Biochemical Pharmacology, Vol. 53, pp. 27–34, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00649-1

Activation of Acyl-CoA: Cholesterol Acyltransferase in Rat Liver Microsomes by 25-Hydroxycholesterol

*Chidambaram Bhuvaneswaran,**
Stella Synouri-Vrettakou† and Kostas A. Mitropoulos‡
LIPID METABOLISM UNIT, MEDICAL RESEARCH COUNCIL, HAMMERSMITH HOSPITAL, DUCANE ROAD, LONDON W12OHS U. K.

ABSTRACT. 25-Hydroxycholesterol stimulated acyl-CoA:cholesterol acyltransferase (ACAT) activity in rat liver microsomes *in vitro* with half-maximal stimulation at 16.8 μM oxysterol and a maximal activity that was three times that in its absence. The current study was conducted to determine the effect of 25-hydroxycholesterol on rates and extent of intervesicular cholesterol transfers within microsomes and to determine whether this activation of ACAT could be accounted for on the basis of increased cholesterol availability for the enzyme. Cholesterol transfer kinetics were assessed in systems that either enriched or depleted microsomal cholesterol. Incubation of microsomes at 37°C with phosphatidylcholine:cholesterol liposomes or purified plasma membranes resulted in enrichment of microsomal cholesterol. Incubation of microsomes with just phosphatidylcholine liposomes resulted in depletion of cholesterol. The extent of cholesterol enrichment or depletion depended on incubation time and the initial concentration of cholesterol in donor and acceptor vesicles. The rate and extent of cholesterol transfer from liposomes to microsomes were slightly increased when 25-hydroxycholesterol was present during the transfer process. Irrespective of the treatment, 25-hydroxycholesterol continued to stimulate the ACAT activity of the treated microsomes. Microsomes that were enriched or depleted of cholesterol in the absence of 25-hydroxycholesterol yielded as much enzyme activities when assayed in the presence of 25-hydroxycholesterol as with the systems that contained 25-hydroxycholesterol during both the transfer process and enzyme assays. The results suggest that a major part of the activation of microsomal ACAT by 25-hydroxycholesterol is not ascribable to increased substrate availability for the enzyme. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:27–34, 1997.

KEY WORDS. 25-hydroxycholesterol; oxysterols; acyl-CoA:cholesterol acyltransferase; ACAT; cholesterol esterification; activation; microsomes; liver

ACAT§ esterifies cholesterol and serves an important function in cholesterol homeostasis *in vivo* [1]. A number of cholesterol oxides alter the activities of ACAT and HMG-CoA reductase, an enzyme involved in cholesterol synthesis, in a variety of tissues *in vivo* and *in vitro* [2–4]. Among these oxidation products, 25-hydroxycholesterol is a potent stimulator of ACAT activity in isolated cells [5–12] and liver microsomal preparations [12–14]. 25-Hydroxycholesterol can be formed endogenously in concentration sufficient to influence ACAT activity [4, 15–18] and can be metabolized through esterification [13, 19], conversion to bile acids [20, 21], and side chain cleavage [22]. These metabolites are less effective than 25-hydroxycholesterol is in altering HMG-CoA reductase activity [22]. An analogous situation, were it to occur with ACAT activity, could endow the cell with an attractive mechanism to regulate this enzyme *in vivo* through synthesis and metabolism of 25-hydroxycholesterol.

It is not clear how 25-hydroxycholesterol exerts it influence on ACAT. Based on the decreased stimulatory effect of 25-hydroxycholesterol on ACAT on substrate saturation of microsomes and cells, the activation found under conditions of substrate unsaturation could be a consequence of increased cholesterol availability for the enzyme through transfer from pools within microsomes [11, 12]. This study evaluates the effects of 25-hydroxycholesterol on ACAT activity and cholesterol transfer rates to and from liver microsomes under conditions where the microsomes are far

* Corresponding author: Dr. C. Bhuvaneswaran, Department of Biochemistry and Molecular Biology, Slot 516, University of Arkansas for Medical Sciences, 4301 West Markham St., Little Rock, AR 72205. TEL: (501) 686-5812; FAX: (501) 686-8169.

† Present address: 3 Davaki Street, Athens 15669, Greece.

‡ Present address: Medical Research Council, Epidemiology and Medical Care Unit, Wolfson Institute of Preventive Medicine, Medical College of St. Bartholomew's Hospital, London EC1M 6BQ, UK. TEL: 071 982 6193; FAX: 071 982 6262.

§ *Abbreviations:* ACAT, acyl-CoA:cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

Received 27 June 1996; accepted 27 June 1996.

from substrate saturation. The results show that the effect of the oxysterol persists irrespective of the microsomal cholesterol content and that the slightly increased cholesterol transfer rates in presence of the oxysterol are inadequate to account for all the ACAT activity elicited by the oxysterol on the basis of substrate enrichment alone.

MATERIALS AND METHODS

Materials

[1α,2α(n)-^{3}H]Cholesterol and [1-^{14}C]oleoyl-CoA were obtained from Amersham International (Bucks, UK). [1α,2α(n)-^{3}H]Cholesteryloleate (47 Ci/mmol) was prepared by coupling oleoyl-chloride with [1α,2α(n)-^{3}H]cholesterol and separating the product by TLC. Oleoyl-CoA, oleoyl-chloride, dithiothreitol, egg yolk phosphatidylcholine, cholesteryloleate and human serum albumin (essentially free of fatty acids) were obtained from Sigma London (Poole, Dorset, UK). No impurities were detected in the phosphatidylcholine by TLC with standard solvent systems. Cholesterol of commercial source was recrystallized three times. [^{3}H]Cholesterol, diluted to the desired specific radioactivity, was purified before use by TLC on Kieselgel H at 5°C with diethyl ether as solvent. 25-Hydroxy-[26,27-^{3}H]cholesterol (New England Nuclear, Boston, MA, USA) of the desired specific radioactivity and 25-hydroxycholesterol (Steraloids, Wilton, NH, USA) were purified before use by TLC on Kieselgel H with chloroform-methanol (95:5, V/V) as solvent.

Preparation of Liposomes

Phosphatidylcholine and cholesterol-phosphatidylcholine (1:1, mol/mol) single bilayer liposomes were prepared as described previously [23]. The washed suspension containing the desired concentration of lipid was used immediately after preparation.

Isolation of Microsomes and Plasma Membranes From Rat Liver

Male Wistar rats, 180–220 g body weight, were housed in a room at 23°C under conditions of controlled lighting and feeding for at least 2 weeks before the experiment. On the day of the experiment, the rats were killed by cervical dislocation at about 0700 hr. The livers were removed immediately, chilled on ice and then perfused with ice-cold 0.25 M sucrose to remove contaminating blood. Microsomes and plasma membranes were isolated from the homogenates of these livers as previously reported [23, 24].

Forward Cholesterol Transfer From Liposomes to Microsomal Vesicles

Portions of liver microsomal fraction (2–4 mg microsomal protein/mL incubation mixture) were incubated at 37°C with different concentrations of [^{3}H]cholesterol/phosphatidylcholine (1:1, mol/mol) liposomes in a medium consisting of 12.3 mM NaCl, 230 mM sucrose and 3 mM imidazole-HCl buffer, pH 7.4, for up to 50 min [23]. For the buffer-treated controls, the liposomes were substituted with an equal volume of 0.15 M NaCl. Where indicated, 25-hydroxycholesterol dissolved in ethanol was added at the start of incubation (zero time). The system without 25-hydroxycholesterol contained an equal volume of ethanol. The ethanol concentration in the final incubation mixture never exceeded 1.5%. At different time intervals, suitable aliquot was withdrawn, cooled in ice-water mixture and centrifuged at 104,000 *g* for 60 min to separate the liposomes from the treated microsomal vesicles. The microsomal pellets were resuspended, and portions were used to assay ACAT activity and to measure radioactivity of [^{3}H]cholesterol in the reisolated microsomal vesicles. The mass of cholesterol transferred to the microsomal vesicles was calculated from the radioactivity incorporated and the specific radioactivity of liposomal cholesterol and equalled the increment of cholesterol determined in the treated microsomal vesicles by GLC [25, 26].

Reverse Transfer of Cholesterol From Microsomal Vesicles to Liposomes

Reverse transfer of cholesterol from microsomal vesicles to liposomal vesicles occurred at incubation of microsomal vesicles with just phosphatidylcholine liposomes [23, 27]. Portions of liver microsomal fraction (about 2 mg microsomal protein/mL incubation mixture) were incubated at 37°C with different concentrations of phosphatidylcholine liposomes in a medium consisting of 12.3 mM NaCl, 230 mM sucrose and 3 mM imidazole-HCl buffer, pH 7.4, for up to 50 min [23]. Where indicated, 25-hydroxycholesterol dissolved in ethanol was added at the start of incubation (zero time). The system without 25-hydroxycholesterol contained an equal volume of ethanol. At the end of the preincubation, the mixtures were centrifuged to isolate the cholesterol-depleted microsomal vesicles from the liposomes. Portions of the reisolated microsomal vesicles were used for ACAT assays.

Assays

The activity of ACAT in the microsomal fraction or the treated and reisolated microsomal vesicles was assayed by using [1-^{14}C]oleoyl-CoA (specific radioactivity 5 Ci/mol) as described elsewhere [23, 25]. The cholesteryl ester formed was extracted and separated, and the rate of product formation was determined as described previously [23, 25]. Total lipids were extracted by using the method of Folch *et al.* [28]. Phospholipid in the total lipid extracts was assayed by using the method of Barlett [29]. Protein was assayed by using the method of Lowry *et al.* [30], with bovine serum albumin as standard. Cholesterol was quantified by GLC [25, 26].

RESULTS

Stimulation of ACAT Activity by 25-Hydroxycholesterol

Without 25-hydroxycholesterol, the ACAT activity of the microsomal fraction is 58.2 pmol of cholesteryl oleate syn-

thesized per min/mg microsomal protein. Consistent with the observations of others [12], the presence of 25-hydroxycholesterol in the assay mixture stimulates this activity; the magnitude of increase is dependent on the concentration of 25-hydroxycholesterol (Fig. 1). A double reciprocal plot (inset in Fig. 1) of increment in ACAT activity (Δv) against concentration of 25-hydroxycholesterol yields 16.8 μM oxysterol for half-maximal stimulation and 98.2 pmol cholesteryloleate synthesized per min/mg microsomal protein for ΔV_{max}. This 3-fold increase in ACAT activity over the control microsomes is not due to synthesis of 25-hydroxycholesterol-[^{14}C]oleate [14, 31]. In a separate experiment using 25-hydroxy-[26,27-^{3}H]cholesterol, its ester with oleic acid did not migrate at the same spot as it did with cholesteryl-[^{14}C]oleate in our TLC system.

Effect of 25-Hydroxycholesterol on Microsomal ACAT Activity in Presence of Different Amounts of Plasma Membrane Vesicles

The preincubation of liver microsomes at 37°C followed by assay for ACAT activity shows a time-dependent linear increase in the activity of the enzyme (Fig. 2, line A). This

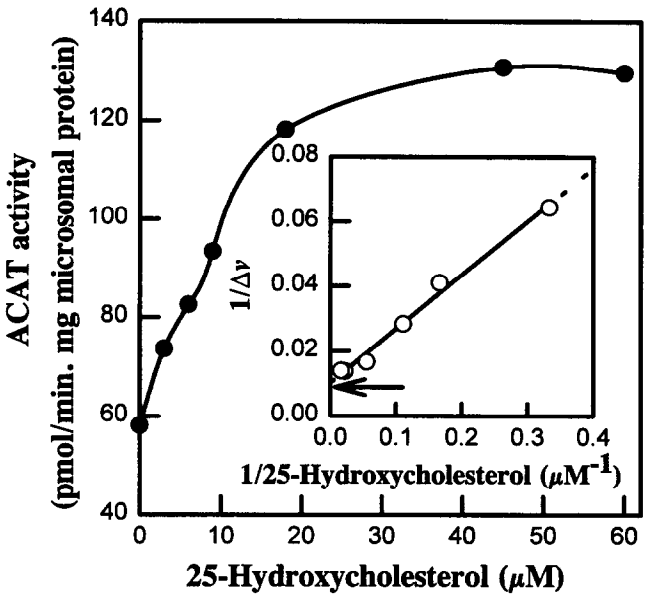

FIG. 1. Effect of 25-hydroxycholesterol on microsomal ACAT activity. ACAT activity was assayed in portions of the microsomal fraction in presence of different concentrations of 25-hydroxycholesterol, as described in Materials and Methods. Enzyme activity is expressed as pmole cholesteryloleate synthesized per min/mg microsomal protein. Inset represents the double reciprocal plot of the increment in ACAT activity (Δv) against the concentration of 25-hydroxycholesterol present in the system. The increment in ACAT activity equals the difference between the activities in the presence and absence of 25-hydroxycholesterol. The line shown is the best fit for the points determined. The correlation coefficient is 0.995 ($P < 0.001$). Extrapolation of the data (dotted line) yielded the ΔV_{max} (arrow), corresponding to 98.2 pmol/min/mg microsomal protein.

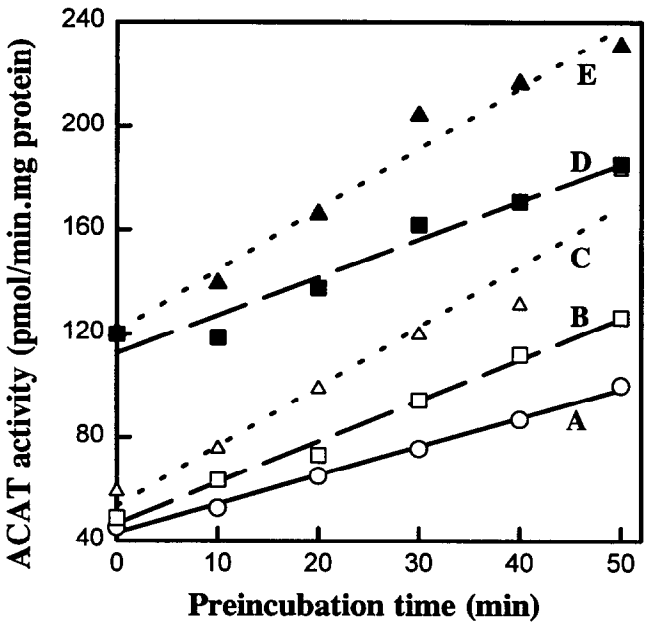

FIG. 2. ACAT activity of microsomes preincubated with purified plasma membranes in the presence or absence of 25-hydroxycholesterol. Aliquots of a purified plasma membrane preparation (175.9 nmol nonesterified cholesterol/mg protein) were added to portions of the microsomal fraction (1.79 mg microsomal protein and 92.45 nmol nonesterified cholesterol/mL of the mixture) to yield plasma membrane cholesterol to microsomal cholesterol ratios of 0.77 (lines B and D) and 4.3 (lines C and E). Line A represents the control microsomes without any added plasma membranes. The mixtures were preincubated as described in Materials and Methods in the presence (lines D and E) or absence (lines A–C) of 60 μM 25-hydroxycholesterol. 25-Hydroxycholesterol was added at zero time as a solution in 95% ethanol. The mixture without 25-hydroxycholesterol contained an equal volume of ethanol. Portions of each mixture were removed at different time intervals and assayed for ACAT activity, as described in Materials and Methods. The enzyme activities (pmole cholesteryloleate synthesized per min/mg microsomal protein) are plotted against the time of preincubation. The lines are the least-squares regressions with correlation coefficients greater than 0.977 ($P < 0.001$).

increase in activity is due to a time- and temperature-dependent transfer of cholesterol within the microsomal preparation from the cholesterol-rich plasma membrane component to the endoplasmic reticular membrane vesicles that contain the ACAT enzyme [23, 25, 32]. Such transfers of cholesterol could be enhanced by adding purified plasma membrane preparation [27]. Figure 2 shows two experiments with added plasma membrane cholesterol to microsomal cholesterol ratios of 0.77 and 4.3. The mixtures were preincubated at 37°C in the absence (Fig. 2, lines B and C) or presence of 60 μM 25-hydroxycholesterol (Fig. 2, lines D and E) and, at intervals, aliquots were taken and assayed for ACAT activity.

The ACAT activity increased linearly with time of preincubation within each mixture. The slopes of the lines in Fig. 2 reflect the rates at which cholesterol is transferred from the plasma membranes to the ACAT substrate site in each system [25, 27]. The slopes and intercepts for several

other ratios of plasma membrane cholesterol to microsomal cholesterol are given in Table 1. With increasing amounts of plasma membrane cholesterol in the preincubation mixture, the slopes progressively increased (Table 1). However, within any one system, the slopes were similar irrespective of the absence or presence of 25-hydroxycholesterol. All of the systems with 25-hydroxycholesterol exhibited similar intercepts (zero-time ACAT activity) irrespective of the amount of added plasma membrane vesicles.

Effect of 25-Hydroxycholesterol on Forward Transfer of Liposomal Cholesterol to Microsomal Vesicles

Microsomal vesicles can also be enriched with additional cholesterol through transfer from cholesterol-rich liposomes when they are incubated together (forward transfer of cholesterol). The extent of cholesterol transfer depends on the concentrations of the acceptor and donor vesicles and on the time and temperature of incubation [23, 25]. The advantage with this system is that the donor and acceptor vesicles can be separated from each other before assay of ACAT activity and the extent of cholesterol transfer can be established directly [23, 25]. The influence of oxysterol on this forward transfer was therefore evaluated for several ratios of cholesterol in liposomes to microsomes (Fig. 3 and Table 2).

The extent of cholesterol transfer was time dependent and followed first-order kinetics under all conditions (Fig. 3). The slope of each line provides the first-order rate constant (k) for that system from which half-time for cholesterol transfer is calculated ($t_{1/2} = \ln 2/k$) (Table 2). At all ratios of cholesterol in liposomes to microsomes, the presence of 25-hydroxycholesterol slightly decreased the half-time for transfer.

A plot of ACAT activities of the reisolated microsomes against the extent of cholesterol transferred (Fig. 4) exhibits a linear relationship for the control (line A) and the system preincubated with 25-hydroxycholesterol (line B). Irrespective of the amount of cholesterol transferred, system B showed a higher ACAT activity than did system A in the reisolated microsomes. This difference, however, is due to the retention of substantial amounts of 25-hydroxycholesterol, added during preincubation, in reisolated microsomes from system B. This reasoning was confirmed in separate experiments using 25-hydroxy-[26,27-^{3}H]cholesterol. When the control (system represented by line A) is assayed for ACAT in the presence of 60 μM 25-hydroxycholesterol (line C), it is indistinguishable from the system preincubated in the presence of the oxysterol (line B).

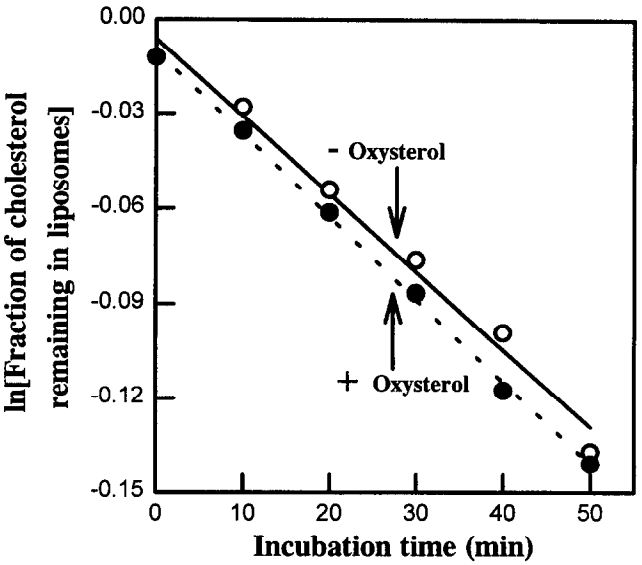

FIG. 3. Effect of 25-hydroxycholesterol on the transfer of cholesterol to microsomes from cholesterol-phosphatidylcholine liposomes. Portions of microsomal fraction (3.48 mg protein and 248.8 nmol nonesterified cholesterol/mL mixture) were incubated with [^{3}H]cholesterol-phosphatidylcholine liposomes (1:1, mol/mol; specific radioactivity = 17.17 dpm/pmol) at 37°C. The ratio of liposomal to microsomal cholesterol at the beginning of incubation was 1.78. Where indicated, 25-hydroxycholesterol was added at zero time as a solution in 95% ethanol to give 60 μM final concentration (solid circle). The mixture without 25-hydroxycholesterol contained an equal volume of ethanol (open circle). At different time intervals, portions of the mixture were centrifuged to isolate the treated microsomal vesicles from the liposomal vesicles. The results are expressed as the fraction of total radioactivity remaining in the [^{3}H]cholesterol-phosphatidylcholine liposomes. The data for the semilogarithmic plot were analyzed by a least-squares method and the best-fitting regression lines drawn. For the system without oxysterol: $y = -(2.46x + 6.2) \times 10^{-3}$ [correlation coefficient is −0.993 ($n = 6$, $P < 0.001$)]; for the system with oxysterol: $y = -(2.62x + 10.2) \times 10^{-3}$ [correlation coefficient is −0.999 ($n = 6$, $P < 0.001$)]. The slopes of the lines give the first-order rate constants (k) for transfer of cholesterol to microsomal vesicles from the liposomal donor.

TABLE 1. Effect of 25-hydroxycholesterol on rat liver microsomal ACAT activity in presence of different amounts of plasma membrane vesicles

Cholesterol ratio (plasma membrane/microsomes)	Slope		Intercept	
	+	−	+	−
0	0.70	1.11	118	43
0.77	1.45	1.58	113	47
1.54	1.65	1.89	122	50
3.08	2.26	2.19	118	51
4.30	2.35	2.30	121	54

Different amounts of a plasma membrane preparation (175.9 nmol nonesterified cholesterol/mg protein) were added to portions of microsomal fraction (1.79 mg microsomal protein and 92.45 nmol nonesterified cholesterol/mL mixture) to yield plasma membrane:microsomal cholesterol ratios of 0.77:4.3. These mixtures were preincubated at 37°C in a medium consisting of 12.3 mM NaCl, 230 mM sucrose and 3 mM imidazole-HCl buffer, pH 7.4, for up to 50 min in the presence (+) or absence (−) of 60 μM 25-hydroxycholesterol. At different time intervals, portions of the mixture were removed and assayed for ACAT activity. The activities (pmol cholesteryl ester formed min^{-1} mg $protein^{-1}$) are plotted against time of preincubation, as shown in Figure 2. The slopes and intercepts were obtained from the least-squares regression lines for each preincubation mixture.

Effect of 25-Hydroxycholesterol on the Reverse Transfer of Cholesterol From Microsomal Vesicles to Liposomes

Preincubation of microsomal vesicles with phosphatidylcholine liposomes resulted in depletion of microsomal cho-

TABLE 2. Effect of 25-hydroxycholesterol on the rate of cholesterol transfer from liposomes to rat liver microsomal vesicles

Cholesterol ratio (liposomes/microsomes)	10^3 × first-order rate constant (k)		$t_{1/2}$ (h)	
	−	+	−	+
Experiment 1				
0.89	ND	2.90	ND	4.0
1.78	2.46	2.62	4.7	4.4
Experiment 2				
0.28	2.57	3.47	4.5	3.3
0.84	2.14	2.85	5.4	4.1

Portions of two different microsomal preparations were incubated with different concentrations of [^{3}H]cholesterol/phosphatidylcholine (1:1, mol/mol) liposomes in a medium consisting of 12.3 mM NaCl, 230 mM sucrose and 3 mM imidazole-HCl buffer, pH 7.4, for up to 50 min in the presence (+) or absence (−) of 60 μM 25-Hydroxycholesterol. For the buffer-treated controls, the liposomes were substituted with an equal volume of 0.15 M NaCl. The microsomal protein concentrations for experiments 1 and 2 are, respectively, 3.48 mg and 2.4 mg/mL of the incubation mixture. The first-order rate constant (k) for each system is calculated from the slope of the least-squares regression lines, as shown in Figure 3. The correlation coefficient for each of the lines is greater than −0.998. Half-times ($t_{1/2}$) were calculated with the equation $t_{1/2} = \ln 2/k$. Other experimental details are given in the caption to Figure 3. ND, not done.

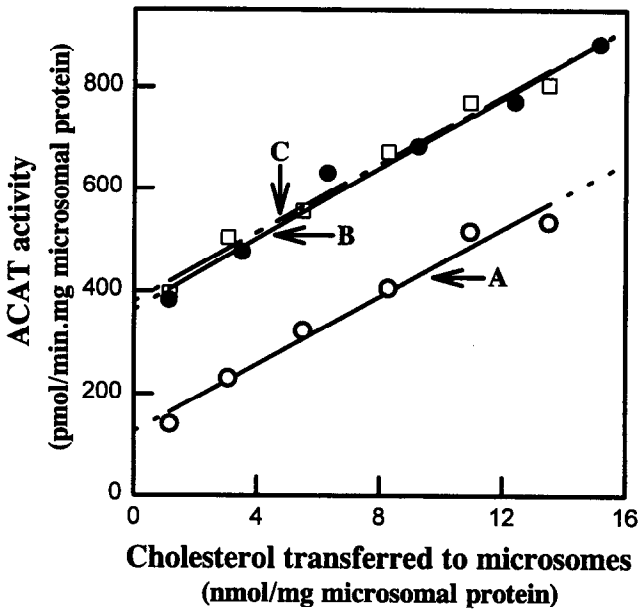

FIG. 4. Relationship between the extent of liposomal cholesterol transferred to microsomal vesicles and ACAT activity. The conditions of preincubation with cholesterol-containing liposomes were as described for Fig. 3. The microsomal vesicles were reisolated by centrifugation and assayed for ACAT activity as described in Materials and Methods. Line A (open circle) represents the control systems that did not contain 25-hydroxycholesterol during either transfer of cholesterol from liposomes to microsomes or subsequent assay for ACAT activity of the reisolated microsomal vesicles. Line B (solid circle) represents systems where 60 μM of 25-hydroxycholesterol were present during the transfer of cholesterol from liposomes to microsomes and the subsequent assay for ACAT activity of reisolated microsomal vesicles. Line C (square) represents the same systems as those represented in line A, except that 60 μM 25-hydroxycholesterol was included during ACAT assays. The best-fitting regression lines are drawn for each system. The equation are: line A, $y = 31x + 145.5$; line B, $y = 34.4x + 364.5$; and line C, $y = 33.5x + 380.7$. The correlation coefficients are greater than 0.990 ($P < 0.001$).

lesterol due to reverse transfer of cholesterol from microsomes to liposomes [23, 25]. Figure 5 shows a reciprocal plot of ACAT activities of microsomes that were subjected to preincubation for 50 min at 37°C with different concentrations of phosphatidylcholine liposomes against the liposomal:microsomal phospholipid ratios used during preincubation. Line A in Figure 5 represents control systems that did not contain 25-hydroxycholesterol during either the cholesterol-depletion process or during the subsequent assays for ACAT activities. Line B represents systems that included 25-hydroxycholesterol (60 μM) during the cholesterol-depletion process and during the subsequent assays for ACAT activities. Line C represents the same systems as line A except for the inclusion of 25-hydroxycholesterol (60 μM) during assays for solely ACAT activities. The plots show straight lines. The progressive decrease in ACAT activity with increases in the liposomal:microsomal phospholipid ratio in the system is a reflection of the extent to which cholesterol is lost from the microsomal vesicles to the liposomal vesicles contained in the preincubation mixture [27]. Although the slopes of lines A and B are distinct, the difference is solely due to the presence of the oxysterol during ACAT assay with system B. Inclusion of 25-hydroxycholesterol only during ACAT assay with system A (Fig. 5, line C) results in activities that are close to those of system B.

DISCUSSION

The microsomes are a heterogeneous population of vesicles; some are derived from the endoplasmic reticulum (containing ACAT enzyme, but minimal nonesterified cholesterol) and others are derived from the plasma membranes (containing most of the nonesterified cholesterol) [12, 32–35]. Thus, the ACAT enzyme has ready access to only a small percentage of the total microsomal cholesterol [27, 33] and, under normal conditions, operates far from substrate saturation [23, 25]. The substrate pool for the enzyme, however, can be expanded through transfer of unesterified cholesterol from plasma membrane vesicles and/or from cholesterol-rich phospholipid liposomes to ACAT-containing vesicles (forward transfer); this expansion results in an increase in ACAT activity [23, 26, 32]. The intervesicular transfer of cholesterol is mediated through preincubation of the microsomal vesicles with the donor vesicles prior to assay for ACAT activity and depends on time, temperature and concentrations of the cholesterol-donor and acceptor vesicles in the preincubation mixture [23, 25, 27]. A few studies have suggested that the stimulatory effect of 25-hydroxycholesterol on ACAT activity observed here (Fig. 1) and by others [5–13] is a consequence of increased substrate availability for the enzyme. This conclusion is based on the observation that the stimulatory effect of the oxy-

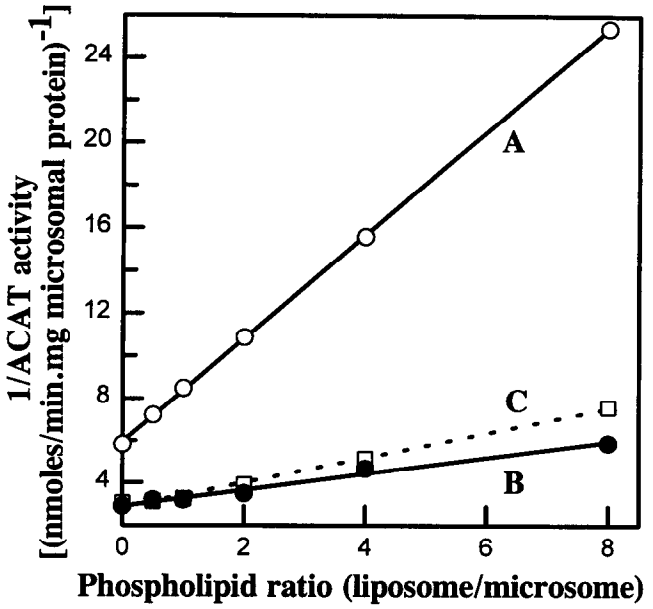

FIG. 5. Effect of 25-hydroxycholesterol and liposomal phospholipid concentration on cholesterol depletion and ACAT activity in microsomal membranes. The microsomal fraction (2.1 mg protein; 946.4 nmol microsomal phospholipid and 122.3 nmol nonesterified cholesterol/mL mixture) was preincubated at 37°C with different concentrations of liposomes made solely of phosphatidylcholine to deplete microsomal cholesterol content. At the end of 50 min of preincubation, the mixtures were centrifuged to isolate the cholesterol-depleted microsomal vesicles. ACAT activities were assayed in portions of the reisolated microsomal vesicles, as described in Materials and Methods. The reciprocal of ACAT activity is plotted against the liposomal:microsomal phospholipid ratio used for the depletion process. Line A (open circle) represents the control system, where both preincubation and ACAT assay were carried out in the absence of 25-hydroxycholesterol. Line B (solid circle) represents the system that contained 60 μM 25-hydroxycholesterol during preincubation and during ACAT assay. Line C (square) is the same as line A except that 60 μM 25-hydroxycholesterol was included only during ACAT assay. The data were analyzed by a least-squares method, and the best-fitting regression lines were drawn. The correlation coefficients are greater than 0.992 ($P < 0.001$).

sterol on ACAT activity progressively decreased and was even abolished as the microsomal cholesterol content was increased [11, 12]. This study evaluates (1) the influence of 25-hydroxycholesterol on cholesterol transfer rates under established conditions favoring an increase and a decrease in the cholesterol substrate pool for ACAT and (2) whether the changes in the extent of cholesterol transferred adequately account for the significant stimulation of the ACAT activity by this oxysterol.

Consistent with our previous findings [27], addition of purified plasma membranes to the microsomal preparation and preincubation results in increased ACAT activity. The time of preincubation and the concentration of the plasma membranes in the preincubation system determine the extent to which the ACAT activity is increased (Fig. 2, Table 1). The slopes, which reflect the rate at which cholesterol is made available to the enzyme for each of the systems [25, 27], are similar irrespective of the presence or absence of 25-hydroxycholesterol in the preincubation system (Table 1). Also, irrespective of the concentration of plasma membrane vesicles in the preincubation system, the intercepts in the presence of 25-hydroxycholesterol remain quite close together (Table 1). A significant involvement of 25-hydroxycholesterol on the transfer process, were it to occur, should have elicited higher slopes and also progressively higher intercepts with increasing amount of added plasma membranes (increasing amount of cholesterol made available for transfer) provided the enzyme is not saturated with respect to cholesterol. The systems evaluated here are not substrate-saturated because the ACAT activity responds linearly to cholesterol transfer even at the highest concentration of added plasma membranes and even after 50 min of preincubation. Hence, for both slopes and intercepts, a significant involvement of 25-hydroxycholesterol in the cholesterol transfer process is not indicated.

This finding is also borne out by the data on the rates of forward transfer of cholesterol to microsomes from cholesterol-rich liposomal donors (Fig. 3, Table 2). Although the presence of 25-hydroxycholesterol gave a slightly higher rate constant for cholesterol transfer, this is far from adequate to explain the substantial increase in the ACAT activity observed in its presence. Analysis of the data in Fig. 4 reveals that the linear responses in ACAT activities to cholesterol transfer have similar slopes for lines A, B and C. The distinct difference between the control (line A) and the one with 25-hydroxycholesterol (line B) is seen in the intercepts. This difference in intercepts amounts to nearly 220 pmol cholesteryl ester synthesized per min/mg microsomal protein. On the basis of the equation for line A, an extra input of nearly 6–7 nmol of cholesterol/mg protein would be necessary to change the ACAT activity of the control system solely through enrichment of cholesterol to comparable ACAT activity found with the system containing the oxysterol (line B). The maximum amount of cholesterol transferred over a 50-min preincubation period in the control (line A) and the one with 25-hydroxycholesterol (line B) are 13.5 and 15.2 nmol/mg protein, respectively. The difference of 1.7 nmol (over a 50-min preincubation period) is not sufficient to account for all of the stimulation in activity seen with system B. Furthermore, this amount of cholesterol must be transferred immediately to account for the ready stimulation of ACAT in the presence of 25-hydroxycholesterol. In addition, the control system, assayed for ACAT activity in presence of 60 μM 25-hydroxycholesterol (line C), is indistinguishable from the system preincubated in the presence of the oxysterol (line B).

Similar results are also seen on reverse transfer of cholesterol from microsomal vesicles to liposomes made of only phosphatidylcholine (Fig. 5). The concentration of the phosphatidylcholine vesicles and the time of preincubation are critical in determining the rate of efflux of microsomal

cholesterol [23, 27]. Consistent with our previous findings [23, 27], the loss of cholesterol from the microsomes is reflected in the loss of ACAT activity in the treated microsomal preparations (Fig. 5, line A). With increasing values for the liposomal:microsomal phospholipid ratio in preincubation, the loss in ACAT activity is progressively greater with the control (line A, no oxysterol is present during preincubation) and with the system that contained 25-hydroxycholesterol (line B) during preincubation. The decreases in ACAT activities are reflections of decreased cholesterol availability to the enzyme. The distinct difference in the slopes of lines A and B is solely due to the presence of the oxysterol during ACAT assay in system B but not in system A. In fact, inclusion of 25-hydroxycholesterol with the control microsomes only during ACAT assay (Fig. 5, line C) results in activities that are close to those of system B. Hence, the presence of 25-hydroxycholesterol during preincubation seems to be inconsequential in the cholesterol depletion process. Taken together, the present data suggest that the significant stimulatory effect on ACAT activity due to the presence of 25-hydroxycholesterol cannot be accounted for by the small changes in the cholesterol transfer rates.

In this context, recent evidence indicates that cholesterol itself can serve as an activator of ACAT, besides its role as a substrate for the enzyme [36]. At saturating substrate concentration, cholesterol may compete with 25-hydroxycholesterol for binding at an allosteric site, which may be the reason for decreased stimulatory effect of the oxysterol on ACAT on saturation of the system with cholesterol [11, 12].

We are indebted to B. E. A. Reeves and J. J. Gallagher for expert technical assistance. We thank Dr. S. Venkatesan for valuable discussions and suggestions. C. B. was on sabbatical from the University of Arkansas for Medical Sciences, Little Rock, and S. S-V. received a scholarship from the British Council.

References

1. Suckling KE and Stange EF, Role of acyl-CoA: cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* **26:** 647–671, 1985.
2. Kandutsch AA, Chen HW and Heiniger HJ, Biological activity of some oxygenated sterols. *Science* **201:** 498–501, 1978.
3. Morin RJ, Hu B and Peng S-K, Effects of cholesterol oxides on cholesterol metabolism. In: *Biological Effects of Cholesterol Oxides* (Eds. Peng S-K and Morin RJ), pp. 103–123. CRC Press, Boca Raton, FL, 1992.
4. Saucier SE, Kandutsch AA, Taylor FR, Spencer TA, Phirwa S and Gayen AK, Identification of regulatory oxysterols, 24(S),25-epoxycholesterol and 25-hydroxy-cholesterol, in cultured fibroblasts. *J Biol Chem* **260:** 14571–14579, 1985.
5. Brown MS, Dana SE and Goldstein JL, Cholesterol ester formation in cultured human fibroblasts. Stimulation by oxygenated sterols. *J Biol Chem* **250:** 4025–4027, 1975.
6. Chang TY and Limanek JS, Regulation of cytosolic acetoacetyl coenzyme A thiolase, 3-hydroxy-3-methylglutaryl coenzyme A synthase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and mevalonate kinase by low density lipoprotein and by 25-hydroxycholesterol in Chinese hamster ovary cells. *J Biol Chem* **255:** 7787–7795, 1980.
7. Chen HW, Cavenee WK and Kandutsch AA, Sterol synthesis in variant Chinese hamster lung cells selected for resistance to 25-hydroxycholesterol. Cross-resistance to 7-ketocholesterol, 20alpha-hydroxycholesterol and serum. *J Biol Chem* **254:** 715–720, 1979.
8. Kandutsch AA and Shown EP, Assay of oxysterol-binding protein in a mouse fibroblast, cell-free system. Dissociation constant and other properties of the system. *J Biol Chem* **256:** 13068–13073, 1981.
9. Schroepfer GJ Jr, Sterol biosynthesis. *Annu Rev Biochem* **50:** 585–621, 1981.
10. Sinensky M, Duwe G and Pinkerton F, Defective regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in a somatic cell mutant. *J Biol Chem* **254:** 4482–4486, 1979.
11. Miller SC and Melnykovych G, Regulation of cholesterol biosynthesis and esterification by 25-hydroxycholesterol in a macrophage-like cell line: uncoupling by progesterone. *J Lipid Res* **25:** 991–999, 1984.
12. Field FJ and Mathur SN, Regulation of acyl CoA:cholesterol acyltransferase by 25-hydroxycholesterol in rabbit intestinal microsomes and absorptive cells. *J Lipid Res* **24:** 1049–1059, 1983.
13. Drevon CA, Weinstein DB and Steinberg D, Regulation of cholesterol esterification and biosynthesis in monolayer cultures of normal adult rat hepatocytes. *J Biol Chem* **255:** 9128–9137, 1980.
14. Erickson SK, Shrewsbury MA, Brooks C and Meyer DJ, Rat liver acyl-coenzyme A: cholesterol acyltransferase: its regulation in vivo and some of its properties in vitro. *J Lipid Res* **21:** 930–941, 1980.
15. Fredriksson DS, The conversion of cholesterol-4-C^{14} to acids and other products by liver mitochondria. *J Biol Chem* **222:** 109–120, 1956.
16. Bjorkhem I and Gustafsson J, Mitochondrial omega-hydroxylation of cholesterol side chain. *J Biol Chem* **249:** 2528–2535, 1974.
17. Morin RJ and Peng S-K, Cholesterol oxides in plasma and tissues. In: *Biological Effects of Cholesterol Oxides* (Eds. Peng S-K and Morin RJ), pp. 89–101. CRC Press, Boca Raton, FL, 1992.
18. Johnson KA, Morrow CJ, Knight GD and Scallen TJ, In vivo formation of 25-hydroxycholesterol from endogenous cholesterol after a single meal, dietary cholesterol challenge. *J Lipid Res* **35:** 2241–2253, 1994.
19. Lichtenstein AH and Brecher P, Esterification of cholesterol and 25-hydroxycholesterol by rat liver microsomes. *Biochim Biophys Acta* **751:** 340–348, 1983.
20. Swell L, Schwartz CC, Gustafsson J, Danielsson H and Vlahcevic ZR, A quantitative evaluation of the conversion of 25-hydroxycholesterol to bile acids in man. *Biochim Biophys Acta* **663:** 163–168, 1981.
21. Zhang J, Larsson O and Sjovall J, 7α-Hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol in human fibroblasts. *Biochim Biophys Acta* **1256:** 353–359, 1995.
22. Taylor FR and Kandutsch AA, Metabolism of 25-hydroxycholesterol in mammalian cell cultures. Side-chain scission to pregnenolone in mouse L929 fibroblasts. *J Lipid Res* **30:** 899–905, 1989.
23. Bhuvaneswaran C and Mitropoulos KA, Effect of liposomal phospholipid composition on cholesterol transfer between microsomal and liposomal vesicles. *Biochem J* **238:** 647–652, 1986.
24. Venkatesan S, Gallagher JJ and Mitropoulos KA, Modulation of the activity of 5′-nucleotidase by the transfer of non-esterified cholesterol to rat-liver microsomal fraction and evidence for regulation of the concentration of non-esterified

cholesterol in plasma membranes in vivo. *Biochim Biophys Acta* **756:** 72–82, 1983.
25. Synouri-Vrettakou S and Mitropoulos KA, Acyl-coenzyme A: cholesterol acyltransferase. Transfer of cholesterol to its substrate pool and modulation of activity. *Eur J Biochem* **133:** 299–307, 1983.
26. Mitropoulos KA, Venkatesan S, Balasubramaniam S and Peters TJ, The submicrosomal localization of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase, cholesterol 7alpha-hydroxylase and cholesterol in rat liver. *Eur J Biochem* **82:** 419–429, 1978.
27. Mitropoulos KA, Venkatesan S, Synouri-Vrettakou S, Reeves BE and Gallagher JJ, The role of plasma membranes in the transfer of non-esterified cholesterol to the acyl-CoA: cholesterol acyltransferse substrate pool in liver microsomal fraction. *Biochim Biophys Acta* **792:** 227–237, 1984.
28. Folch J, Lees M and Sloane-Stanley GH, A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226:** 497–509, 1957.
29. Bartlett GR, Phosphorous assay in column chromatography. *J Biol Chem* **234:** 466–468, 1959.
30. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin-phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
31. Erickson SK, Matsui SM, Shrewsbury MA, Cooper AD and Gould RG, Effects of 25-hydroxycholesterol on rat hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in vivo, in perfused liver, and in hepatocytes. *J Biol Chem* **253:** 4159–4164, 1978.
32. Lange Y, Strebel F and Steck TL, Role of the plasma membrane in cholesterol esterification in rat hepatoma cells. *J Biol Chem* **268:** 13838–13843, 1993.
33. Balasurbramaniam S, Venkatesan S, Mitropoulos KA and Peters TJ, The submicrosomal localization of acyl-coenzyme A-cholesterol acyltransferase and its substrate, and of cholesteryl esters in rat liver. *Biochem J* **174:** 863–872, 1978.
34. Balasubramaniam S, Mitropoulos KA and Venkatesan S, Rat-liver acyl-CoA: cholesterol acyltransferase. *Eur J Biochem* **90:** 377–383, 1978.
35. Venkatesan S, Mitropoulos KA, Balasubramaniam S and Peters TJ, Biochemical evidence for the heterogeneity of membranes from rat liver endoplasmic reticulum. Studies on the localization of acyl-CoA: cholesterol acyltransferase. *Eur J Cell Biol* **21:** 167–174, 1980.
36. Cheng D, Chang CC, Qu X and Chang TY, Activation of acyl-coenzyme A:cholesterol acyltransferase by cholesterol or by oxysterol in a cell-free system. *J Biol Chem* **270:** 685–695, 1995.