.14 GBq/mmol), [9,10-^{3}H]oleate and (370 MBq/mmol) [4-^{14}C]cholesterol (2.2 GBq/mmol) were purchased from Amersham International plc (Little Chalfont, U.K.). Oleoyl CoA was synthesized by the mixed anhydride method [11] and its purity checked by TLC on polyester backed type 100 cellulose with 254 nm fluorescent indicator, (Sigma Chemical Co., Poole, U.K.). The TLC was developed in butan-1-ol: acetic acid: H_2O (5:2:3 v/v/v). The [^{3}H]oleoyl CoA gave the same identical spot when detected by (i) iodine vapour; (ii) nitroprusside/alkali spray; (iii) quenching of 254 nm fluorescence; and (iv) by scanning for radioactivity. [4-^{14}C]Cholesteryl oleate was synthesized chemically [12]. All the tissue culture media and sera were from Imperial Labs. (Europe) Ltd (Salisbury, U.K.). Cyclandelate was a gift from Gist-Brocades n.v. Delft, Holland and all other reagents were purchased from commercial sources.

Rat liver microsomal fractions were prepared as described previously [13] and stored in aliquots in assay buffer at −20° until required. Under these conditions the ACAT activity was stable for at least 7 days. The same procedure was followed for the preparation of adrenal microsomes. Protein was assayed by the microbiuret assay [14].

Preparation of J774 homogenates and microsomal

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fractions. Cells from confluent monolayers were scraped from culture dishes, pooled and after centrifugation at 2000 *g* for 10 min were resuspended in hypotonic buffer at pH 7.4. They were subjected to five cycles of freezing and thawing using liquid nitrogen to produce a cell homogenate. When microsomal fractions were required the cell homogenate was centrifuged at 2000 *g* × 10 min to remove the nuclear and mitochondrial fractions. A microsomal pellet was obtained by centrifugation of the post mitochondrial supernatant fraction at 100,000 *g* for 1 hr. This pellet was resuspended in 10 mM potassium phosphate buffer pH 7.4 containing 2 mM dithiothreitol (DTT) at a protein concentration of 1000 μg/mL.

Preparation of a cholesterol enriched microsomal fraction. Cholesterol/phospholipid liposomes were prepared by the method of Suckling *et al.* [15]. Phosphatidylcholine, phosphatidylserine and cholesterol were mixed in a molar ratio of 3:1:4 in chloroform in a small round bottom flask and the solvent was evaporated under reduced pressure to leave a thin film of lipid. Potassium phosphate buffer (100 mM) pH 7.4 was added such that the final concentration of cholesterol was 8 mg/mL. The mixture was sonicated to yield an opaque dispersion the cholesterol content of which was determined using the Boehringer-Mannheim cholesterol assay kit.

The cholesterol content of rat liver microsomes was increased by incubating microsomal membranes (500 μg protein) with increasing volumes of the cholesterol-liposome dispersion at 37° for 90 min. Aliquots of the microsomal fraction were then assayed for ACAT activity in the presence or absence of cyclandelate. The cholesterol content of the loaded microsomal membranes was determined using the Boehringer-Mannheim test combination kit.

Assay of acylcoenzyme A: cholesterol acyltransferase. The procedure followed was essentially that described by Lichtenstein and Brecher [16]. The final incubation volume (200 μL) contained 100 mM phosphate buffer, pH 7.4, 1 mg fatty acid free BSA, 100 μM [^{3}H]oleoyl CoA (60 dpm/pmol) and 100–200 μg microsomal protein. Reactions were started by addition of protein and stopped after 5 min by addition of chloroform:methanol (1:2 v/v; 3.75 mL). Under these conditions the rate was linear with time and with added protein up to 3 mg/mL. Half maximum saturation with oleoyl CoA was found at around 20 μM. [4-^{14}C]Cholesteryl oleate (2000 dpm) was added as recovery standard and lipids were extracted by the method of Bligh and Dyer [17]. Lipid extracts were evaporated to dryness and redissolved in chloroform (100 μL) containing approximately 50 μg of unlabelled cholesteryloleate. Extracts were applied to silica gel impregnated paper (SG81) and the chromatograms were developed in hexane:diethyl ether:acetic acid (98:2:1 v/v/v). Lipids were visualized by staining with iodine vapour and radioactivity on the plate was detected using a Panax scanner. Areas on the chromatogram corresponding to cholesteryloleate were cut out and assayed for radioactivity by liquid scintillation spectrometry. When the effects of cyclandelate on ACAT were measured the drug was added as a solution in dimethylsulphoxide (DMSO) to a final concentration of 2.5% (v/v). Controls always contained the same volume of DMSO alone.

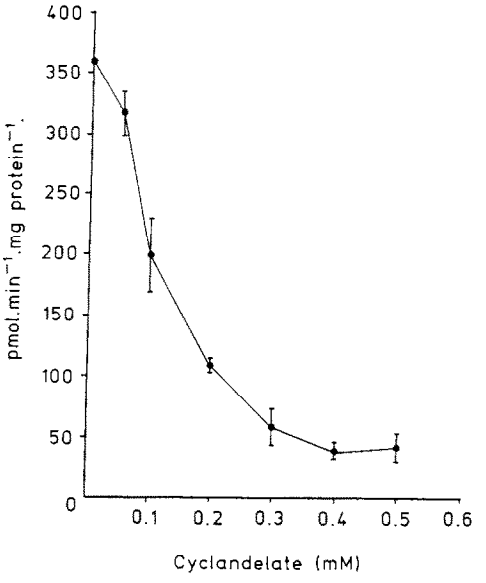

Fig. 1. The effect of increasing concentrations of cyclandelate on ACAT activity on rat liver microsomes. ACAT activity was measured in rat liver microsomes using [^{3}H]oleoyl CoA in the presence of increasing concentrations of cyclandelate. Activities are expressed as pmol cholesteryl oleate produced per mg microsomal protein per min and are means ± range of two separate determinations.

RESULTS AND DISCUSSION

The inhibitory effects of cyclandelate were investigated initially using rat hepatic microsomal ACAT assayed under optimal conditions. As the concentration of drug in the assay increased there was a concomitant rise in the inhibition of the rate of cholesterol esterification (Fig. 1). The apparent inhibitor concentration required to reduce activity by 50% (IC_{50}) was 80 μM and at concentrations greater than 100 μM esterification was virtually abolished. In these experiments the assay was started by addition of microsomal enzyme to incubation medium containing the drug.

In an experiment to determine whether preincubation of the microsomal fraction with cyclandelate might enhance its inhibitory action the drug (100 μM) was incubated with microsomes for 15 min at 37 or 0° prior to addition of oleoyl CoA to start esterification. As shown in Fig. 2, while preincubation at 0° resulted in little change in inhibition, preincubation at 37° produced hardly any inhibition at all. This suggests that the parent drug is being metabolized in the preincubation at 37° giving rise to non inhibitory products. Neither of the products of hydrolysis of cyclandelate, mandelic acid and 3,3,5-trimethylcyclohexanol, are inhibitory at concentrations up to 200 μM (Fig. 3). We have reported previously on the rapid hydrolysis and metabolism of a dual-radiolabelled cyclandelate in rat hepatocytes [18].

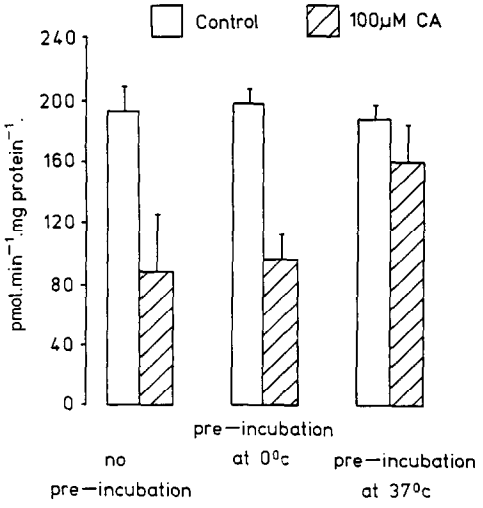

Fig. 2. The effect of preincubation of cyclandelate with microsomal fractions on its inhibition of microsomal ACAT. ACAT activity was measured, as described in Materials and Methods, in microsomes which had undergone either no preincubation, preincubation at 0° for 15 min or preincubation at 37° for 15 min in the presence of ethanol (2.5% v/v) as control (open column) or cyclandelate (100 μM) (shaded columns). Results are expressed as pmol cholesteryl [^{3}H]oleate per mg microsomal protein per min and represent mean ± range of two separate determinations.

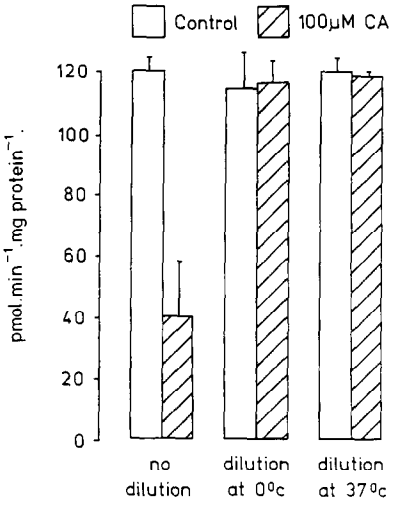

Fig. 4. The effect of dilution of microsomes preincubated with cyclandelate on ACAT activity. Microsomes (1 mg protein) were preincubated with either DMSO (10 μL) or cyclandelate (100 μM in DMSO 10 μL) at 0 and 37° for 1 min. Aliquots of protein were then assayed for ACAT activity in an incubation volume which caused dilution of cyclandelate to 5 μM. Results are expressed as pmol cholesteryl oleate produced per mg protein and are means of duplicate determinations with the range shown.

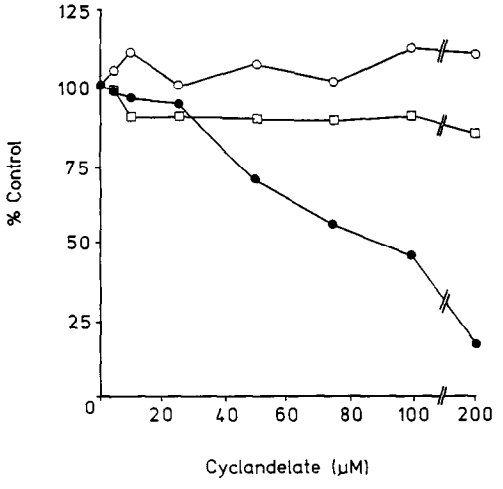

Fig. 3. The effects of mandelic acid and trimethylcyclohexanol on hepatic microsomal activity. ACAT activity was assayed in the presence of increasing concentrations of cyclandelate (●), trimethylcyclohexanol (□) or mandelic acid (○) as described in Materials and Methods. Results are expressed as a percentage of the control activity which was 386 ± 37 pmol per mg protein per mg and are means of duplicate determinations which vary by less than 5%.

Similar inhibitions of ACAT activity (approximately 50% at 100 μM cyclandelate) were seen in microsomal fractions isolated from rabbit and human liver.

Cyclandelate *in vivo* caused a marked inhibition of rat hepatic HMGCoA reductase measured 17 hr after dosing [8] but no such inhibition of ACAT was seen in the present study either at 17 hr or at times earlier than this. However, cyclandelate added to hepatic microsomal fractions prepared from animals which had been dosed previously with the drug, still inhibited ACAT. The failure to detect *in vivo* inhibition is probably due to two factors, (i) hydrolysis of the drug by the liver and (ii) removal of the drug from membrane preparations during fractionation. The loose affinity of the drug for the enzyme is shown in Fig. 4. In this experiment microsomal protein (1 mg) was preincubated at 0 or 37° with 100 μM cyclandelate, a concentration shown to inhibit markedly ACAT. After 1 min of preincubation aliquots (10 μL) of this mixture were added to the assay system for measurement of ACAT. In such a system the cyclandelate concentration was diluted 20-fold. Whereas cyclandelate inhibited ACAT during the preincubation period dilution into the assay mixture relieved the inhibition suggesting that the action of the drug, while rapid, is also rapidly reversible so that no covalent interaction with ACAT is involved.

Suckling *et al.* [15] enhanced rat hepatic ACAT activity by raising the cholesterol substrate concentration in microsomal preparations. Incubation of cholesterol-rich liposomes with microsomal membranes caused the transfer of cholesterol from the liposomes into the membranes. Enrichment of microsomal membranes with cholesterol in the present experiments caused a progressive increase in cholesterol esterification with maximal activity (2.5 times the control) at a cholesterol:protein ratio of 0.25 (i.e. 250 μg cholesterol per mg protein). At

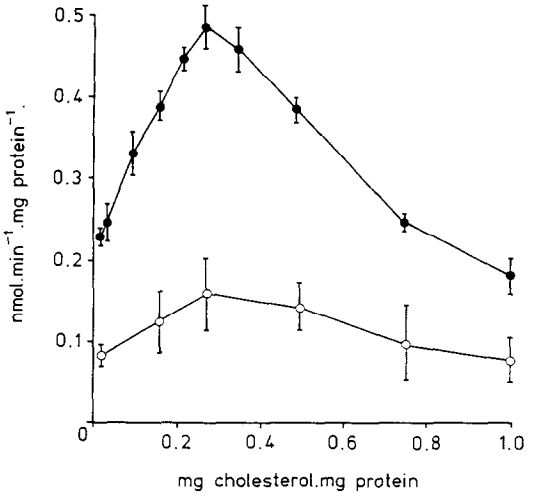

Fig. 5. The effect of cyclandelate on ACAT activity in cholesterol loaded microsomes. Microsomal membranes were loaded with cholesterol from cholesterol-rich liposomes as described in Materials and Methods and ACAT activity was measured in the presence of ethanol (2.5%) or cyclandelate (100 μM). Results are expressed as nmol cholesteryl oleate per mg protein per min and represent the mean ± SD of three separate determinations.

inhibition and a Dixon plot from these data gave a K_i of 102 μM for cyclandelate.

The inhibitory action of cyclandelate on ACAT is not restricted to the liver. The optimal conditions for assay of ACAT in homogenates prepared from the permanent mouse macrophage line J774 are shown in Fig. 7. The conditions were similar to those for the rat liver enzyme and the routine assay system contained 100 μM oleoyl CoA, 1 mg BSA and 100 μg protein in a final volume of 200 μL, incubated at 37° for 5 min. Cyclandelate at concentrations greater than 100 μM inhibited completely and 50% inhibition was seen at a concentration of 30 μM (Fig. 8). This lower IC_{50} for the J774 enzyme probably reflects the lack of hydrolysis of the drug in this system. Incubation of dual labelled cyclandelate with either J774 microsomes or whole cells gave rise to neither [^{14}C]mandelate nor [^{3}H]trimethylcyclohexanol [18]. Addition of increasing amounts of protein to the assay at constant drug concentration also had no effect on the inhibition. This increased ACAT sensitivity to the drug was also seen in rabbit adrenal microsomes where 100 μM cyclandelate caused an 80% inhibition of the enzyme. Again there was no hydrolysis of the drug in this tissue.

Thus cyclandelate, a drug with no apparent side effects in man even at high doses (1.5 g/day) is a

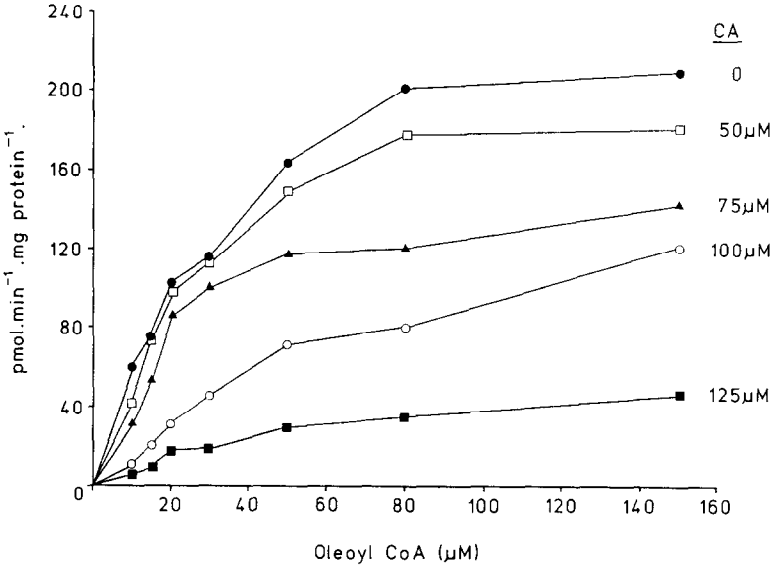

Fig. 6. The effect of increasing cyclandelate concentrations on ACAT activity assayed with increasing concentrations of [^{3}H]oleoyl CoA. ACAT activity was measured as described in Materials and Methods with increasing concentrations of oleoyl CoA and cyclandelate. Results are expressed as pmol cholesteryl oleate per mg protein per min and represent the mean ± SD of three separate determinations.

ratios greater than this the ACAT activity decreased and returned to control values at a ratio of 1 (Fig. 5). Inclusion of cyclandelate in the assay using cholesterol loaded microsomal membranes caused an inhibition of 70% irrespective of the cholesterol : protein ratio. The effect of increasing the concentration of the second substrate, oleoyl CoA, on the inhibitory action of cyclandelate is shown in Fig. 6. Increasing oleoyl CoA concentration did not relieve the potent inhibitor of cholesterol esterification in both hepatic and extra hepatic tissues. Its effectiveness in the liver is however decreased due to rapid hydrolysis of the drug to inactive mandelic acid and trimethylcyclohexanol. The lack of effect of these latter compounds indicates the specificity of the inhibition of cholesterol esterification by cyclandelate. Furthermore, cyclandelate does not affect synthesis of oleoyl-CoA or the hydrolysis of cholesteryl oleate.

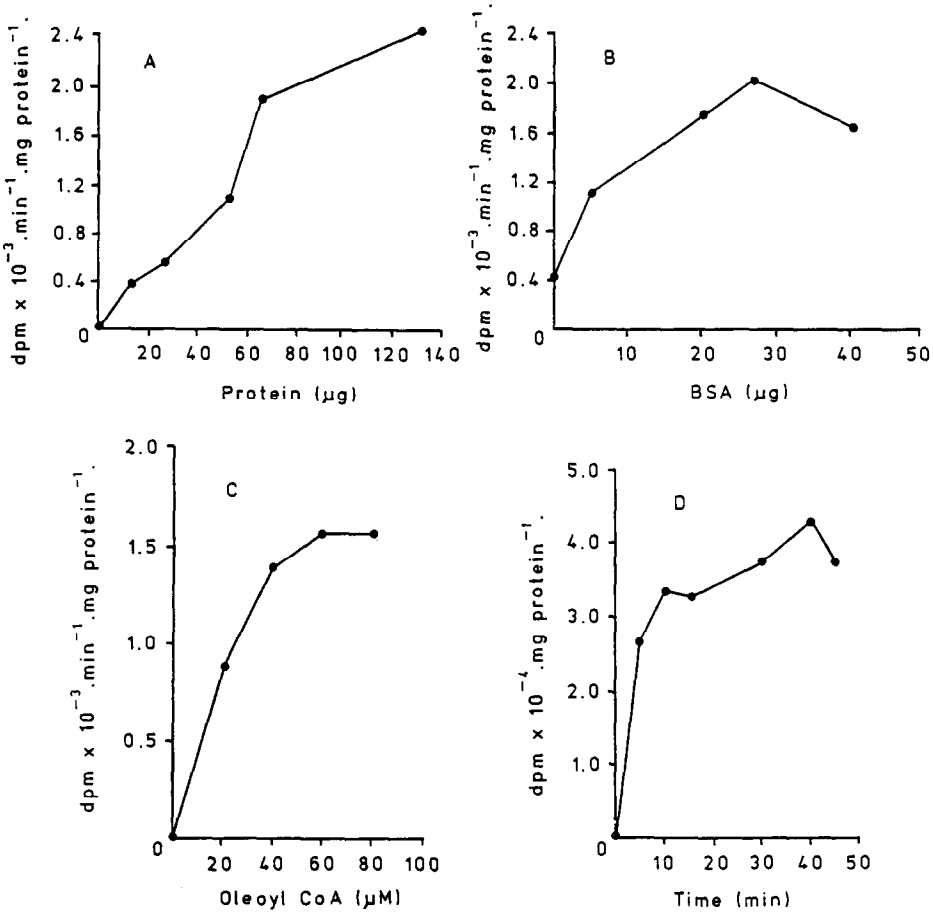

Fig. 7. Optimal conditions for assay of ACAT in J774 cell homogenates. ACAT was assayed as described in Materials and Methods in the presence of increasing concentrations of protein (A), BSA (B), oleoyl CoA (C) and for increasing incubation times (D). Assays normally contained 100 μg protein, 100 μM oleoyl CoA and 1 mg BSA for 5 min. Activities are expressed as dpm cholesteryl [^{3}H]oleate per mg protein per min and values represent means of duplicate determinations which varied by less than 5%.

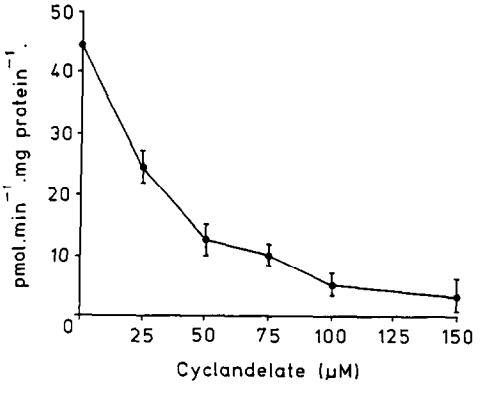

Fig. 8. The effect of increasing concentrations of cyclandelate on ACAT activity in J774 cell microsomes. ACAT activity was assayed as described in Materials and Methods. Increasing concentrations of cyclandelate dissolved in DMSO (0.5% v/v) were included in the incubation. Results are expressed as pmol cholesteryl oleate produced per mg protein per min and represent mean ± range of two separate determinations.

The mechanism of the inhibition of ACAT by the drug is unclear since it is not competitive with either oleoyl CoA or cholesterol, and its effects are rapidly reversed on dilution. No evidence was found for increased cholesterol esterase activity in the presence of the drug. It is also unlikely that cyclandelate exerts its action by stimulating dephosphorylation of the enzyme. Dephosphorylation of the hepatic enzyme would lead to inactivation [1] but to activation of the adrenal enzyme [15]; cyclandelate is equally inhibitory to the ACAT activity of both tissues.

The drug therefore would seem to have an effect at a site other than the active site of the enzyme. Its lipophilic properties would lead to its partition in cellular membranes, such as has been shown for a variety of terpenes [13], and it may prevent the intramembrane movement of cholesterol to the active site of the enzyme. Middleton [19] has shown that cyclandelate and progesterone both inhibit ACAT activity in human fibroblasts and also down regulate LDL binding whereas 58035 a potent ACAT inhibitor, had no effect on LDL receptor number. It

was suggested that two pools of free cholesterol exist in microsomal membranes with only one exerting a regulatory effect on LDL receptor number. Cyclandelate may increase the cholesterol content of this pool (A) by inhibiting its movement into the ACAT substrate pool (B). This would lead to a fall in ACAT activity due to decreased substrate concentration in B and to a down regulation of LDL receptor number through the increase in A. Interestingly evidence for two distinct pools of free cholesterol has been provided by Synouri-Vrettakou and Mitropoulos [20]. Furthermore, the inhibition of hepatic HMGCoA-R caused by the drug *in vivo* is comparable with the proposed increase in a regulatory pool of cellular cholesterol.

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