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# In vitro modulation of rat adipocyte ghost membrane fluidity by cholesterol oxysterols

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*Received 28 July 1994; accepted 9 January 1995*

**Abstract.** The effects of cholesterol and cholesterol-derived oxysterols (cholestanone, cholestenone, coprostanone and epicoprostanol) on adipocyte ghost membrane fluidity were studied using a fluorescence depolarization method. The fluorescence anisotropy of the treated membranes was determined using 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH). Cholestanone and cholesterol decreased membrane fluidity at both the concentrations tested (10 & 50 μM) while the rest of the sterols did not exert any significant effect on membrane fluidity. In the presence of epinephrine, cholestanone partitioned more towards the lipid core but cholesterol partitioning was not affected. The fusion activation energies (ΔE) obtained for membranes preincubated with cholestanone (8.6 kcal/mol) and cholesterol (8.2 kcal/mol) were not significantly different from that of untreated membranes (8.3 kcal/mol). Membranes preincubated with cholestanone and cholesterol did not exhibit any change in lipid phase throughout the temperature range (10–45 °C) tested. The sterols were found to inhibit fisetin-induced phospholipid methylation in isolated rat adipocytes in the rank order of cholesterol > epicoprostanol > cholestanone = cholestenone = coprostanone, while basal methylation was unaffected. When adipocytes were preincubated with the sterols before the addition of fisetin, cholestanone and cholestenone showed 74% and 66% inhibition of maximal methylation respectively. These results indicated that cholesterol oxysterols interact differently with rat adipocyte membranes, with cholestanone interacting more with phospholipids located at the inner lipid bilayer (e.g. phosphatidylethanolamine) while cholesterol interacts more with phosphatidylcholine located at the outer lipid bilayer. This differential interaction may cause selective changes in membrane fluidity at different depths of the bilayer and thus may modulate the activities of membrane-bound proteins such as enzymes and receptors.
**Key words.** Rat adipocyte; adipocyte ghost membrane; membrane fluidity; cholesterol oxysterols; fluorescence anisotropy.

Biological membranes are known to function not merely as a physical boundary to the living cells. They also play a pivotal role in the regulation of cellular functions. Many membrane-associated proteins (receptors, enzymes, transporters) contain hydrophobic transmembrane sequences which are anchored to the lipid bilayer, thus requiring the presence of specific lipids for their actions[1–4]. Membrane fluidity or the thermal motion of membrane components is now believed to affect the mobility[5,6] and conformation of receptors embedded in the bilayer[7–10]. Therefore, any gross membrane fluidity changes will affect the physiological functions of cells in normal and abnormal physiological states such as primary lipoprotein lipase deficiency[11], lymphoma[5] and leukemia[12].

The most important factors influencing membrane fluidity are the cholesterol and phospholipid composition of the membrane[13,14]. However, other cholesterol oxysterols, such as 3β-hydroxysterols and ketosterols, have also been reported to influence cellular functions[15–17]. The presence of these sterols in normal cells may not be prominent but their content in pathological conditions may be high enough to change normal cell functions. Abnormal plasma lipid levels are known to be caused by dietary intake[18] and metabolic disorders such as diabetes[19], hypertriglyceridaemia[20], and hypobetalipoproteinaemia[21]. Changes in membrane fluidity can also be elicited by the binding of drugs[22] and hormones[23,24] to their receptors or by methylation of membrane phospholipids[25].

Membrane fluidity has been studied by various methods; the most commonly used method is fluorescence polarization. In this study, two lipophilic probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethylamino-diphenylhexatriene (TMA-DPH), were used to study the effect of several cholesterol oxysterols on adipocyte ghost membrane fluidity. These two probes were chosen for their established usage and good theoretical documentation[26,27]. They are known to survey different environments in the bilayer, as DPH partitions deep into the lipid core of the bilayer[13] while TMA-DPH reports the motion of phospholipid acyl chains at the interface and head group region of the bilayer[28]. The oxysterols used in this study (cholesterol, cholestanone, cholestenone, coprostanone and epicoprostanol) have

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been shown to have differential effects on lipolysis in isolated rat adipocytes in our laboratory[29]. We are now interested to see if their effects involve any changes in membrane fluidity. The data obtained from this study may provide a better understanding of the effects of cholesterol oxysterols on lipid metabolism in individuals with abnormal serum oxysterols.

## Materials and methods

**Chemicals.** Cholesterol (5-cholestene-3$\beta$-ol), cholestanone (5$\alpha$-cholestan-3-one), cholestenone (4$\alpha$-cholesten-3-one), coprostanone (5$\beta$-cholestan-3-one), epicoprostanol (5$\beta$-cholestan-3$\alpha$-ol), collagenase type II (E.C. 3.4.24.3), (-)-isoproterenol-HCl, (-)-epinephrine, 2,7-dichlorofluorescein, bovine serum albumin and Coomassie brilliant blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fisetin was obtained from Extrasynthese (Genay, France). 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). S-adenosyl-L-[methyl-$^3$H]methionine (83 Ci/mmol) and biodegradable scintillant (BSC) were purchased from Amersham (Amersham Far East, Singapore). Silica gel G-60 TLC plates were obtained from Merck (Germany). Other chemicals used were of analytical grade.

**Animals.** Male Wistar rats (180–220 g) were obtained from the Animal Laboratory Centre, National University of Singapore and fed on a rat diet (Glen Forrest Stockfeeders, WA, Australia) and water ad libitum.

**Test compounds.** Cholesterol and its oxysterol derivatives were found to be chromatographically pure and were freshly prepared in absolute ethanol to give 20 mM stock solutions. Other test compounds were solubilized either in fluorescence assay buffer (pH 7.4) containing 120 mM NaCl, 10 mM $MgCl_2$, and 25 mM Tris-HCl or in modified Krebs-Henseleit-Hepes (MKHH) buffer (pH 7.4) consisting of 130 mM NaCl, 10 mM $MgCl_2$, 5 mM KCl, 0.1 mM Na-EDTA and 25 mM Hepes. For fluorescence studies, oxysterol stock solutions were injected into assay buffer (pH 7.4) to yield a 100× diluted suspension and sonicated in a sonication bath for 15 min before use.

**Adipocyte isolation and preparation of adipocyte suspension.** Male Wistar rats (180–220 g) were killed by cervical dislocation. Epididymal fat pads were removed and kept in MKHH buffer (pH 7.4) before being transported to the laboratory. Main blood vessels were excised and the fat pads cut into smaller pieces and randomly distributed into 25 ml polycarbonate conical flasks containing collagenase (2 mg/ml) in MKHH buffer and supplemented with 3% BSA. Digestion was carried out under 95% $O_2$ and 5% $CO_2$ atmosphere at 37 °C for 34 min in a shaking water bath. The adipocytes were isolated by filtration through four layers of cotton gauze and centrifuged at 1200 rpm for 30 s. The infranatant and cell pellet (stromal cells) were removed by aspiration and the remaining packed fat cells were washed with 10 ml of MKHH buffer by gently inverting the centrifuge tube repeatedly. This washing step was repeated three times. After the final wash, the packed adipocytes were suspended in MKHH buffer supplemented with 3% BSA in a 1 to 3 ratio (by volume). Aliquots (0.5 ml) of this cell suspension were used in subsequent methylation studies.

**Preparation of rat adipocyte ghost membranes.** Adipocytes were isolated as described above. After the final wash, the isolated adipocytes were packed by centrifuging at 1200 rpm for 1 min using a Jouan BR 3.11 centrifuge. The packed fat cells were transferred to 50 ml centrifuge tubes and lysed with an equal volume of hypotonic lysing buffer composed of 2.5 mM $MgCl_2$, 1 mM $KHCO_3$, 2 mM Tris-HCl, pH 7.4 and protease inhibitors: phenylmethylsulfonyl fluoride [200 µM], leupeptine [1 µg/ml] and EGTA [3 mM][30]. The lysing buffer was maintained at 20 °C to reduce trapping of membrane in the congealed fat cake. The suspended fat cells were lysed either by vortexing or by vigorous shaking for 3 min. The lysing mixture was centrifuged at 2500 rpm (900 *g*) for 5 min and the infranatant was separated from the fat cake and kept on ice. An equal volume of lysing buffer was added to the fat cake in the centrifuge tubes and vortexed for 5 min to lyse the remaining cells. The tubes were then re-centrifuged as before. The infranatant was aspirated using a glass pasteur pipette and pooled with the previously obtained infranatant. The pooled infranatant was centrifuged (100,000 *g*) for 15 min at 4 °C using a Beckman L8-80M model ultracentrifuge. The pellet obtained was washed in ice-cold lysing buffer and pelleted as before. The washing was repeated twice and the final pellet was resuspended in lysing buffer. The membrane suspension was aliquoted into 300 µl fractions and stored at −70 °C. The protein content was estimated using the method of Bradford[31].

**Fluorescence labelling and measurement.** Prior to labelling, ghost membrane suspension at a final concentration of 50 µg/ml was preincubated with sterol (at a final concentration of either 10 µM or 50 µM) at 37 °C for 30 min. Thereafter, the membranes were pelleted at 85004 *g* for 10 min using an Eppendorf microfuge. The membranes were resuspended in assay buffer (pH 7.4) by vortexing at high speed and subjected to the appropriate probe labelling. Ghost membranes were labelled with either DPH or TMA-DPH for fluorescence studies. For DPH labelling, the DPH solution (4 mM in tetrahydrofuran) was injected into a vigorously stirred assay buffer (pH 7.4) to yield a 2000-fold diluted dispersion solution. This dispersion solution was stirred for 15 min in the dark and then mixed with pre-treated ghost

membrane suspension (100 µg/ml) in a 1:1 ratio (v/v). The labelling was carried out by incubating at 37 °C for 30 min. The labelled ghost membranes were equilibrated to 25 °C and fluorescence polarization was measured using a ISS K2 multifrequency phase fluorometer (ISS Inc., Champaign, IL, USA) with a temperature-programmable circulation bath attached to the cuvette holder. An L-format measurement was employed with the excitation wavelength set at 360 nm and the emission wavelength set at 430 nm for both DPH and TMA-DPH fluorescence measurements. The excitation and emission slit widths were 1 mm and 2 mm respectively. Labelling with TMA-DPH was as for DPH except that the incubation time was reduced to 2 min for labelling of the outer leaflet of the bilayer[28]. TMA-DPH stock was prepared in methanol. A suspension of unlabelled ghost membranes was used as a blank in the reference cuvette to correct for light scattering. The blank values were automatically subtracted from all readings. The final sample absorbance at the emission wavelength was less than 0.15 and at this concentration, depolarization due to sample turbidity was approximately 8% of the total polarization values and was thus not corrected for in subsequent calculations.

**Theoretical consideration.** Steady-state depolarization experiments were expressed in terms of fluorescence anisotropy $r_s$, with

$$r_s = \frac{I_\parallel - I_\perp(G)}{I_\parallel + 2I_\perp(G)}$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensity observed with the analysing polarizer parallel and perpendicular to the polarized excitation beam respectively and (G) is the wavelength-dependent grating factor, which was set at a value of 1. The steady-state anisotropy ($r_s$) can be resolved into two components: the fast decaying or kinetic component ($r_f$), and the slow decaying or static component ($r_\infty$). The static component is more predominant in biological membranes and is determined by the molecular packing (orientational order parameter, $S$) in the vicinity of the fatty acyl chain and cholesterol entities of the lipid bilayer[26,32].

The equivalent microviscosity determined using DPH can be calculated using the approximate relationship derived by Shinitzky and Barenholz[13] as:

$$\tilde{n}\ (\text{poise}) = \frac{2.4r_s}{0.364 - r_s}$$

The fusion activation energy for viscosity, $\Delta E$, can be determined from the slope of the Arrhenius plot, ln $\tilde{n}$ vs 1/T, fitted by the least square method. The term microviscosity was used in this study for ease of discussion as it is by definition an operational term and is therefore only of conceptual importance as pointed out by Shinitzky and Barenholz[13].

**Methylation of membrane phospholipid.** Adipocytes were pre-incubated with [$^3$H]S-adenosyl-methylmethionine (8 µM, 2 µCi) in MKHH buffer (pH 7.4) in 15 ml polyethylene tubes for 15 min at 37 °C as described earlier[33]. Various test compounds were then added to the mixture separately and incubated for another 30 min at 37 °C. The reaction was terminated by adding 3.5 ml of chloroform:methanol:hydrochloric acid (2:1:0.2, vol/vol) to the reaction mixture. The mixture was vortex mixed at high speed for 1 min and centrifuged at 2500 *g* for 10 min to hasten phase separation. The aqueous layer (top layer) was aspirated out and the chloroform layer was gently washed twice with KCl in 50% methanol. The washed chloroform layer was transferred to pre-weighed glass scintillation vials and evaporated to dryness under reduced pressure. The weight of the lipid extract was noted and the lipid redissolved in 100 µl of chloroform:methanol (2:1, vol/vol) mixture.

**Thin layer chromatography (TLC).** Aliquots (30 µl) were taken for TLC separation using 250 µm thick, 20 × 20 cm silica G-60 gel TLC plates. The plates were developed using the following solvent system: n-hexane:diethyl ether:acetic acid; 80:20:1 (vol/vol/vol). The developed plates were either sprayed with 2,7-dichlorofluorescein so that lipids appeared as orange coloured bands under long wavelength UV light, or placed in an iodine chamber, when lipid appeared as brown bands. The phospholipid bands (which stayed at the origin of application) were scraped off and transferred into scintillation vials. The radioactivity was measured after the addition of a biodegradable liquid scintillant. The counts per gram lipid (CPM/g lipid) were calculated and expressed as percentage of basal level or percentage of inhibition, where appropriate.

**Protein determination.** The protein content of the ghost membranes was determined using Coomassie blue binding assay as described by Bradford[31].

**Statistical analysis.** Values were obtained from at least three separate experiments carried out in triplicate and expressed as means ± SD. Student's *t*-test[34] was used to evaluate the significance of differences between the mean values.

## Results

The steady-state fluorescence anisotropy ($r_s$) for adipocyte ghost membranes treated with various cholesterol oxysterols showed differential effects with the polyene hydrocarbon probes DPH and TMA-DPH. As shown in the table, the ghost membranes exhibited significant increases in $r_s$ values after preincubation with either 10 or 50 µM of cholestanone or cholesterol but not with cholestenone, coprostanone or epicoprostanol, using either DPH or TMA-DPH probes. Upon incubation with 1 µM epinephrine, the $r_s$ value of the un-

Table. Effect of various cholesterol oxysterols on DPH and TMA-DPH steady-state fluorescence anisotropy in adipocyte ghost membranes.

| Membrane incubated with[a] | Concentration μM | Anisotropy ($r_s$)[b] | |
|---|---|---|---|
| | | DPH | TMA-DPH |
| Membrane | 50 μg/mL | 0.142 (0.166)** | 0.146 (0.148) |
| Cholestanone | 10 | 0.170 (0.186)** | 0.162 (0.159) |
| | 50 | 0.200 (0.219)** | 0.161 (0.157)** |
| Cholesterol | 10 | 0.171 (0.174) | 0.165 (0.161) |
| | 50 | 0.202 (0.202) | 0.177 (0.182)* |
| Cholestenone | 10 | - | 0.149 (0.158) |
| | 50 | - | 0.144 (0.147) |
| Coprostanone | 10 | 0.146 (0.145) | 0.147 (0.156) |
| | 50 | 0.161 (0.167) | 0.148 (0.141) |
| Epicoprostanol | 10 | 0.146 (0.149) | 0.155 (0.152) |
| | 50 | 0.156 (0.157) | 0.147 (0.147) |

[a]Ghost membranes were incubated with various oxysterols in the presence or absence of 1 μM epinephrine at 37 °C for 30 min. After that the membranes were labelled with either DPH or TMA-DPH as described in 'Materials and methods'. Membranes were allowed to equilibrate to 25 °C in a thermostated water bath before fluorescence measurements were taken at 25 °C using an ISS K2 multifrequency phase fluorometer.

[b]Values presented are mean values obtained from three separate experiments determined in triplicate with SD in the range of 0.001 to 0.005. Values in parenthesis are determined in the presence of 1 μM epinephrine.

*$p < 0.1$, using unpaired $t$-test; **$p < 0.05$, using unpaired $t$-test.

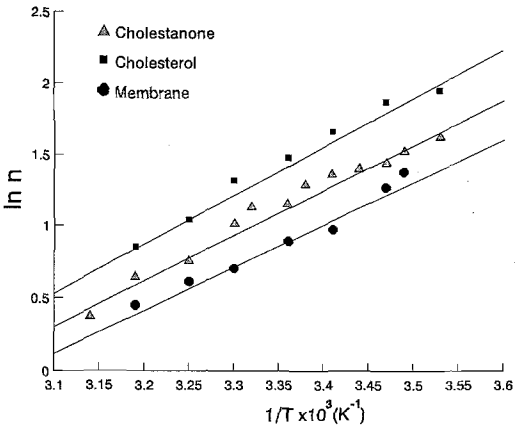


Figure 1. Temperature dependence of microviscosity in the lipid core of ghost membranes. Arrhenius Plot (ln ñ vs 1/T) was plotted using equivalent microviscosity (ñ) values calculated from DPH anisotropy data for membranes preincubated (37 °C, 30 min) with either 50 μM cholestanone or cholesterol. The fluorescence depolarization readings were taken at a temperature range of 10 °C to 45 °C using ISS K2 multifrequency phase fluorometer fitted with a water-jacketed cuvette holder. The sample temperature was monitored using a thermocoupled probe attached to a Fluke 52 thermometer and the sample temperature was kept at ±0.2 °C of the indicated temperature. The fusion activation energy, ΔE was calculated from the slope of the plot.

treated membrane increased from 0.142 to 0.166 ($p < 0.05$) for DPH but with TMA-DPH it was not significantly affected. A similar trend was also observed for membranes preincubated with cholestanone, where the DPH probe anisotropy increased from 0.170 to 0.186 ($p < 0.05$) and from 0.200 to 0.219 ($p < 0.05$) at 10 and 50 μM cholestanone respectively. This effect of epinephrine was not observed with cholesterol. The $r_s$ found for the other oxysterols tested were also not significantly different in the absence or presence of epinephrine when probed with either DPH or TMA-DPH.

When $r_s$ was determined using TMA-DPH, only cholestanone and cholesterol caused significant decreases in membrane fluidity (increased $r_s$) compared to that of non-treated membranes. However, in the presence of epinephrine, only membrane preincubated with cholesterol showed a decrease in membrane fluidity ($r_s$ increased from 0.177–0.182, $p < 0.1$) when compared to membranes preincubated with epinephrine alone. This change in TMA-DPH anisotropy was not observed for membranes preincubated with cholestanone and epinephrine (table).

When studies were carried out using DPH, membranes preincubated with cholestanone and epinephrine showed an increase in $r_s$ value compared to that without epinephrine. However, no significant difference was seen for membranes preincubated with cholesterol and epinephrine. With the DPH and TMA-DPH results taken together, cholestanone seems to affect specifically the bilayer component probed with DPH in the presence of epinephrine while cholesterol did not show any preferential effect on the DPH and TMA-DPH anisotropy whether in the presence or absence of epinephrine.

In order to evaluate how cholestanone and cholesterol influence the physical properties of the membrane, the change in 'equivalent microviscosity' (ñ) with temperature for membranes preincubated with cholestanone and cholesterol was determined and the fusion activation energy for viscosity (ΔE) calculated from the slope of the Arrhenius Plot (ln ñ vs 1/T) (fig. 1). As expected for biomembranes, the graph obtained was linear over the temperature range studied (10–45 °C), indicating the absence of phase transition. The ΔE value obtained for membranes preincubated with cholestanone was 8.6 kcal/mol and that preincubated with cholesterol was 8.2 kcal/mol. The ΔE value obtained for untreated membranes was 8.3 kcal/mol.

In order to distinguish whether the effects seen were due to the interaction of oxysterols (cholesterol and cholestanone) with membrane components or to the direct effect of oxysterols on the physical state of the membrane, the effect of the sterols on membrane methyltransferase was examined, since membrane phospholipid methylation has been shown to affect membrane fluidity[25]. From figure 2 it can be clearly seen that none of the oxysterols tested exhibited any effect on basal membrane phospholipid methylation either alone or in the presence of epinephrine. When methylation

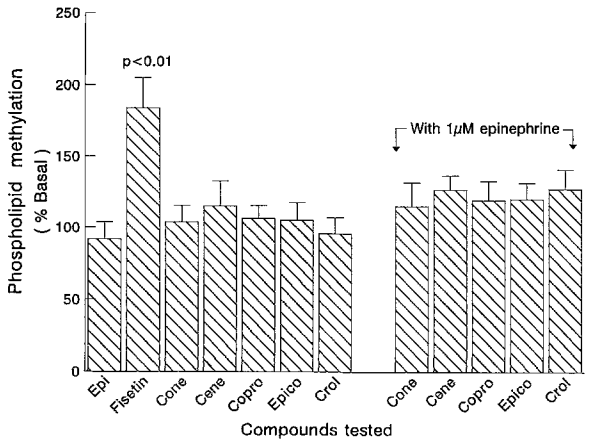


Figure 2. Effect of oxysterols on basal membrane phospholipid methylation. Adipocyte membrane phospholipid methylation was carried out using $^3$H-S-adenosyl-methionine (8 μM, 2 μCi) as described in 'Materials and methods'. Adipocytes were incubated with 50 μM of the test sterols in the presence or absence of 1 μM epinephrine (Epi) at 37 °C for 30 min and the results were expressed as percentage of basal level. Basal methylation was determined in the absence of any test compounds and was arbitarily set as 100%. The results presented were mean values obtained from three separate experiments carried out in triplicate with the error bars representing the SD of the means. (Cone = cholestanone; Cene = cholestenone; Crol = cholesterol; Copro = coprostanone; Epico = epicoprostanol).

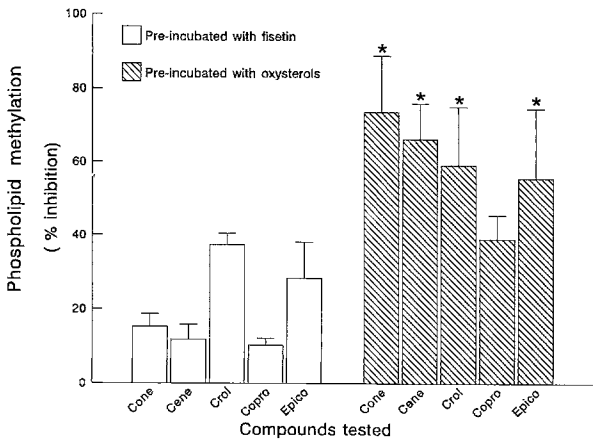


Figure 3. The inhibitory effect of sterols on fisetin-induced methyltransferase activity was studied using adipocytes pre-loaded with $^3$H-S-adenosyl-methionine (8 μM, 2 μCi). Adipocytes were then incubated with 50 μM of the various test sterols at 37 °C for 15 min followed by 250 μM of fisetin, and the incubation carried on for another 30 min at 37 °C. The assay was repeated with adipocytes first incubated with fisetin followed by the addition of sterols. The methylated phospholipids were quantitated as described in 'Materials and methods'. The results shown were obtained from three separate experiments performed in triplicate with the error bars representing the SD of the means. Asterisk (*) sign denotes level of significance for data showing more than 50% inhibition, $p < 0.01$. (Cone = cholestanone; Cene = cholestenone; Crol = cholesterol; Copro = coprostanone; Epico = epico-prostanol).

was induced using fisetin[33], cholesterol showed 37% inhibition but the rest of the sterols exhibited similar inhibition effects ranging from 10 to 18% (fig. 3). However, when adipocytes were pre-incubated with sterols before fisetin addition, all the sterols showed substantial inhibition of the stimulatory effect of fisetin, with cholestanone and cholestenone showing the highest inhibition at 74% and 66% respectively. Cholesterol and epicoprostanol inhibited the fisetin-stimulated methyltransferase activity to about the same extent (55–60%), while coprostanone has the lowest inhibitory effect (39%) (fig. 3).

## Discussion

In the present study, we examined the effects of cholesterol and its oxysterol-derivatives on the membrane fluidity of rat adipocyte ghost membranes. This is in view of our previous findings that cholesterol and its oxysterol-derivatives have differential effects on lipolysis in rat adipocyts[29]. Yeagle[16] also suggested that sterols can modify membrane fluidity and thus play an important role in controlling the functions of membrane proteins and receptors. Recent reports showed that some 3β-hydroxysterols and ketosterols have effects on ($Ca^{2+}$-$Mg^{2+}$)ATPase[17] and protein kinase C activity[4]. Our results demonstrated that cholestanone and cholesterol were able to decrease membrane fluidity in adipocyte ghost membranes (increase in fluorescent anisotropy, $r_s$) upon insertion into the bilayer. However, this effect was not observed for the ketosterols (cholestenone and coprostanone) and 3α-hydroxysterol (epicoprostanol) tested. Such compound selectivity has also been reported by Demel and co-workers[35] on the permeability of liposomes in the presence of various 3β-hydroxysterols, 3α-hydroxysterols and ketosterols. This differential effect was attributed to the selective interaction of the sterols with the phospholipid (phosphatidylcholine) found in the liposomes, based on the functional groups of the sterols. The presence of a planar sterol nucleus, an intact side chain and a 3β-hydroxy group was said to be necessary for optimum sterol effects. Michelangeli et al.[17] also reported that the 3β-hydroxysterols bind better to phospholipids than the 3α-hydroxy isomers. We have also observed a similar selective effect for the sterols tested. However, several groups have cautioned that even though the effects of sterols are generally structurally specific, exceptions have been noted[36–38].

Cholestanone and cholesterol were shown to partition into different lipid environments in the presence of epinephrine, as indicated by their effects on DPH anisotropy and TMA-DPH anisotropy. Both compounds were shown to insert into the bilayer in the absence of epinephrine, as indicated by the increase in DPH anisotropy. In the presence of epinephrine, cholestanone inserted deeper into the bilayer, shown by the increase in DPH anisotropy. Surprisingly, the partitioning of cholesterol was not significantly affected by

epinephrine binding. This difference could be due to the greater hydrophobicity of cholestanone exerted by the keto group at C-3 where cholesterol has a hydroxyl group. The lack of a 3$\beta$-hydroxyl group in cholestanone may also cause it to interact better with the more saturated phospholipids (e.g. phosphatidylethanolamine) found in the inner leaflet of the bilayer[39]. The presence of cholestanone could increase the acyl chain ordering in that region and thus affect the microviscosity there. This distinctive behaviour may have significant physiological effects and may partially explain the effects of different oxysterols on epinephrine-induced lipolysis in rat adipocytes reported recently[29].

Membranes preincubated with either 50 μM cholestanone or cholesterol did not show any lipid phase transition, indicated by the continuity of the Arrhenius Plot (fig. 1), and this could be interpreted as the absence of any change in the lipid phase of the bilayer. This is expected because biomembranes have a very heterogeneous lipid composition. The fusion activation energy ($\Delta E$) determined for membranes preincubated with cholesterol (8.2 kcal/mol) and cholestanone (8.6 kcal/mol) did not differ significantly from that of untreated membranes (8.3 kcal/mol). These values fall in the $\Delta E$ range for biomembranes (6.5–8.5 kcal/mol) as reported by Shinitzky and Inbar[40]. This lack of change in $\Delta E$ values for the treated membrane may reflect a compensatory mechanism in which the membrane proteins were displaced vertically to a level in the bilayer which balances the effect of the inserted sterols, presumably towards the aqueous environment as proposed by Shinitzky and Inbar[40]. Stable $\Delta E$ values have also been reported for lymphocytes and lymphoma cells, in which differences in bilayer microviscosity are also observed. This was attributed to the difference in cholesterol content in the two cell types by the authors[41]. However, the authors did not determine the presence of any sterol substitute in the lymphoma cells. Thus, the contribution of other cholesterol oxysterols, if any, could not be ascertained.

Cholestanone and cholesterol have a similar effect on the lipid environment probed by DPH on a molar-to-molar comparison in the absence of epinephrine. However, in the presence of epinephrine, cholestanone (50 μM) caused a more pronounced decrease in membrane fluidity as shown by the increase in anisotropy ($r_s$) from 0.200 to 0.219 ($p < 0.05$). Cholesterol did not exhibit a similar effect in the presence of epinephrine. The increase in anisotropy (microviscosity) reflects an increase in lipid-lipid interactions and packing density, thereby reducing protein-lipid interactions and hence the solubilization capacity. Thus, it would cause a vertical displacement of proteins into the more aqueous surroundings[42]. This observation suggests that cholestanone can 'force' membrane proteins to be more exposed to the aqueous environment and therefore promote better interaction with their substrates, provided that the protein (receptor, enzyme) conformation is not perturbed sufficiently to render it non-functional.

The increase in membrane acyl chain ordering has been shown to activate basal adenylate cyclase activity[43]. Our recent report has also shown that rat adipocytes incubated with cholestanone have increased epinephrine-induced lipolysis with a concomitant increase in adenylate cyclase activity[29]. The effect could well be due to the increase in acyl chain ordering in the region where cholestanone is situated (as probed by DPH).

In order to see if the oxysterols used in this study exert their effect by interfering with the conversion of PE to PC in adipocyte membranes, the effect of the sterols on methyltransferase was examined. It is known that membrane methyltransferases can convert phosphatidylethanolamine (PE) to phosphatidylcholine (PC) which is then transported to the outer leaflet of the bilayer, thus increasing membrane fluidity[25]. Inhibition of this conversion pathway will invariably produce higher microviscosity in the inner leaflet of the bilayer since the fatty acyl chain of PE is normally less saturated than that of PC and less saturated phospholipids are known to cause greater acyl ordering[4]. Fisetin was used as a positive control since this flavonoid has been shown to increase methyltransferase activity in rat adipocytes[33]. Results (fig. 2) showed that the sterols in general did not act by inhibiting the basal methyltransferase activity but may exert their effects by changing the physicochemical properties of the bilayer (e.g. changing the microviscosity at the site of insertion).

However, in the presence of a suitable inducer such as fisetin, the sterols were shown to inhibit stimulated methyltransferase activity in the order of cholesterol > epicoprostanol > cholestanone = cholestenone = coprostanone (fig. 3). This may indicate that the insertion site of cholesterol is near the inserted fisetin, thereby inhibiting the stimulatory effect of fisetin. This result may also allow us to infer that fisetin binds to the outer leaflet of the bilayer since its activity is affected more by cholesterol, which is known to bind preferentially to the outer bilayer, than by cholestanone. When the adipocytes were preincubated with sterols before the addition of fisetin, the stimulatory effect of fisetin was inhibited by about 50% for most of the sterols. Cholestanone and cholestenone showed the highest inhibitions at 73.6% and 66.2% respectively. Thus, cholestanone could prevent the activation of methyltransferase I that is located in the inner leaflet of the bilayer through modification of the microviscosity at that region. Alternatively, cholestanone could be exerting its effect through its interaction with PE, since PE is said to play a more important regulatory role than PC[39]. The results support the hypothesis that cholestanone can insert deeper into the lipid core than cholesterol and has a tendency to interact with less

saturated phospholipids than cholesterol, which tends to interact with PC at the outer leaflet of the bilayer. Data obtained in this study favour the long-established view that sterols interact with specific membrane phospholipids and exhibit selective effects on biological systems[35,36,44] which are dependent on the complex nature and composition of the membrane and the active ligand that binds to it. This report also shows that sterols in the aqueous phase can insert into biological membranes and supports the suggestion that cholesterol/sterol transfer in biological systems involves an aqueous pool of sterols[45,46].

Acknowledgements. The authors thank Dr. H. E. Khoo for her advice in the revision of this manuscript and the National University of Singapore for the research scholarship awarded to W. F. Lau. This work was supported by a research grant GR 6091 from the Shaw Foundation. This paper is dedicated to the memory of Dr. N. P. Das who passed away in September 1994.

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