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Effects of novel synthetic sterol probes on enzymes of cholesterol metabolism in cell-free and cellular systems

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Abstract

A series of novel sterols was synthesised as probes for the enzymatic and cellular functions of two important enzymes of intracellular cholesterol metabolism, acyl-CoA:cholesterol acyltransferase (ACAT) and cholesterol 7α-hydroxylase. The compounds were 6-fluoro-5-cholesten-3β-ol (6-fluorocholesterol), 7-cholesten-3β-ol (7-cholestenol), 6β-fluorocholestan-3β-ol (6β-fluorocholestanol), 3-acetoxy-6-fluorocholestan-3β-ol (3-acetoxy-6-fluorocholestanol) and 7-methoxy-5-cholesten-3β-ol (7-methoxycholesterol). They were designed to reveal the effect of small changes in sterol structure, particularly reactivity of certain parts of the ring structure and polarity, on enzyme activity and intracellular cholesterol metabolism. The 3β-hydroxy group was essential for interaction with both enzymes since 3-acetoxy-6-fluorocholestanol did not affect any of the enzyme-catalysed reactions. 6-Fluorocholesterol and 7-cholestenol had no effect on cholesterol esterification but did inhibit the hydroxylation of cholesterol, as did the other compounds with groups that could influence the 7 position, namely 6β-fluorocholestanol and 7-methoxycholesterol. The fluorocholestanols were all competitive substrates for cholesterol esterification in cell-free and cellular assays of ACAT activity. 7-Methoxycholesterol was a surprisingly effective inhibitor of ACAT for a simple sterol. However, 6-fluorocholesterol did not have any effect on ACAT, suggesting that interactions between the enzyme and the region around C-6 and C-7 of the sterol are important. These results show that it is possible to dissect components of cholesterol metabolism using simple, specifically substituted sterols and thus define a new approach to studying the relationships between the various enzymes that catalyse intracellular cholesterol metabolism.

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1. Introduction

The steroid ring structure has provided in nature a rigid framework on which to attach an array of functional groups of varying polarity and orientation. Steroid hormones and vitamin D are perhaps the most striking examples of the versatility of such a structure for biological signalling processes, both inter- and intra-cellular. The structure of cholesterol itself, the precursor of many of these molecules, is also precisely related to its biological function, for example in the way in which it stabilises many biological membranes, a property of the overall shape and the distribution of polarity over the molecule [1]. Further, derivatives of cholesterol that have not been well defined, loosely called oxysterols, are believed to participate in the regulation of cholesterol and isoprenoid synthesis, which is one of the most tightly and comprehensively regulated processes in the cell [2].

The metabolism of cholesterol to its oxidation products, bile acids and steroid hormones, and to its storage form of cholesteryl ester requires that the respective enzymes abstract the cholesterol molecule from the surrounding phospholipid/cholesterol bilayer and recognise the precise site of the molecule for transformation. Over 20 years ago, we and others showed that the requirement for the oxidation of cholesterol to bile acids by cholesterol 7α-hydroxylase is quite precise: only molecules of the same length and polarity as cholesterol can act as substrates [3]. Similar conclusions were reached by others for the enzyme that catalyses the intracellular esterification of cholesterol, acyl-coenzyme A:cholesterol acyltransferase (ACAT) [4].

More recently, we attempted to explore in a more subtle way the nature of the interactions of cholesterol with the active site of these two enzymes. Specifically designed steroids were synthesised with functional groups situated in positions that could be expected to participate in some way in the enzyme catalysed reaction [5]. We showed that 5α,6α-methanocholestan-3β-ol was a substrate for cholesterol 7α-hydroxylase. The cyclopropyl ring did not open during oxidation, implying that any free radical formed at C-7 during the hydroxylation reaction was very sterically shielded and hence inaccessible for further reaction or was short-lived [6]. A 6-aza cholesterol was found to be an inhibitor of cholesterol 7α-hydroxylase but had no effect on cholesterol esterification [7]. This compound allowed some conclusions to be drawn about the movement of cholesterol between different metabolic pools in the hepatocyte [7]. The reactivity and substrate binding of cholesterol to cholesterol 7α-hydroxylase and ACAT was probed more closely using 6,6-difluorocholestanol and 7,7-difluorocholestanol [8]. Both sterols were shown by a rigorous biophysical study to partition into a phospholipid bilayer in a manner identical to cholesterol [9]. 6,6-Difluorocholestanol was found to be an inhibitor of cholesterol 7α-hydroxylase but not a substrate, whereas 7,7-difluorocholestanol did not affect cholesterol 7α-hydroxylase in any way. 6,6-Difluorocholestanol was also found to be a competitive substrate for ACAT. Thus, the introduction of the highly polar fluorine atoms did not greatly affect binding of the sterol to ACAT in particular, but the electronic influence of the fluorines on C-7 probably had a significant effect on the reactivity of that position towards hydroxylation.

None of the compounds described so far had any direct effect on 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme of cholesterol and isoprenoid synthesis that is regulated by the so-called oxysterols in intact cells [10].

These results suggested that more information about substrate interactions with ACAT and cholesterol 7α-hydroxylase and their role in cells could be obtained using further compounds designed with the molecular interactions of enzyme and substrate in mind. This paper describes the synthesis of 5 additional analogues of cholesterol, mostly bearing very small structural modifications, and their effects on the activity of these enzymes and on intracellular cholesterol metabolism.

2. Materials and methods

2.1. Synthesis of sterols

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WH 250 spectrometer operating at 250 MHz. $CDCl_3$ was used as solvent and tetramethylsilane as the internal standard. Syntheses of sterols were from cholesterol (Aldrich, Gillingham, UK) and based upon established procedures [11–13]. 6,6-difluoro- and 7,7-difluorocholestanols used in these studies were samples prepared as previously described [8].

2.2. Standard procedure for fluorinating steroidal ketones

The 3-acetoxyketone (1 g) was dissolved in glyme (15 ml) to which diethylaminoethyl sulphur trifluoride (DAST, 2.5 ml) and fuming sulphuric acid (1 drop) were added. The solution was kept sealed under nitrogen for 4–14 days. At the end of the reaction, the solution was poured into dilute aqueous sodium bicarbonate solution and the steroid extracted with dichloromethane. The product was purified by chromatography on silica gel and by recrystallisation from methanol or ethanol.

2.3. Standard procedure for fluorinating steroidal alcohols [11]

The 3-acetoxyalcohol (0.5 g) was dissolved in dichloromethane (dry, 3 ml) to which a solution of DAST (0.5ml) in dry dichloromethane (5 ml) was added over 20 minutes with stirring. The reaction was allowed to continue for 30 min to 2 h and water (5 ml) was then added slowly. Dichloromethane (5 ml) was added and the organic layer separated. The organic layer was washed with dilute aqueous sodium bicarbonate solution and water, dried ($MgSO_4$) and evaporated to dryness. The fluorosteroid was purified by recrystallisation from methanol or ethanol or by chromatography on silica gel if necessary.

2.4. Standard procedure for the hydrolysis of acetate esters

The sterol ester (0.1 g) was dissolved in a solution of potassium hydroxide in methanol (10 ml, 0.1% w/v) and allowed to stand at room temperature until hydrolysis was complete (tlc). The solution was concentrated and water added to precipitate the product which was recrystallised from methanol.

3β-Acetoxy-7β-fluorocholest-5-ene: Prepared from the corresponding 7-β-ol (m.p. 106–108°C, lit. m.p. [12] m.p. 108–112°C), (0.5 g); Yield 0.24 g (49%) m.p. 128–129°C (from methanol). δ_H5.63 (1H, bd s, H-6); 4.69 (1H, m, H-3); 4.63 (1H, bd d, $J_{HF} = 50.2$ Hz, H-7); 2.37 (2H, m, H-4); 2.04 (3H, s, MeCO); 1.08 (3H, s, Me-10); 0.97 (3H, d, J = 6.5 Hz, Me-18); 0.88 (6H, d, J = 6.5Hz, Me-25); 0.68 (2H, s, Me-13).

Attempted preparation of 7β-fluorocholest-5-en-3β-ol: Under the conditions of hydrolysis and recrystallisation in methanol, the above compound underwent allylic substitution affording 7β-methoxycholest-5-en-3β-ol: m.p. 159–160°C. δ_H 5.16 (1H, d, J = 7 Hz H-6); 3.60 (1H, m, H-3); 3.36 (3H, s, OMe); 3.29 (1h, bd s, H-7); 1.02 (3H, d, J = 6.5 Hz, Me 18); 1.04 (6H, d, J = 6.5 Hz, Me-25); 0.98 (3H, s, Me-10); 0.54(3H, s, Me-13). Found m/z 416.3631, $C_{28}H_{48}O_2$ requires 416.3654.

Attempted preparation of 3β-acetoxy-7β-fluorocholestane: The corresponding 7β-ol (0.3 g m.p. 110–112°C, lit. m.p. 116–117°C, prepared by reduction of the corresponding ketone with $LiAl(OBut)_3H$ [12]) underwent elimination on treatment with DAST under standard fluorinating conditions to afford 3β-acetoxycholest-7,8-ene, yield 0.1 g (33%): m.p. 120–122°C (from methanol). δ_H 5.14 (1H, bd s, H-7); 4.70 (1H, m, H-3); 2.03 (3H, s, MeCO); 1.04 (3H, d, J = 6.5 Hz, Me 18); 0.91 (6H, d, J = 6.5 Hz, me-25); 0.81 (3H, s, Me-10); 0.54 (3H, s, Me-13).

Cholest-5-en-3β-ol was obtained by hydrolysis of the above acetate (0.05 g), Yield 0.038 g (75%); m.p. 120–121°C (from methanol). δ_H 5.16 (1H, s, H-7); 3.60 (1H, m, H-3); 2.03 (2H, m, H-6); 1.02 (3H, d, J = 6.5 Hz, Me-18); 1.04 (6H, d, Me-25); 0.98, (3H, s, Me-10); 0.54 (3H, s, Me-13). Found m/z 386.3535, $C_{27}H_{46}O$ requires 386.3549.

3β-Acetoxy-6-fluorocholest-5-ene: Prepared from 3β-acetoxycholest-6-one (0.7 g, m.p. 126–128°C, lit. m.p. 128–129°C) [13], yield 0.52 g (73%) m.p. 110–112°C (from methanol) lit m.p. [13] 110–112°C. δ_H 4.59 (1H, m, H-3); 3.03 (2H, dd, J = 6.5 and 14 Hz, H-7; 2.04 (3H, s, Me CO); 1.02 (3H, s, Me-10); 0.97 (3H, d, J = 6.5 Hz, Me 18); 0.87 (6H, d, J = 6.5 Hz, Me-25); 0.68 (3H, s, Me-13).

6-Fluorocholest-5-en-3β-ol: Prepared from the corresponding acetate (0.25 g), yield 0.22 g (97%) m.p. 138–139°C (from methanol). δ_H 3.51 (1H, m, H-3); 3.02 (1H, dd, J = 6 and 13 Hz, H-7β); 2.12 (2H, m, H-4); 2.01 (1H, d, J = 13Hz. H-7α); 0.95 (3H, d, J = 6.5 Hz, Me 18); 0.88 (6H, d, J = 6.5 Hz, Me-25); 0.98 (3H, s, Me-10); 0.68 (3H, s, Me-13). Found C, 79.8; H, 11.3%. $C_{27}H_{45}FO$ requires C, 80.1; H, 11.2%. Found m/z 404.3448, $C_{27}H_{45}FO$ requires 404.3454.

3β-Acetoxy-6β-fluorocholestane: This compound was prepared by hydrogenation of the corresponding 5-ene above (0.28 g) using Pd-C (10%) in glacial acetic acid solution at atmospheric pressure. Yield 0.18 g (64%) m.p. 95–96°C (from methanol). δ_H 4.71 (1H, m, H-3); 4.54 (1H, d, J_{HF} = 50.0 Hz, H-6); 2.03 (3H, s, MeCO); 1.08 (3H, d, J = 1.5 Hz, Me-10); 1.06 (3H, d, J = 6.5 Hz, Me 18); 0.87 (6H, d, J = 6.5 Hz, Me-25); 0.65 (3H, s, Me-13). Found m/z 448.3724, $C_{29}H_{49}FO_2$ requires 448.3717.

6β-Fluorocholestan-3β-ol from the above acetate (0.14 g), yield 0.02 g (10%): m.p. 122–124°C (from methanol/water). δ_H 4.57 (1H, d, J_{HF} = 50.0 Hz, H-6); 3.59 (1H, m, H-3); 1.07 (3H, d, J = 1.5Hz, Me-10); 0.98 (3H, d, J = 6.5 Hz, Me 18); 0.88 (6H, d, J = 6.5 Hz, Me-25); 0.65 (3H, s, Me-13). Found 406.3603, $C_{27}H_{47}FO$ requires 406.3611.

2.5. Biochemical studies

2.5.1. Materials

L-α-Phosphatidylcholine (type V-E from egg yolk), cholesterol, Ficoll (Type 400), cholestyramine, sodium deoxycholate, dimethylsulphoxide (DMSO), oleoyl-CoA and oleic acid were from Sigma (Poole, Dorset). [1-^{14}C]oleoyl-CoA (52.7 mCi/mmol), [1α, 2α(n)-^{3}H]cholesteryl oleate (49.6 mCi/mmol), [9,10(n)-^{3}H]oleic acid (10 mCi/mmol) [n^{14}C]acetate and cholesteryl [1-^{14}C]oleate (each 52 mCi/mmol) were supplied by Amersham (Amersham, Bucks.). Fatty acid-free bovine serum albumin (BSA, fraction V) was from Boehringer Mannheim (Lewes, East Sussex). Techelut silica gel columns were from HPLC Technology (Macclesfield, Cheshire). Gibco BRL (Uxbridge, Middlesex) supplied foetal calf serum (FCS), trypsin-ethylenediamine tetraacetic acid (EDTA) and L-glutamine. ICN Flow (High Wycombe, Bucks.) supplied Dulbecco's modified Eagle's medium (DMEM), DMEM(*N*-2-hydroxyethylpiperadine-*N'*-2-ethansulfonic acid (HEPES)) and phosphate buffered saline (PBS). Protein assay dye concentrate was supplied by BioRad laboratories (Hemel Hempstead, Herts.). CaCo-2 cells were from the European Collection of Animal Cell Cultures. Purified HMG-CoA reductase from human liver was a gift from Ruth Mayer, SmithKline Beecham, USA. Other chemicals were obtained from BDH Ltd., Poole, Dorset or from Fisons Scientific Equipment, Loughborough, Leics.

2.5.2. Animals

Male Sprague-Dawley rats were maintained on a standard laboratory diet supplemented with 2% cholesterol to stimulate ACAT activity or 4% cholestyramine to stimulate cholesterol 7α-hydroxylase activity.

2.5.3. Preparation of microsomes

Sprague-Dawley rats were anaesthetised with diethyl ether delivered by a vaporiser and the livers were perfused with 0.104 M KCl/0.05 M NaF, pH 7.4. Microsomes were then prepared by the method of Boyd et al. [3] and were stored freeze-dried at −20°C. Protein was measured using the method of Bradford [14].

2.5.4. Microsomal assay of ACAT

The microsomal assay was that used by Suckling, Boyd and Smellie [15]. Assays contained 0.5 mg microsomal protein, 0.5 mg fatty acid-free BSA and 0.5 mg reduced glutathione in 0.05 M KPO_4/0.05 M NaF buffer (pH 7.4). After a preincubation of 5 min at 37°C with compounds added

in DMSO, 38.8 nmoles of [^{14}C]oleoyl-CoA was added to start the reaction (final concentration 78 μM, 0.020 μCi/ml). The total assay volume was 0.5 ml. Assays were carried out for a further 3 min and the reactions were then stopped by the addition of 1 ml methanol. [^{3}H]Cholesteryl oleate (approximately 25 000 dpm) was added as a recovery marker and the lipids extracted with dichloromethane:methanol (2:1, v/v). Extracts were dried down under nitrogen and the products were separated on silica gel columns.

2.5.5. Solubilisation and reconstitution of ACAT activity

This method was based on that used by Cadigan and Chang [16] as developed by Harte et al. [17]. ACAT activity was solubilised from rat liver microsomes using deoxycholate and reconstituted in cholesterol-phosphatidylcholine vesicles [18] by a dilution procedure.

2.5.6. Assay of reconstituted ACAT activity

Assays contained 5 μg fatty acid-free BSA, 0.1 mM dithiothreitol and 3.3 μg reconstituted protein in 50 mM Tris-HCl, 1 mM EDTA (pH 7.7). The assays were started by the addition of 1 nmole [^{14}C]oleoyl-CoA (final concentration 10 μM, 0.5 μCi/ml) in a total volume of 0.1 ml. Incubations were for 10 min at 37°C. Compounds were added in 5 μl dimethylsulphoxide and the mixture perincubated for 5 min. Reactions were terminated by the addition of propan-2-ol/heptane (4:1 v/v, 1 ml) with a recovery marker of [^{3}H]cholesteryl oleate (approximately 25 000 dpm). Lipids were then extracted by the addition of heptane (0.4 ml) and water (0.6 ml), removing 0.4 ml of the upper layer. Products were separated as described below.

2.5.7. Culture of CaCo-2 cells

CaCo-2 cells were cultured in DMEM supplemented with 20% FCS and 2 mM glutamine in 75 cm^2 flasks. Cells were passaged at a dilution of 1 to 10 using 0.05% trypsin-0.02% EDTA for cell detachment, and then cells were collected by centrifugation. Cells were grown for 5–6 days until confluent and then plated out (2 ml per well) on 12 well Costar plates and grown again for 7 days until confluent before use in an experiment.

2.5.8. Measurement of cholesteryl oleate formation in CaCo-2 cells

This was based on the method of Murthy et al. [19]. Medium was replaced with 600 μl DMEM(HEPES) containing 1% (w/v) fatty acid-free BSA and supplemented with 2 mM glutamine (which had been filtered through a 0.2 μm filter); plates were initially incubated for 20 min at 37°C. Compounds were then added in 10 μl DMSO to wells in triplicate; control wells either contained DMSO or no additions. After an incubation of 1 h, 300 μl [^{3}H]oleate medium was added containing [^{3}H]oleic acid (900 nmoles, 5 μCi/ml) in DMEM(HEPES) with 2 mM glutamine and 10.5% (w/v) fatty acid-free BSA. Plates were further incubated for 2 h. Each well was washed with 1 ml PBS, cells were removed into 1 ml 0.05% trypsin-0.02% EDTA and lipids were extracted using dichloromethane/methanol (2:1 v/v). Extracts were dried down under nitrogen and redissolved in hexane (1 ml). For protein determination, cells were removed from wells with 0.5 ml 0.2 M NaOH and diluted 1 in 3 with distilled water before protein measurement by the method of Lowry et al [20].

2.5.9. Separation of reaction products of cholesterol esterification assays

The dissolved reaction products were added to Techelut silica gel columns and eluted with 3 ml hexane:diethyl ether (95:5 v/v, microsomal and reconstituted assays) or with hexane:diethyl ether (95:5 v/v, cellular assays). The radioactivity of the eluate was detected by liquid scintillation counting. Where tlc was used to separate the lipids, samples were applied to silica gel F_{254} plates (5x20 cm) in 0.3 ml hexane and the plates developed with hexane:diethyl ether:acetic acid (95:5:0.5 v/v, microsomal and reconstituted assays) or hexane:diethyl ether:acetic acid (95:5:0.5 v/v cellular assays). Plates were scanned for radioactivity on a Rita 6800 tlc scanner before scraping the required regions into 10 ml scintillation fluid for counting.

2.5.10. Measurement of cholesterol synthesis in CaCo-2 cells [19]

Cells were preincubated for 20 min with 900 μl DMEM(HEPES) supplemented with 2 mM glu-

tamine. Compounds were added in 15 μl DMSO and preincubated for 1 h. Cholesterol synthesis was initiated by the addition of [^{14}C]acetate in 20 μl volume (final concentration, 500 mM). After an incubation of 2 h, wells were washed with PBS. The cells were removed into 2 M NaOH and then incubated at 95°C for 1 h to allow saponification of lipids. A recovery marker of [^{3}H]cholesterol (approximately 20 000 dpm/sample) was added, followed by extraction with dichloromethane:methanol (2:1 v/v, 5 ml). Samples were applied to aminopropyl Bondelut columns (equilibrated with 1 ml hexane) in 4 ml hexane. Radioactivity was determined in the eluate by liquid scintillation counting.

2.5.11. Preparation of cell extracts for HMG-CoA reductase assays

Cells were incubated overnight with DMEM(HEPES) containing 2 mM glutamine and 5% lipoprotein-deficient serum (LPDS). Compounds were then added to the cells in 10 μl DMSO and incubated for 2 h. The medium was removed from the cells and the wells were washed with PBS. To each well, 100 μl buffer A (0.1 M sucrose, 40 mM potassium dihydrogen phosphate, 30 mM EDTA, 50 mM potassium chloride and either 100 mM potassium fluoride or 100 mM potassium chloride, pH 7.4) containing dithiothreitol (5 mM) and Brij (0.25%, v/v) was added to solubilise the cells [21]. After 20 min at room temperature, resulting cell suspensions were homogenised. The homogenates were removed into microfuge tubes and the wells were washed with 100 μl buffer A containing 5 mM dithiothreitol (without Brij). Samples were centrifuged for 2 min at 13 000 × *g* and the supernatants were retained. Protein was measured by the method of Bradford [14]. Inclusion of potassium fluoride allowed measurement of expressed HMG-CoA reductase activity. To measure total HMG-CoA reductase activity, potassium fluoride was replaced by potassium chloride.

2.5.12. Measurement of HMG-CoA reductase activity in CaCo-2 cell extracts

The assay for HMG-CoA reductase was based on those used by Balasubramaniam et al. [22] and Smith et al. [23]. To 50 μl of the supernatant from the above preparation, buffer A containing dithiothreitol (5 mM) was added to give a final volume of 90 μl. The positive control contained purified human liver HMG-CoA reductase (initial concentration of 1 mg/ml) diluted with 1% BSA. Samples were assayed in duplicate. NADPH generating cocktail (containing 60 mM glucose-6-phosphate, 7.5 mM NADP, 5 mM dithiothreitol and 10 U/ml glucose-6-phosphate dehydrogenase in buffer A), 50 μl, was added and tubes were incubated at 37°C for 10 min. The reaction was started by the addition of 15 nmoles [^{14}C]HMG-CoA (final concentration 100 μM; total assay volume was 150 μl) and samples were incubated for a further 45 min. The assay was terminated by the addition of 10 μl of 6 M HCl, followed by 10 μl of [^{3}H]mevalonate as a recovery marker. Samples were incubated for a further 30 min to ensure complete production of mevalonic acid lactone. Denatured protein was removed by centrifugation at 13 000 × *g* for 5 min. Silica Techelut columns were pre-equilibrated with 1 ml toluene:acetone (3:1) and 100 μl of each of the supernatants was added to the columns. After the addition of 3 ml toluene:acetone, the eluate was discarded. Mevalonic acid lactone was then eluted with 4 ml toluene:acetone and radioactivity was determined.

2.5.13. Assay for microsomal cholesterol 7α-hydroxylase activity

Each assay contained 3 mM cysteamine HCl (final concentration), 0.1 mg microsomal protein, a trace amount of [^{14}C]cholesterol (250 000 dpm in 10 μl acetone), in 0.1 M Tris-HCl/0.05 M NaF buffer (pH 7.4) in a total volume of 1 ml. Compounds were added in 10 μl DMSO (controls were present both with and without this vehicle) and the mixture preincubated for 10 min at 37°C. Assays were started by the addition of NADPH to give a final concentration of 1 mM. After an incubation of 60 min, assays were terminated with 5 ml chloroform:methanol (2:1, v/v). Two extractions were carried out and the combined organic extracts applied to silica gel tlc plates in 0.2 ml chloroform:methanol (2:1, v/v). Plates were eluted with ethyl acetate:toluene (70:30, v/v). Radioactive peaks corresponding to [^{14}C]7α-hydroxy-

cholesterol and [^{14}C]cholesterol were scraped and counted [24]. Enzyme activity was expressed as the % conversion of cholesterol to 7α-hydroxycholesterol.

3. Results

Five sterols were synthesised for use in the following studies (Fig. 1): 6-fluoro-5-cholesten-3β-ol (6-fluorocholesterol, 1), 7-cholesten-3β-ol (7-cholestenol, 2), 6β-fluorocholestan-3β-ol (6β-fluorocholestanol, 3), 3-acetoxy-6-fluorocholestan-3β-ol (3-acetoxy-6-fluorocholestanol, 4) and 7-methoxy-5-cholesten-3β-ol (7-methoxycholesterol, 5). The shortened names in parentheses are used in the text, with the reference number used in Fig. 1.

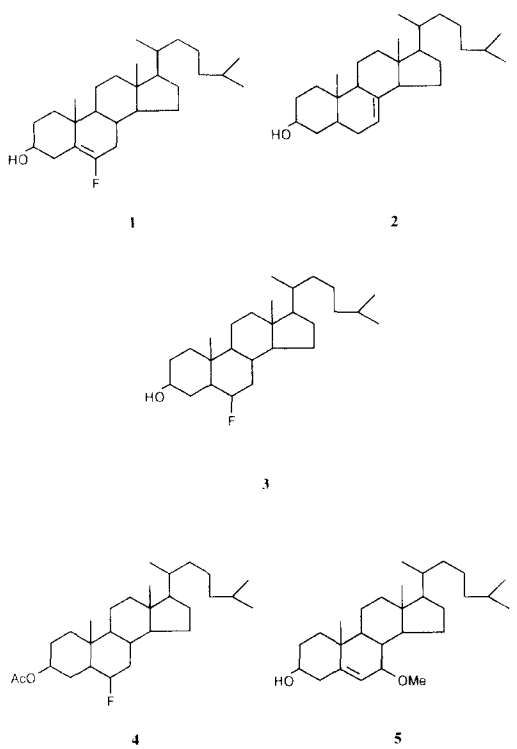


Fig. 1. Structures of sterols used in these studies. 1. 6-Fluorocholesterol; 2. 7-cholestenol; 3. 6β-fluorocholestanol; 4. 3-acetoxy-6-fluorocholestanol; 5. 7-methoxycholesterol.

3.1. Effects on cholesterol esterification in two assay systems

The five compounds were added in DMSO to two assay systems for ACAT activity, the commonly used rat liver microsomal assay [25] and a new development [17] of previously described reconstituted preparations in which the ACAT activity has been released from the microsomal membrane by treatment with detergent followed by reconstitution in a cholesterol-phospholipid vesicle [16]. This latter preparation allowed the study of the effects of the added compounds in a defined lipid environment. Because in our previous studies, 6,6-difluorocholestanol had been found to be a substrate for ACAT [8], extracts of incubations containing the present sterols were analysed on tlc to identify any products other than cholesteryl esters. The control activities for cholesterol esterification in the studies with the reconstituted system were variable and less than the microsomal activity, but the % change in activity caused by the added sterols was reproducible whatever the control value.

The two compounds that showed the greatest effect on ACAT activity in both the microsomal and the reconstituted assays were 6β-fluorocholestanol (3) and 7-methoxycholesterol (5) (Fig. 2). The inhibition due to 6β-fluorocholestanol was very small, but we found a concentration-dependent increase in a new product which migrated on tlc at a similar position to 6,6-difluorocholesteryl oleate, a product which had been found in our previous work [8]. In the reconstituted assay, 6β-fluorocholestanol (3) competitively inhibited the esterification of cholesterol and produced a similar concentration-dependent increase in a product with the tlc mobility of 6β-fluorocholestanyl oleate.

In contrast, 7-methoxycholesterol (5) was found to be one of the most potent sterol inhibitors of ACAT known, giving an IC_{50} of 10 ± 1 μM. No ester of this compound could be detected, although its mobility on tlc would be expected to be very similar to cholesteryl oleate, making its detection without differential radioactive labelling difficult. On the other hand, in the reconstituted system, where there is a greater molar excess of

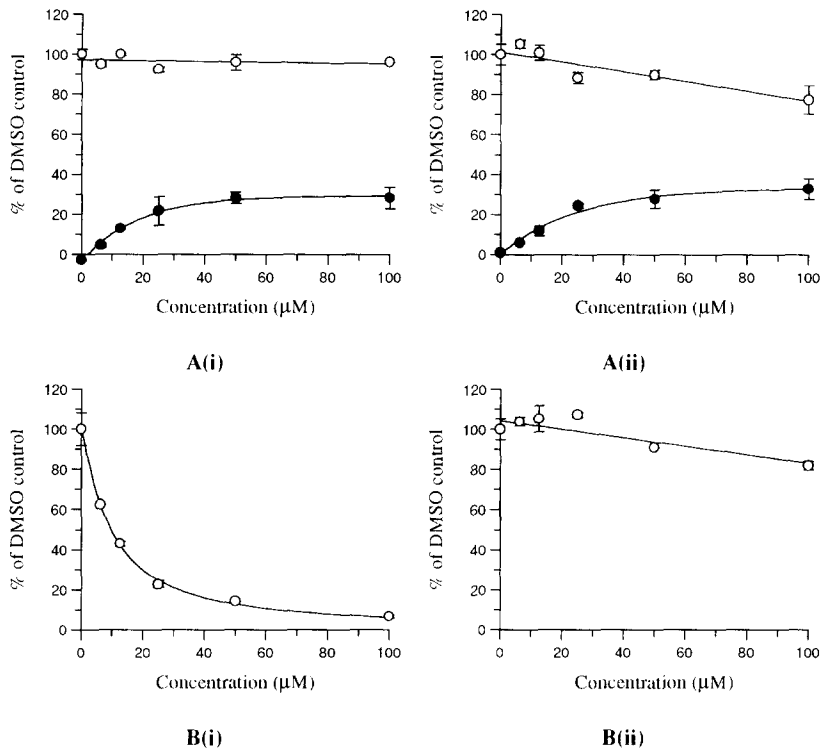


Fig. 2. Effect of cholesterol analogues on ACAT activity in the microsomal and reconstituted assay systems. Compounds were added in dimethylsulphoxide to assays which were carried out as described in Materials and methods. Open circles represent cholesteryl oleate produced and closed circles represent the amount of ester of the cholesterol analogue produced. Points are shown as the mean ± standard error (S.E.) of duplicate assays for microsomal assays and at least duplicate assays for the reconstituted assays for one representative example of two experiments. Values quoted are as a % of the control. Control rates were (pmol/min/mg cholesteryl oleate formed) A(i) 1025 ± 27 ($n = 2$), A(ii) 410 ± 21 ($n = 4$), B(i) 1101 ± 90 ($n = 2$), B(ii) 410 ± 21 ($n = 4$)A. 6-fluorocholesterol (1); B. 7-methoxycholesterol (5). (i) denotes a microsomal assay and (ii) denotes a reconstituted assay.

cholesterol, 7-methoxycholesterol showed very little effect (Fig. 2).

6-Fluorocholesterol (1) showed some inhibition of ACAT in the microsomal assay (39 ± 6% at 100 μM) but none in the reconstituted assay (not shown). The effects of 7-cholestenol (2) were similar but the ester, 3-acetoxy-6-fluorocholestanol (4) had no effect in either system.

The difluorocholestanols studied in our previous work [8,9] were examined again in order to see if they had any effect in the reconstituted assay. Both compounds were found to be weak inhibitors of ACAT activity in microsomes, causing inhibition of 26 ± 3% and 33 ± 5% respectively at 100 μM (Fig. 3). Unlike in our earlier work [8], where no product from 7,7-difluorocholestanol was detected, both were also competitive substrates in the reconstituted assay. The more sensitive tlc scanner used in the present work and scintillation counting of the whole tlc plate scraped off in 1 cm bands allowed the detection of product from 7,7-difluorocholestanol, in contrast to our previous report.

3.2. Effects of compounds on cholesterol esterification in CaCo-2 cells

The effects of inhibition of ACAT activity have frequently been studied in the human intestinal cell line CaCo-2, which is an in vitro model for the absorptive cells in the intestine and is thought to reflect the role of intestinal cholesterol esterifi-

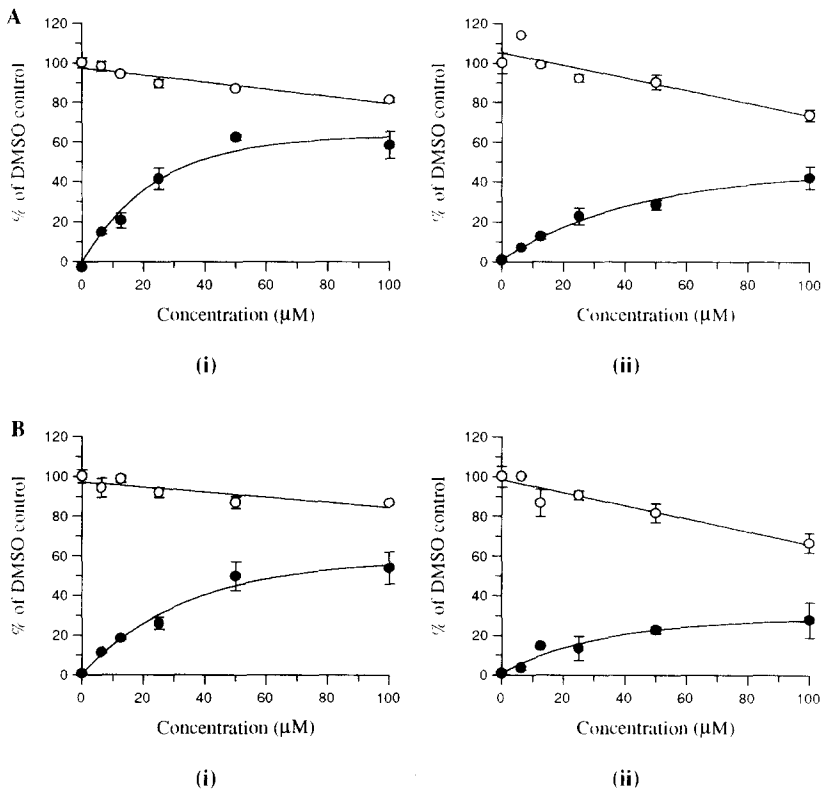


Fig. 3. Effect of difluorosterols on ACAT activity in the microsomal (i) and reconstituted assay (ii) systems. Compounds were added in dimethylsulphoxide and assays carried out as described in Materials and methods. Open circles show the cholesteryl oleate produced and closed circles the fluorocholesteryl ester. Points are shown as the mean ± standard error (S.E.) of duplicate assays for microsomal assays and at least duplicate assays for the reconstituted assays for one representative example of two experiments. The control values were (pmol/min/mg cholesteryl oleate formed): A(i) 1155 ± 42 ($n = 2$), B(i) 1378 ± 22 ($n = 2$) and A(ii) and B(ii) 410 ± 21 ($n = 4$). A. 6,6-difluorocholestanol; B. 7,7-difluorocholestanol.

cation during its absorption. CaCo-2 cells have also been widely used to study ACAT activity and have been a convenient screen for ACAT inhibitors. 6-Fluorocholesterol (1), 7-cholestenol (2) and 3-acetoxy-6-fluorocholestanol (4) had no effect. In contrast, 6β-fluorocholestanol (3) and 7-methoxycholesterol (5) increased the incorporation of oleic acid into cholesteryl ester (Fig. 4). 6β-Fluorocholestanol caused a linear concentration-dependent increase of cholesterol esterification (up to 94 ± 32% at 100 μM) as well as presumably acting as a substrate itself in the cells, since an additional product was detected by tlc, as with the studies in the microsomal preparations.

Fig. 5 shows the results obtained with the difluorosterols. 6,6-Difluorocholestanol increased cholesteryl oleate synthesis in a concentration-dependent manner (up to 3.6-fold at 100 μM) and was also a substrate for esterification. 7,7-Difluorocholestanol was also esterified and increased esterification of cholesterol.

3.3. Effect of 6β-fluorocholestanol (3) and 7-methoxycholesterol (5) on cholesterol synthesis

It is possible that the increase in cholesterol esterification observed in the CaCo-2 cells in the presence of the compounds was due to a greater supply of substrate cholesterol resulting from an increased rate of intracellular synthesis. To test

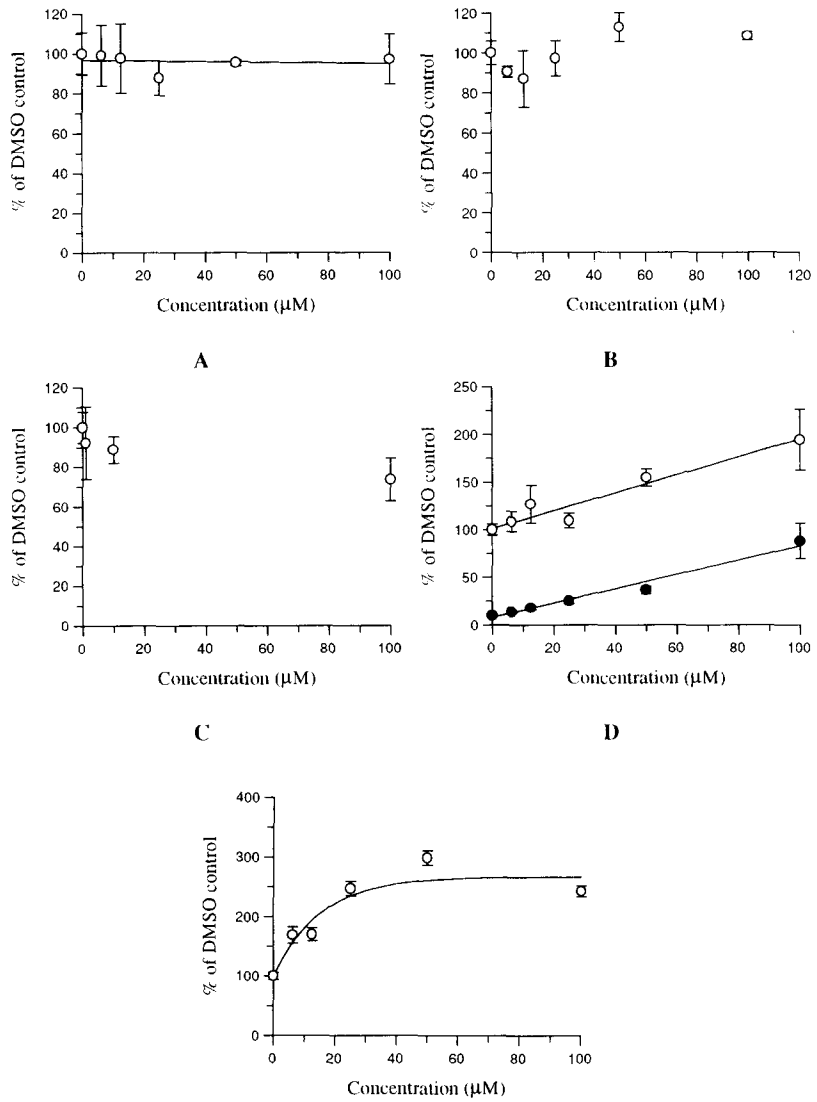


Fig. 4. Effect of cholesterol analogues on [^{3}H]oleate incorporation into [^{3}H]cholesteryl oleate in CaCo2 cells. Cells were preincubated with DMEM(HEPES) containing 2 mM glutamine for 20 min at 37°C. Compounds were added in dimethylsulphoxide for 1 h before the addition of the [^{3}H]oleate substrate for a further two hour incubation. Assays were carried out as described in the Materials and methods. Open circles represent cholesteryl oleate produced and closed circles represent the amount of ester of the cholesterol analogue produced. Points are shown as the mean ± standard error (S.E.) of triplicate assays of one representative example of two experiments. Controls, containing dimethylsulphoxide alone, were the mean of 3 assays for A and B, and of 9 assays for C, D and E. Control values (pmoles/h/mg cholesteryl oleate produced) were A 282 ± 30 ($n = 3$), B 300 ± 18 ($n = 3$), C 966 ± 46 ($n = 9$), D and E 520 ± 31 ($n = 9$).A. 6-fluorocholesterol (1); B. 7-cholestenol (2); C. 3-acetoxy-6-fluorocholestanol (4); D. 6β-fluorocholestanol (3); E. 7-methoxycholesterol (5).

this possibility, the compounds were added to CaCo-2 cells and incubated in the presence of [^{14}C]acetate. 25-Hydroxycholesterol was used as a model compound since it is known to affect both cholesterol synthesis and esterification in several types of cell in culture [25].

6β-Fluorocholestanol (3) had little effect on cholesterol synthesis from acetate, but 7-methoxycholesterol (5) increased cholesterol synthesis by 1.5-fold at 25 μM (Fig. 6). This increase might be sufficient to drive the observed additional synthesis of cholesteryl ester. 25-Hydroxycholesterol produced the expected decrease in cholesterol synthesis (50% at 100 μM).

To examine the effect of these compounds further, HMG-CoA reductase activity was measured in cell extracts after incubation with the sterols. Measurements were carried out in the presence of fluoride, to inhibit the effect of dephosphorylation by protein phosphatases, as well as in its absence. 6β-Fluorocholestanol (3) had little effect on HMG-CoA reductase activity in the presence or absence of fluoride (Table 1). 7-Methoxycholesterol (5) was found to decrease HMG-CoA reductase activity by 44%–56% although it surprisingly increased overall incorporation of acetate into cholesterol in the intact cells (Fig. 6B). 7-Methoxycholesterol was found to have no effect on purified human HMG-CoA reductase at 6.25–100 μM (data not shown).

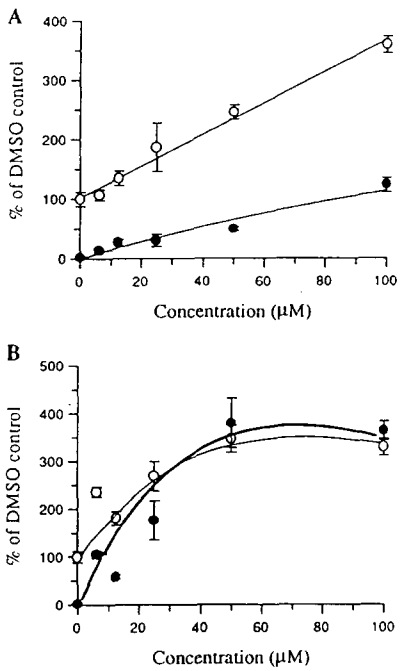


Fig. 5. Effect of difluorocholestanols on [^{3}H]oleate incorporation into [^{3}H]cholesteryl oleate in CaCo-2 cells. Cells were preincubated with DMEM(HEPES) containing 2 mM glutamine for 20 min at 37°C. Compounds were added in dimethylsulphoxide for 1 h before the addition of the [^{3}H]oleate substrate for a further two hour incubation. Assays were carried out as described in the Materials and methods. Open circles represent cholesteryl oleate produced and closed circles represent the amount of ester of the cholesterol analogue produced. Points are shown as the mean ± standard error (S.E.) of triplicate assays for one representative example of two experiments. Results are shown as % of control. Control values (pmol/h/mg cholesteryl oleate formed) were 85 ± 15 ($n = 3$).A. 6,6-difluorocholestanol; B. 7,7-difluorocholestanol.

3.4. Effect of compounds on liver microsomal cholesterol 7α-hydroxylase activity

The compounds were incubated with rat liver microsomes at concentrations up to 50 μM. Concentration-response data were only obtained for 7-cholestenol (2) , which was the most potent compound, and gave an IC_{50} of 18 ± 2 μM (Fig. 7). Inhibition of hydroxylation was observed for 7-methoxycholesterol (5) and 6β-fluorocholestanol (3) at 50 μM (Table 2).

4. Discussion

Table 3 summarises our results and shows that relatively small changes in structure make a large change in the sensitivity of the enzymes to the sterols, a sensitivity that relates to the nature of the reaction being catalysed. Of the compounds tested, only the cholesteryl ester was unable to influence any of the enzyme-catalysed reactions studied.

7-Cholestenol (2, also known as lathosterol) has been shown to be a substrate for ACAT in a detergent-solubilised assay system [26]. In this procedure, the sterols were added in excess over cholesterol, allowing the individual esters to be measured. It is possible that 7-cholestenol was also esterified in our studies, but, since the tlc mobility of the oleate ester of cholesterol and 7-cholestenol are the same, it would not have been possible to detect in our experiments.

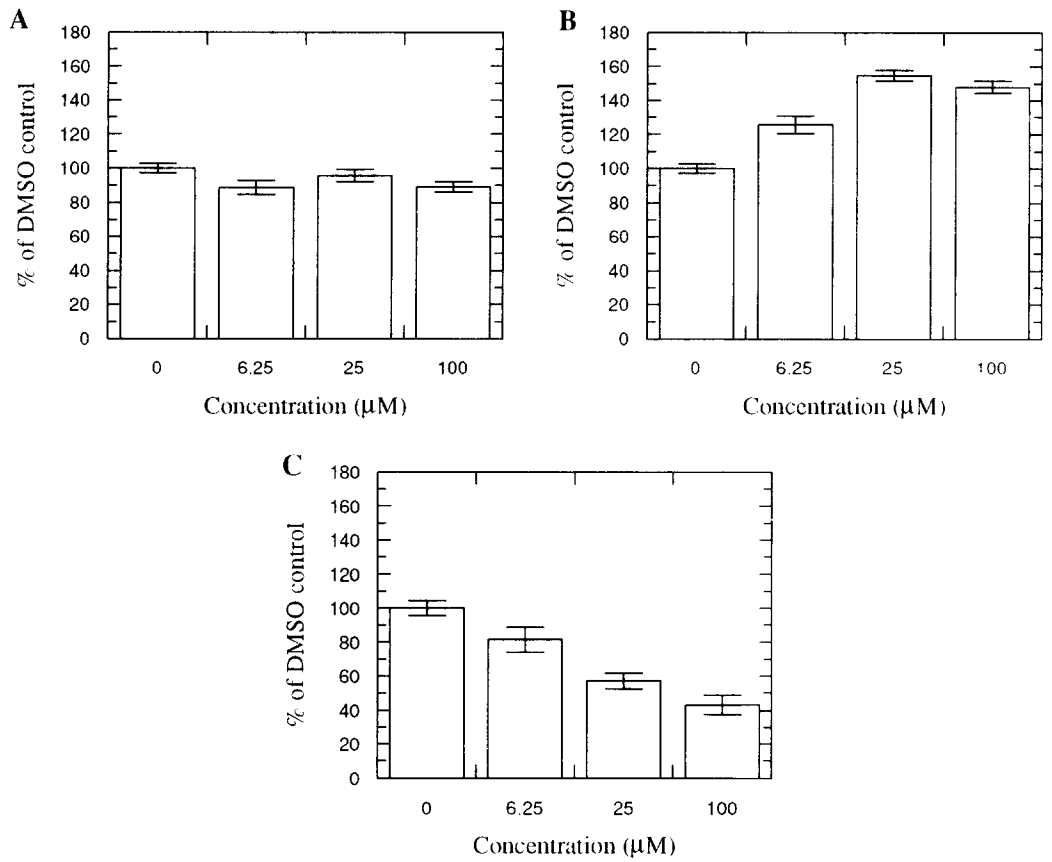


Fig. 6. Effect of cholesterol analogues on [^{14}C]acetate incorporation into [^{14}C]cholesterol. Cells were preincubated with DMEM(HEPES) containing 2mM glutamine for 20 min at 37°C. Compounds were added in dimethylsulphoxide for 1 h before the addition of the [^{14}C]acetate substrate for a further two hour incubation. Assays were carried out as described in the Materials and methods. Reaction products were separated on aminopropyl columns or by tlc on silica gel plates. Bars are shown as the mean ± standard error (S.E.) of triplicate assays for one representative example of two experiments. Controls, containing dimethylsulphoxide alone, were the mean of 5 assays. The control values were (pmoles acetate incorporated) A and B 1776 ± 46 ($n = 5$) and C835 ± 36 ($n = 5$).A. 6β-fluorocholestanol (3); B. 7-methoxycholesterol (5); C. 25-hydroxycholesterol.

7-Cholestenol (2) inhibited the hydroxylation of cholesterol, as did 6β-fluorocholestanol (3) and 7-methoxycholesterol (5). In agreement with our previous work [8], cholesterol 7α-hydroxylase seems to be sensitive to the presence of strongly electron-withdrawing groups on C-6 and the presence of a double bond at the site of hydroxylation also leads to some inhibition. Substitution with fluorine at C-7 led to a compound that showed no activity against the enzyme [8]. Because of the relatively crude assay that is available for cholesterol 7α-hydroxylase, in which the microsomal concentration of cholesterol and added sterols in the region of the enzyme is unknown, further definition of the nature of the interaction between the test sterols and the enzyme is not possible.

The fluorocholestanols were all substrates for cholesterol esterification in the microsomal, reconstituted and cellular assays. It is likely that the fluorosterols were able to penetrate into the substrate pool of cholesterol readily and hence compete at the active site of ACAT. They also stimulated cholesterol esterification in CaCo-2 cells, perhaps by a similar mechanism to 25-hydroxycholesterol [25,27]. 7-Methoxycholesterol (5) inhibited microsomal ACAT surprisingly effec-

tively for a simple sterol, but probably was diluted out by the excess phospholipid and cholesterol in the reconstituted system, leading to no observable effect. In CaCo-2 cells, it stimulated cholesterol esterification.

The most subtle effect observed in our series of compounds was the fact that 6-fluorocholesterol (1) did not influence ACAT activity, whereas 6β-fluorocholestanol (3) and 6,6-difluorcholestanol were both competitive substrates for cholesterol. The interaction of the fluorosterol with the cholesterol binding site may be influenced by the fact that the orientation of the fluorine atom in 6-fluorocholesterol is markedly changed by the presence of the 5,6 double bond compared with the two fluorocholestanols. In 6-fluorocholesterol (1), the electron density associated with the fluorine is in the plane of the sterol ring system, whereas in 6β-fluorocholestanol (3) and 6,6-difluorocholestanol, the 6β-fluorine provides substantial electron density out of the plane. Such a difference may be enough to affect interaction with a sterol binding site.

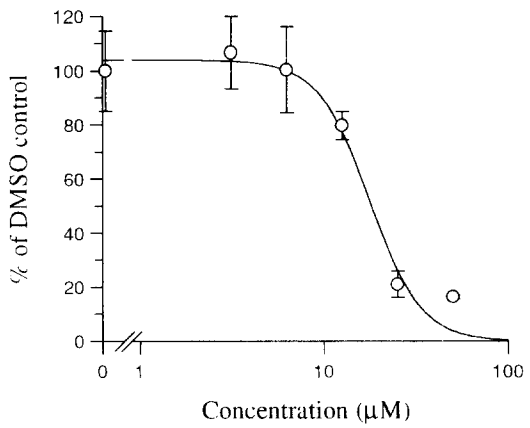
Unlike 25-hydroxycholesterol, which inhibits cholesterol synthesis, there was some evidence that 7-methoxycholesterol (5) may actually stimulate cholesterol synthesis. Direct effects on purified HMG-CoA reductase were not observed, which implies that the effects depend upon the environment of the enzyme in the membrane or on the integrated regulatory system of the cell.

Recently, it has become clear that compounds that have been regarded as ACAT inhibitors in the literature for many years, such as progesterone [28], may not interact directly with the

Table 1
Effect of 6β-fluorocholestanol (3) and 7-methoxycholesterol (5) on HMG-CoA reductase activity in CaCo-2 cells

Additions	Specific activity (pmoles/min/mg)	% of DMSO control
No fluoride		
None	61.3 ± 2.8	114 ± 5
Dimethylsulphoxide	53.8 ± 1.0	100 ± 2
6β-Fluorocholestanol (3)	47.5 ± 1.0	88 ± 2
7β-Methoxycholesterol (5)	23.9 ± 1.0	44 ± 2
25-Hydroxycholesterol	5.3 ± 0.7	10 ± 1
With fluoride (100 mM)		
None	33.6 ± 2.2	97 ± 7
Dimethlysulphoxide	34.6 ± 2.8	100 ± 8
6β-Fluorocholestanol (3)	36.7 ± 2.7	106 ± 8
7β-Methoxycholesterol (5)	19.5 ± 2.7	56 ± 8
25-Hydroxycholesterol	8.8 ± 2.7	25 ± 8

Three wells of a plate were treated in the same manner with 6β-fluorocholestanol and 7-methoxycholesterol using 25-hydroxycholesterol as a control and they were incubated as described in Materials and methods. Compounds were added at a concentration of 100 μM. Cells were harvested and duplicate assays were carried out for each well as described in Materials and methods. Values are shown as mean ± standard error (S.E.) for one representative example of two experiments. The mean values for activity of purified HMG-CoA reductase (2 ng per assay) added as a control to each set of assays was 2.1 ± 0.1 pmoles/min of mevalonic acid produced.



$IC_{50} = 18 \pm 2\ \mu M$

Fig. 7. Inhibition of cholesterol 7α-hydroxylase activity by 7-cholestenol (2). Assays were carried out as described in the Materials and methods. Assays were carried out in triplicate. Points represent the mean ± standard error (S.E.) of a single experiment. The control containing DMSO alone was 1.16 ± 0.17% ($n = 3$) nd the control with no additions was 1.50 ± 0.10% ($n = 3$), expressed as % conversion of labelled cholesterol to 7α-hydroxycholesterol. IC_{50} was calculated by the Grafit software package, Version 3.01, with a defined endpoint at 0 and simple weighting.

Table 2
Effects of compounds on microsomal cholesterol 7α-hydroxylase activity

Compound (50μM)	% Conversion (mean ± SD, n = 3)	% of DMSO control
Dimethylsulphoxide	1.16 ± 0.03	100
6-Fluorocholesterol (1)	1.11 ± 0.03	96
7-Cholestenol (2)	0.17*	15
6β-Fluorocholestanol (3)	0.50 ± 0.14	43
7-Methoxycholesterol (5)	0.26 ± 0.07	22

Rat liver microsomes were incubated with the compounds shown as described in Materials and methods. Results are shown as a % conversion of the added [^{14}C]cholesterol to 7α-hydroxycholesterol for triplicate determinations. See Fig. 7 for the full data for 7-cholestenol.

enzyme but may in fact inhibit the movement of substrate cholesterol to the enzyme [28]. HMG-CoA reductase inhibitors that have been reported to inhibit cellular cholesterol esterification may well also do this by a similar mechanism [29]. It is likely that our fluorosterols, since they are substrates for ACAT, and probably also 7-methoxycholesterol (5), do not interact with this enzyme at the same site as many of the recently described amide and urea-based ACAT inhibitors [30]. The stimulation of cholesterol esterification in the CaCo-2 cells by our sterols and also by 25-hydroxycholesterol is probably due to a reorganisation of the distribution of cellular cholesterol, promoting its delivery to ACAT in the endoplasmic reticulum. The mechanism by which this process occurs is not known, although evidence has been presented that a threshold concentration of cholesterol must be exceeded in cells before ACAT activity is stimulated [31]. Cholesterol itself has been shown to activate ACAT in cells expressing the cloned enzyme, but naturally devoid of cholesterol [27]. One possibility, though difficult to prove, is that the cholesterol-like sterol concentration in the membrane could influence the enzyme activity through a biophysical mechanism involving membrane fluidity.

Bearing in mind the unfunctionalised, highly hydrophobic nature of cholesterol, it might have been expected that analogues such as those described here would behave identically. However, our results show that the use of specifically designed sterols based on considerations of enzyme-catalysed reactions for which cholesterol is substrate can yield details of interactions at both the enzyme and cellular level. This approach has not been extensively developed rationally. In principle, it can provide both molecular and metabolic

Table 3
Summary of the effect of cholesterol analogues on cholesterol esterification in different assay systems and on cholesterol 7α-hydroxylase activity

Compound	Effect on cholesterol esterification in different assay systems			
	Microsomal	Reconstituted	CaCo-2	Cholesterol 7α- hydroxylase
6-Fluorocholesterol (1)	→	→	→	→
7-Cholesterol (2)	→	→	→	↓↓
6β-Fluorocholestanol (3)	substrate	substrate	substrate, ↑	↓↓
3-Acetoxy-6-fluorocholestanol (4)	→	→	→	—
7-Methoxycholesterol (5)	↓↓	→	↑↑	↓
6,6-Difluorocholestanol	substrate	substrate	substrate, ↑	↓*
7,7-Difluorocholestanol	substrate	substrate	substrate, ↑	→*
25-Hydroxycholesterol	↑(normal fed) →(cholesterol fed)	→	↑	—

* ref [8].

information through the spectroscopically sensitive fluorine atoms and product analysis, respectively. These studies could be extended further to model cell systems in which the enzymes examined in the present work are expressed under gene regulatory control to investigate the effect of the interaction of specific sterols with binding sites in regulatory proteins controlling gene expression.

Acknowledgements

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