Conditions that may result in (de-)phosphorylation of hepatic acyl-CoA:cholesterol acyltransferase result also in modulation of substrate supply *in vitro*

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The present experiments were designed to study intervesicular transfer of cholesterol in rat liver microsomal fraction and modulation of the activity of acyl-CoA:cholesterol acyltransferase (ACAT) under conditions that are expected to result in the covalent modification (phosphorylation/dephosphorylation) of the enzyme. Preincubation of rat liver microsomal fraction followed by assay of ACAT showed a time-dependent increase in activity. This rate was temperature-dependent. Preincubation in the presence of cholesterol/phospholipid liposomes resulted in a time-dependent transfer of cholesterol from liposomal to the microsomal vesicles and in an increase in the rate of ACAT change owing to the preincubation. Both these rates were dependent on liposomal cholesterol concentration and on temperature. The presence of cytosol in the preincubation mixture increased the rate of change of ACAT activity in the absence or in the presence of cholesterol/phospholipid liposomes. In the latter case the presence of cytosol also increased the rate of transfer of cholesterol from liposomal to the microsomal vesicles. Activation energies of the rate of this transfer and of the rate of increase of ACAT activity were similar in the presence and in the absence of cytosol. Both in the absence and in the presence of cytosol, the presence of NaF (50 mM) in the preincubation mixture considerably decreased the rate of transfer of cholesterol from liposomal to microsomal vesicles and the rate of increase of ACAT activity. The presence of Mg^{2+} in the preincubation mixture produced no effect on the rate of transfer of cholesterol from liposomal to the microsomal vesicles, although under most conditions it decreased the rate of increase of ACAT activity caused by the preincubation. These results are discussed in relation to the molecular mechanism involved in this intervesicular transfer of cholesterol and to the modulation of ACAT activity by substrate supply, and also in relation to the hypothesis that ACAT activity can be modulated by a mechanism involving the phosphorylation/dephosphorylation of the enzyme.

The mechanism involved in modulation of ACAT activity by cholesterol is the subject of investigation. One possibility is that the enzyme operates below substrate saturation and that changes in the availability of cholesterol result in changes in the rate of synthesis of cholesteryl esters (Nilsson, 1975; Mathur *et al.*, 1981). However, the fact that *in vivo* or *in vitro* a small increment of non-esterified cholesterol can produce a many-fold

increase in the activity of ACAT has been seen as an indication that changes in the availability of cholesterol is not the only factor responsible for the modulation of the esterification rate (Hashimoto *et al.*, 1974; Brecher & Chan, 1980). Nevertheless, if the pool of cholesterol available to ACAT is very small and the increment is preferentially channelled to that pool, then a small increase in cellular non-esterified cholesterol content may represent a large increment in substrate availability. Consistent with this possibility, studies of the kinetics of cholesterol transfer from cholesterol/phospholipid unilamellar vesicles to the rat liver microsomal

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26); HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

fraction and to the ACAT substrate pool have demonstrated that the rate of transfer of liposomal cholesterol to the microsomal fraction is considerably lower than the rate of transfer of cholesterol to the ACAT substrate pool (Synouri-Vrettakou & Mitropoulos, 1983a,b). The latter rate increased fast with increasing amounts of liposomal cholesterol transferred to the microsomal fraction (Synouri-Vrettakou & Mitropoulos, 1983a), and it has been suggested that the intermediate donor of cholesterol in this intervesicular transfer is plasma-membrane vesicles that are also present in the microsomal fraction (Mitropoulos *et al.*, 1984).

Several reports have shown that rat liver or intestinal ACAT in the microsomal fraction from these tissues can be reversibly inactivated or activated by preincubation under conditions that favour dephosphorylation or phosphorylation respectively (Basheeruddin *et al.*, 1982; Suckling *et al.*, 1983a,b; Gavey *et al.*, 1983). Thus, in the presence of NaF at concentrations that inhibit the action of phosphatases, preincubation of the microsomal fraction with cytosol (100 000 *g*-supernatant fraction) and ATP/MgCl₂ resulted in a preincubation-time-dependent increase in the activity of ACAT. Preincubation of pre-activated or non-treated microsomal fraction in the presence of cytosol and MgCl₂ resulted in a progressive decline in the activity of ACAT. It is significant that similar conditions *in vitro* can result in inactivation of HMG-CoA reductase by phosphorylation, whereas ACAT activity is increased. It has therefore been proposed that the co-ordinate regulation of ACAT and HMG-CoA reductase by phosphorylation/dephosphorylation can be relevant to the intracellular short-term cholesterol homeostasis (Scallen & Sanghvi, 1983).

In the present paper we have studied the transfer of cholesterol from unilamellar cholesterol/phospholipid liposomes to the rat liver microsomal fraction and to the ACAT substrate pool on preincubation of the microsomal fraction in the absence and in the presence of some factors that may be involved in the proposed covalent modification of ACAT. Moreover, the effect of the same factors on the intervesicular cholesterol transfer were studied in the absence of an external donor of cholesterol.

Materials and methods

[1 α ,2 α -³H₂]Cholesterol, [1 α ,2 α -³H₂]cholesteryl oleate and [1-¹⁴C]oleoyl-CoA were obtained from Amersham International (Amersham, Bucks., U.K.). Oleoyl-CoA, dithiothreitol, human serum albumin (essentially free of fatty acids), egg-yolk phosphatidylcholine and cholesteryl oleate were

from Sigma (Poole, Dorset, U.K.). No impurities could be detected in the phosphatidylcholine by t.l.c. with standard solvent systems. [³H]Cholesterol was purified before use by t.l.c. on Kieselgel H at 5°C with diethyl ether as solvent. Cholesterol was from a commercial source and was recrystallized three times before use.

Preparation of liposomes

[³H]Cholesterol/phosphatidylcholine (1:1, mol/mol) single-bilayer liposomes of the desired specific radioactivity were prepared as described previously (Batzri & Korn, 1973; Kremer *et al.*, 1977). The dilute suspension of vesicles in 0.15M-NaCl was concentrated and washed free of ethanol by ultrafiltration on an Amicon (Woking, Surrey, U.K.) ultrafiltration device with an XM-100A membrane with rapid stirring under N₂ pressure (103 kPa).

Preparation of liver subcellular fractions and treatment of the microsomal fraction

Male Wistar rats weighing 180–250 g were used for all experiments. They were kept under conditions of controlled lighting and feeding (Mitropoulos *et al.*, 1973), for at least 2 weeks before the experiment. All rats were killed at 07:00 h on the day of the experiment, and the livers were removed at once. The livers were chilled on ice and perfused with ice-cold 0.25M-sucrose to remove contaminating blood. Unless otherwise indicated, the minced tissue was homogenized in 4 vol. of 0.25M-sucrose/1.5 mM-EDTA buffer (Buffer C), pH 7.4. The microsomal pellet was obtained as described previously (Venkatesan *et al.*, 1980) and was resuspended in 0.25M-sucrose/3 mM-imidazole/HCl buffer (Buffer C'), pH 7.4. The microsomal fraction contained 2–5 mg of protein/ml. In some experiments the minced liver was homogenized in either 0.1M-sucrose/40 mM-potassium phosphate/30 mM-EDTA/50 mM-NaF, pH 7.4 (Buffer A), or 0.1M-sucrose/40 mM-potassium phosphate/50 mM-KCl/5 mM-dithiothreitol, pH 7.4 (Buffer B). The presence of 30 mM-EDTA is expected to inhibit protein kinases (Buffers A and B), and 50 mM-NaF can inhibit phosphatases (Buffer A). Dithiothreitol was previously shown to inhibit the preincubation-time-dependent increase in the activity of ACAT (Synouri-Vrettakou & Mitropoulos, 1983a). The microsomal pellets obtained from the two liver homogenates were resuspended in Buffer A, Buffer B or Buffer C' to obtain the various microsomal preparations. The cytosol was the supernatant after centrifugation of the post-mitochondrial fraction for 60 min at 104 000 *g*. The floating lipid layer was removed, and the clear supernatant, containing 9–12 mg of protein, was obtained. The

microsomal fraction was incubated in the presence of [^3H]cholesterol/phosphatidylcholine liposomes or in the presence of other additions at the concentrations indicated. In incubations in the absence of liposomes, the volume of liposomes was replaced with an equal volume of 0.15M-NaCl. All incubations contained the same amount of microsomal protein, and at the end of the incubation period the mixture was cooled in ice. When indicated, the treated microsomal preparation was centrifuged at 104000g for 60 min, the supernatant was removed and the microsomal pellet was resuspended in the same buffer to give the volume of the original mixture used.

Assay of ACAT

The activity of ACAT was assayed in portions of the microsomal fraction or of the preincubation mixture at the concentration of cholesterol available to the enzyme (Synouri-Vrettakou & Mitropoulos, 1983a,b). Activity was identical whether assayed directly in a portion of the preincubation mixture or in the treated and re-isolated preparations obtained therefrom.

[^3H]Cholesteryl oleate was used as an internal standard to correct for losses during the extraction and separation of the cholesteryl [$1\text{-}^{14}\text{C}$]oleate formed. In the incubations where [^3H]cholesterol-labelled microsomal preparations were used, only non-radioactive cholesteryl oleate was used as carrier, so that the [^3H]cholesteryl oleate formed during the assay or the specific radioactivity of the product could be calculated. In all such cases, recovery was better than 90% and was corrected from the average recovery in several assays in non-labelled microsomal fraction that received internal standard. The latter assays were treated identically with those without internal standard. In all experiments described, zero-time controls and incubations containing all reagents except microsomal fraction (blanks) were used as controls, and in these assays no esterification was observed.

Other assays

Cholesteryl esters and non-esterified cholesterol (Mitropoulos *et al.*, 1978), total phospholipid (Bartlett, 1959) and protein (Lowry *et al.*, 1951) in the various subcellular fractions or the fractions obtained after their treatment were determined by methods described previously.

Results

Modulation of activity under conditions that are expected to result in the covalent modification of the enzyme

The microsomal fraction was preincubated at 37°C in the presence of 50 mM-NaF and in the

absence or the presence of various factors expected to increase the ratio of phosphorylated to dephosphorylated enzyme. At various time intervals a portion of the mixture was taken to assay ACAT activity. Fig. 1 shows the results of this experiment. Under all conditions the relation between activity and preincubation time was linear and highly significant. The slope of this relation was highest ($11.2\text{ pmol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}/\text{min}$) in the preincubation mixture containing cytosol + ATP + Mg^{2+} . Cytosol alone or cytosol + Mg^{2+} gave rates that were higher than that observed in the preincubation in the absence of these factors (Fig. 1). These results are in agreement with previous observations (Suckling *et al.*, 1983a; Gavey *et al.*, 1983) and can be consistent with a preincubation-time-dependent increase in the ratio of phosphorylated/dephosphorylated enzyme caused by the action of a putative protein kinase present in the cytosol.

A similar experiment was conducted in the absence of NaF (Fig. 2). The preincubation-time-dependent rate of increase of activity was highest in the mixture containing cytosol ($19.2\text{ pmol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}/\text{min}$), intermediate in the preincubation containing only microsomal fraction ($6.0\text{ pmol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}/\text{min}$) and lowest in the mixture containing cytosol + Mg^{2+} ($1.7\text{ pmol}\cdot$

Fig. 1. Preincubation-time-dependent increase in the activity of ACAT in the presence of NaF

Rat liver microsomal fraction (2.85 mg of protein/ml of mixture) was preincubated at 37°C in the presence of 50 mM-NaF and other additions. At the end of the preincubation period, portions of the mixture were removed to assay ACAT activity. The results are plotted against the preincubation time: ●, microsomal fraction; △, microsomal fraction + cytosol (2.5 mg of protein/ml of mixture); ○, microsomal fraction + cytosol + MgCl_2 (4 mM); ■, microsomal fraction + cytosol + Mg^{2+} + ATP (2 mM). The relation between activity and preincubation time was in all cases highly significant.

min⁻¹·ml⁻¹/min). Activity also increased progressively with preincubation time in the presence of cytosol+ATP, cytosol+ATP+Mg²⁺ or cytosol+ATP+cyclic AMP+Mg²⁺, but this increase was not linearly related to the preincubation time. At all preincubation times activity was similar under the last three conditions specified (results not shown), and activity after 50min of preincubation was lower in the preincubation containing cytosol+ATP+Mg²⁺ (Fig. 2) than that in that containing only cytosol. In all preincubation mixtures that contained ATP either in the absence or in the presence of NaF, microsomal vesicles showed aggregation at early times during the preincubation.

Effects of the buffer used to homogenize the liver and to resuspend the microsomal pellet on the activity of ACAT

Liver homogenization in Buffer A or Buffer B followed by resuspension of the microsomal pellet

Fig. 2. Preincubation-time-dependent increase in the activity of ACAT in the absence and presence of various additions. The composition of the preincubation mixture and other experimental details are as in Fig. 1.

in any of the three buffers resulted in lower activity of ACAT compared with those in the preparations derived from liver that was homogenized in Buffer C (Table 1). The homogenization of liver in Buffers

Fig. 3. Effect of cytosol on preincubation-time-dependent transfer of cholesterol from [3H]cholesterol/phosphatidylcholine liposomes to microsomal vesicles. The microsomal fraction (2.5mg of protein/ml of mixture) was incubated at 37°C in the presence of [3H]cholesterol/phosphatidylcholine liposomes (sp. radioactivity of cholesterol 8.13 d.p.m./pmol) and in the absence or the presence of cytosol (0.51 mg of protein/ml of mixture). Portions of the mixture were removed at the times shown to separate the treated microsomal vesicles from the liposomal vesicles and to determine the radioactivity associated with the microsomal fraction. Each line is the best fit for the points determined (the relation was in all cases highly significant). R is the ratio of liposomal to microsomal cholesterol at the beginning of the incubation. The broken lines represent incubations in the presence of cytosol.

Table 1. Effect of liver-homogenization and microsomal-resuspension buffer on the activity of ACAT assayed before or after preincubation at 37°C

The minced liver was homogenized in the buffer shown, and each of the microsomal pellets was resuspended in the indicated buffer. The compositions of the various buffers are given in the Materials and methods section. Values given are the average of duplicate assays.

Homogenization buffer	Buffer used to resuspend the microsomal pellet ...	ACAT activity without or with preincubation (pmol/min per mg of protein)					
		A		B		C'	
		0 min	40 min	0 min	40 min	0 min	40 min
A		19	45	17	17	18	41
B		20	51	16	20	17	53
C		42	74	26	27	39	104

A, B or C and resuspension of the microsomal pellets in Buffer B resulted in no activity increase after preincubation. However, there was an increase in the activity after the preincubation of the preparations obtained by resuspension of the microsomal pellet in buffers A or C, irrespective of the buffer used initially to homogenize the liver (Table 1).

Effects of cytosol, of NaF and of Mg^{2+} on the transfer of cholesterol from cholesterol/phosphatidylcholine liposomes to the microsomal vesicles and to the pool of cholesterol available to ACAT

To investigate whether the conditions that have been assumed to result in the covalent modification of the enzyme are also associated with different intervesicular transfer of cholesterol, we have studied under these conditions the kinetics of cholesterol transfer in the presence of cholesterol donor and the modulation of ACAT activity.

To study the effect of cytosol on the preincubation-time-dependent transfer of liposomal cholesterol to the microsomal vesicles, microsomal fraction was preincubated at 37°C for various times in the presence of various concentrations of liposomal [3H]cholesterol and in the absence or the presence of various concentrations of cytosolic protein. At the end of the desired period, a portion of the preincubation mixture was centrifuged to isolate the treated microsomal vesicles in the pellet and the liposomal fraction in the supernatant. Portions of the re-isolated microsomal vesicles were removed to assay the radioactivity associated with this fraction. We have shown previously that under these conditions transfer of cholesterol from the liposomal to microsomal vesicles is unidirectional and that the amount of cholesterol transferred, calculated from the radioactivity incorporated and the specific radioactivity of the liposomal cholesterol, is equal to the increment of cholesterol

Table 2. *Effect of liver cytosol on the intervesicular transfer of cholesterol and the activity of ACAT in liver microsomal fraction*

The liver microsomal fraction was incubated at 37°C for various periods of time in the absence or the presence of the additions shown. All preincubations in Expts. I and II contained 2.5 and 2.2 mg of microsomal protein/ml of the mixture respectively. In the presence of [3H]cholesterol/phosphatidylcholine (1:1, mol/mol; sp. radioactivity 8.13 d.p.m./pmol), *R* denotes the ratio of liposomal to microsomal cholesterol at the beginning of preincubation. In preincubations in the presence of cytosol, *C* denotes the ratio of cytosolic to microsomal protein. At the end of the preincubation period, portions of the mixture were removed to assay ACAT activity and other portions to measure 3H radioactivity associated with the treated microsomal vesicles. [3H]Cholesteryl oleate was calculated, and rates of increase owing to preincubation are from slopes of the linear relations (see Fig. 4). The specific radioactivity of cholesteryl oleate was calculated from the 3H radioactivity associated with the product on assay of ACAT. The half-time of equilibration of liposomal cholesterol with the pool of cholesterol available to ACAT was determined from the latter values as described previously (Synouri-Vrettakou & Mitropoulos, 1983a). The first-order rate constant (*k*) is the slope of the relation shown in Fig. 3, and $t_1 = \ln 2/k$ was calculated from this slope. All values given were derived from linear regression analysis of the observations at five or six preincubation times (0–50 min), and correlation coefficients were better than 0.990: * significant difference from the appropriate value in the absence of cytosol at $P \leq 0.05$.

Composition of preincubation mixture	Half-time of equilibration of liposomal cholesterol with		Preincubation-time-dependent increase in synthesis [activity (pmol · min ⁻¹ · mg ⁻¹ of protein)/min]	
	Microsomal vesicles (h)	ACAT substrate pool (min)	Total cholesteryl oleate	[3H]Cholesteryl oleate
Expt. I.				
A. Microsomal fraction	—	—	3.6	—
B. A + cytosol (<i>C</i> = 0.2)	—	—	4.5*	—
C. A + liposomes (<i>R</i> = 1)	3.8	22.0	4.9	1.0
D. A + liposomes (<i>R</i> = 2)	4.8	10.0	6.2	1.9
E. A + liposomes (<i>R</i> = 4)	7.0	9.0	9.3	4.0
F. D + cytosol (<i>C</i> = 0.2)	3.9*	9.8	9.3*	3.1*
G. E + cytosol (<i>C</i> = 0.2)	5.5*	8.4*	11.0*	4.9*
Expt. II.				
A. Microsomal fraction	—	—	1.5	—
B. A + cytosol (<i>C</i> = 0.5)	—	—	3.9*	—
C. B + liposomes (<i>R</i> = 1)	2.6	31.0	7.5	2.1
D. B + liposomes (<i>R</i> = 2)	3.5	8.0	9.7	3.6
E. A + liposomes (<i>R</i> = 2) + cytosol (<i>C</i> = 0.27)	3.6	16.0	7.8	3.0
F. A + liposomes (<i>R</i> = 2) + cytosol (<i>C</i> = 1)	2.3	7.0	12.1	4.6

determined in the treated microsomal vesicles by g.l.c. (Synouri-Vrettakou & Mitropoulos, 1983a).

Fig. 3 shows the relation between preincubation time and the radioactivity associated with the microsomal vesicles, plotted as fraction of the radioactivity in the donor at the beginning of the preincubation. The first-order rate constant (k) is the slope of the relation, and from this the half-time ($t_{1/2}$) of this transfer can be calculated [$t_{1/2} = \ln 2/k$]. The rate of transfer decreased with increasing concentration of liposomal cholesterol, but in all cases increased in the presence of cytosol (Fig. 3 and Table 2).

The preincubation-time-dependent transfer of [^3H]cholesterol to the treated microsomal vesicles is associated with an increase in the rate of ACAT change and results in a preincubation-time-dependent increase in the incorporation of [^3H]cholesterol into the cholesteryl oleate synthesized during the assay. The amount of cholesteryl oleate derived from liposomal cholesterol ([^3H]cholesteryl oleate) can be calculated from this incorporation and the specific radioactivity of [^3H]cholesterol in liposomes. As shown previously, the rates of increase in total cholesteryl oleate and in [^3H]cholesteryl oleate owing to preincubation increased with increasing concentration of liposomal cholesterol (Synouri-Vrettakou & Mitropoulos, 1983a,b), and both these values increased further when cytosol was present in the preincubation mixture (Fig. 4 and Table 2). Furthermore, both these values increased progressively with increasing cytosolic protein.

The ^3H specific radioactivity of cholesteryl oleate synthesized during assay of ACAT can be assumed to be equal to the specific radioactivity of the pool of cholesterol that acts as substrate for the enzyme. A double-reciprocal plot of the specific radioactivity of cholesteryl oleate versus preincubation time was linear and could provide the half-time of equilibration of liposomal cholesterol with the substrate pool available to ACAT (Synouri-Vrettakou & Mitropoulos, 1983a). Such treatment of the present results shows that this half-time decreased with increasing concentration of liposomal cholesterol and with the concentration of cytosolic protein present in the preincubation mixture (Table 2).

To investigate the effect of NaF on the inter-vesicular transfer of cholesterol, the microsomal fraction was preincubated in the presence of cytosol and in the absence or presence of various concentrations of cholesterol/phosphatidylcholine (1:1, mol/mol) liposomes with NaF (50mM) or without this salt. In the absence of liposomes, NaF considerably decreased the preincubation-time-dependent rate of ACAT increase (Table 3). In the presence of liposomes NaF increased the half-time

Fig. 4. Effect of cytosol on the preincubation-time-dependent increase in the rate of synthesis of cholesteryl oleate in microsomal vesicles treated in the absence or the presence of liposomes

Experimental details are given in the legend to Fig. 3. ACAT activity was directly assayed in the portion removed from the preincubation mixture at the times shown. The [^3H]cholesteryl oleate synthesized during ACAT assay was determined from the radioactivity incorporated into cholesteryl esters and the specific radioactivity of [^3H]cholesterol in the liposomes. (a) Preincubation-time-dependent increase in the synthesis of total cholesteryl oleate; (b) preincubation time-dependent increase in the rate of synthesis of [^3H]cholesteryl oleate. The ratio of liposomal to microsomal cholesterol present at the beginning of the preincubation (R) is indicated beside each line. The broken lines represent preincubations in the presence of cytosol.

of equilibration of liposomal cholesterol with the microsomal vesicles, decreased the rate of increase of total cholesteryl oleate and [^3H]cholesteryl oleate and has increased the half-time of equilibration of liposomal cholesterol with the pool of cholesterol available to ACAT (Table 3).

The effect of Mg^{2+} , present in the preincubation mixture in the absence or in the presence of cytosol and in the presence of liposomes, on the inter-vesicular transport of cholesterol is shown in Table

Table 3. *Effect of NaF on the intervesicular transfer of cholesterol and the activity of ACAT in rat liver microsomal fraction*
Experimental details are given in Table 2. Preincubations were in the absence or the presence of 50 mM-NaF.
* Significant difference from the appropriate value in the absence of NaF at $P \leq 0.05$.

Composition of preincubation mixture	Half-time of equilibration of liposomal cholesterol with		Preincubation-time-dependent increase in synthesis [activity (pmol·min ⁻¹ ·mg ⁻¹ of protein)/min]	
	Microsomal vesicles (h)	ACAT substrate pool (min)	Total cholesteryl oleate	[³ H]Cholesteryl oleate
A. Microsomal fraction	—	—	1.5	—
B. A + cytosol ($C = 0.5$)	—	—	3.9	—
C. B + NaF	—	—	1.4*	—
D. B + liposomes ($R = 1$)	2.6	31.0	7.6	2.1
E. D + NaF	4.8*	100.0*	2.5*	0.6*
F. B + liposomes ($R = 2$)	3.5	8.0	9.7	3.6
G. D + NaF	5.4*	32.0*	4.0*	1.3*

Table 4. *Effect of Mg²⁺ on the intervesicular transfer of cholesterol and the activity of ACAT in rat liver microsomal fraction*
The microsomal fraction was incubated at 37°C for various periods of time in the presence of the additions shown. All preincubations contained 1.75 mg of microsomal protein/ml of the mixture. [³H]Cholesterol/phosphatidylcholine (1:1, mol/mol; sp. radioactivity 7.28 d.p.m./pmol) liposomes were added to give the ratio of liposomal/microsomal cholesterol shown. The ratio of cytosolic to microsomal protein was 0.5 and [MgCl₂] was 4 mM. Other experimental details are given in Table 2 legend. * Significant difference from the appropriate value in the absence of Mg²⁺ at $P \leq 0.05$.

Composition of preincubation mixture	Half-time of transfer of cholesterol to microsomal vesicles (h)	Preincubation-time-dependent increase in synthesis [activity (pmol·min ⁻¹ ·mg ⁻¹ of protein)/min]	
		Total cholesteryl oleate	[³ H]Cholesteryl oleate
A. Microsomal fraction	—	2.5	—
B. A + Mg ²⁺	—	2.0*	—
C. A + cytosol	—	4.2	—
D. C + Mg ²⁺	—	2.5*	—
E. A + liposomes ($R = 1.0$)	3.5	5.2	1.6
F. A + liposomes ($R = 2.0$)	5.3	7.2	3.1
G. A + liposomes ($R = 4.0$)	8.2	8.9	4.2
H. F + Mg ²⁺	5.6	4.1*	1.1*
J. H + cytosol	4.4	4.3	1.7

4. The presence of 4 mM-MgCl₂ in the preincubation mixture had no effect on the rate of transfer of liposomal cholesterol to the microsomal vesicles either in the absence or in the presence of cytosol. However, the addition of Mg²⁺ considerably decreased the preincubation-time-dependent increase in synthesis of total cholesteryl oleate either in the presence of cytosol or liposomes or in the absence of these additions. Similarly, in preincubations in the presence of liposomes, the presence of Mg²⁺ resulted in a considerable decrease in the preincubation-time-dependent increase in synthesis of [³H]cholesteryl oleate, irrespective of the presence of cytosol (Table 4).

Effects of cytosol on the temperature-induced kinetics of transfer of liposomal cholesterol to the microsomal vesicles and to the ACAT substrate pool

Rat liver microsomal fraction was incubated for various periods of time (0–50 min) at various temperatures (25–37°C) in the absence or presence of cytosol and in the absence or presence of [³H]cholesterol/phosphatidylcholine (1:1, mol/mol) liposomes. At the end of the incubation period, portions of the mixture were removed to assay ACAT activity at 37°C, and other portions were taken to separate the treated microsomal vesicles from the liposomal vesicles in the super-

nant. From the assay of ACAT, the synthesis of total cholesteryl oleate and that of [^3H]cholesteryl oleate were calculated. To determine rates of transfer of liposomal cholesterol to the microsomal vesicles, the radioactivity associated with this fraction was plotted against the preincubation time (see Fig. 3). The rates obtained at the various temperatures were plotted against the incubation temperature (Fig. 5). The Arrhenius plot shows that, irrespective of the presence of cytosol, activation energies are constant between 37 and 25°C. This activation energy determined in the presence of cytosol (79.8 kJ/mol) is similar to that determined in its absence (60.1 kJ/mol). In another experiment of similar design, activation energies in the presence and in the absence of cytosol were correspondingly 83.2 and 88.2 kJ/mol.

Fig. 5. Arrhenius plots of the rate of transfer of liposomal cholesterol to microsomal vesicles on incubation in the absence or the presence of cytosol

Portions of the microsomal fraction (2.0 mg of protein/ml of mixture) were incubated, in the presence of [^3H]cholesterol/phosphatidylcholine (1:1, mol/mol; cholesterol sp. radioactivity 7.28 d.p.m./pmol) liposomes ($R = 2$) and in the absence or presence of cytosol (1.33 mg of protein/ml of mixture) at the temperatures indicated for various time intervals (0–50 min). Samples were removed at each time, the treated microsomal vesicles were re-isolated and the fraction of radioactivity remaining in the liposomal fraction was determined. The relation similar to that shown in Fig. 3 was thus obtained for each temperature, and from it the rate constant could be obtained. The lines shown are the best fit for the points determined (both the relations determining the first-order rate constant and the relations shown in this Figure were highly significant). Activation energies (kJ/mol) were calculated from the slope of the relation and are given beside each line.

Plots of total cholesteryl oleate and of [^3H]cholesteryl oleate, obtained from the assay of ACAT at 37°C, versus the preincubation time were prepared for each temperature and each preincubation mixture (see Fig. 4). The slopes of these relations provide the rate of increase in total cholesteryl oleate and that of increase in [^3H]cholesteryl oleate owing to the preincubation. Arrhenius plots of the rate of increase in total cholesteryl oleate gave a constant activation energy between 37 and 25°C both in the presence of cytosol and in its absence, as well as in the presence of liposomes (Fig. 6a). In the absence of liposomes, activation energies in the presence and the absence of cytosol were correspondingly 62.2 and 54.6 kJ/mol, whereas in the presence of liposomes the corresponding values were 43.7 and 57.1 kJ/mol. In another experiment of similar design, activation energies in the absence of liposomes were 71.8 and 79.4 kJ/mol correspondingly for the preincubation in the presence and the absence of cytosol, whereas in the presence of liposomes the respective values were 68.5 and 52.9 kJ/mol.

Activation energies for the rate of synthesis of [^3H]cholesteryl oleate were also constant between 37 and 25°C (Fig. 6b). Values in the presence and the absence of cytosol were correspondingly 59.2 and 81.9 kJ/mol. In a different experiment the respective values were 76.4 and 73.5 kJ/mol.

Discussion

The pool of cholesterol that acts as substrate for ACAT is the cholesterol in the environment of the enzyme (Venkatesan *et al.*, 1980) or that confined to vesicles of the microsomal fraction that are derived from endoplasmic-reticular membranes with a high density of ribosomes (Balasubramaniam *et al.*, 1978). The present results and our previous work (Synouri-Vrettakou & Mitropoulos, 1983a,b; Mitropoulos *et al.*, 1984) suggest that the increase in ACAT activity that is observed on preincubation of the rat liver microsomal fraction either in the absence or in the presence of cholesterol/phospholipid liposomes is due to an expansion in the size of this pool of cholesterol resulting from the time- and temperature-dependent transfer of cholesterol from other vesicles in the microsomal fraction and/or from liposomal vesicles to the vesicles that contain the enzyme. In the preincubations in the absence of cholesterol/phospholipid liposomes, the time-dependent increase in ACAT activity is due to the flow of cholesterol from plasma-membrane vesicles to the ACAT substrate pool, and this flow can be diverted to an alternative cholesterol acceptor if present in the preincubation mixture (Mitropoulos *et al.*, 1984). In preincubations in the presence of

Fig. 6. Arrhenius plots of the rate of increase in cholesteryl oleate synthesis on preincubation of the microsomal fraction in the absence or the presence of cytosol

Experimental details are described in the legend to Fig. 3, except that additional preincubations were set up in the absence of liposomes and in the absence or presence of cytosol. Samples were removed at each time interval to assay ACAT activity at 37°C and determine the ³H radioactivity incorporated into the cholesteryl oleate during the assay (where applicable). The values thus obtained for the various preincubation intervals were used to determine the rate of increase of total cholesteryl oleate and that of [³H]cholesteryl oleate synthesis at each temperature (the slope of the relations shown in Fig. 4). The lines shown are the best fit for the points thus determined, and in all cases the relation was highly significant. Activation energies (kJ/mol) were calculated from the slope of these lines. (a) Plots of the rate of the preincubation-time-dependent increase in synthesis of total cholesteryl oleate: ●, the preincubation mixture contained cytosol; ■, preincubations in the absence of cytosol. The ratio (*R*) of liposomal to microsomal cholesterol present at the beginning of preincubation is also shown beside

the additional cholesterol donor (cholesterol/phospholipid liposomes), transfer of cholesterol to microsomal vesicles (plasma membranes) results in a higher rate of cholesterol transfer from these vesicles to the vesicles that contain ACAT and therefore in a higher rate of increase in the synthesis of cholesteryl oleate. The kinetics of cholesterol transfer between microsomal and artificial membrane vesicles, observed in the present and in previous studies (Synouri-Vrettakou & Mitropoulos, 1983a,b; Mitropoulos *et al.*, 1984), are compatible with a mechanism that involves desorption of cholesterol molecules from the donor membranes, diffusion through the aqueous phase and absorption of these molecules by the acceptor membranes (see also McLean & Phillips, 1981; Backer & Dawidowicz, 1981). Desorption of cholesterol from the donor membranes is the rate-determining event at high ratios of acceptor to donor vesicles (Phillips *et al.*, 1980; Rothblat & Phillips, 1982; Lund-Katz *et al.*, 1982). It is likely that the same molecular mechanism is involved in the transfer of cholesterol from plasma membranes to the vesicles that contain ACAT. However, in this case the ratio of donor to acceptor vesicles remains constant and independent of the concentration of liposomal vesicles. In this transfer of cholesterol, rates increased with increasing concentration of cholesterol in the liposomal fraction (Tables 2–4; see also Synouri-Vrettakou & Mitropoulos, 1983a,b). The absolute amount of cholesterol transferred to the plasma-membrane vesicles increased with increasing concentrations of liposomal cholesterol, and this presumably results in a higher chemical potential difference for cholesterol between these vesicles and the endoplasmic-reticular membrane vesicles that contain ACAT.

The presence of cytosol during the preincubation considerably increased the rate of transfer of liposomal cholesterol to the microsomal vesicles and the rate of transfer to the vesicles that contain ACAT (Table 2). Since activation energies of both these processes are not affected by the presence of cytosol, it is unlikely that cytosol mediates these effects by increasing the desorption rate of cholesterol from the donor membranes. It is more likely that either a factor in the cytosol (Gavey *et al.*, 1981; Poorthuis & Wirtz, 1982) promotes higher concentration of desorbed cholesterol in the aqueous phase, or that such a factor by its inter-

each line. (b) Plots of the rate of increase in synthesis of [³H]cholesteryl oleate in the presence of liposomes (*R* = 2) and in the absence (□) and the presence of cytosol (○). Rates are expressed in units of pmol · min⁻¹ · ml⁻¹ · min.

action with the acceptor vesicles facilitates absorption of cholesterol by these vesicles.

The presence of NaF in the preincubation mixture produces effects opposite to those of cytosol (Table 3), and in the presence of NaF and cytosol the effects of cytosol on the intervesicular transfer of cholesterol are abolished. It is unlikely that this factor or Mg^{2+} has an influence on these processes at the level of cholesterol desorption from the donor or on the concentration of cholesterol in the aqueous phase. It is more likely that these factors influence transfer of cholesterol at the acceptor level. However, Mg^{2+} has no effect on the rate of transfer of liposomal cholesterol to the microsomal vesicles (Table 4).

The present results do not necessarily contradict the hypothesis of modulation of ACAT by covalent modification. However, they offer an alternative explanation for the evidence that led to this hypothesis. Thus the present results demonstrate that preincubation conditions that are expected to alter the ratio of phosphorylated to dephosphorylated enzyme result also in considerable modulation of the cholesterol intervesicular transport and that such modulation can be responsible for the observed activation/inactivation. Unfortunately we have failed to study intervesicular transfer of cholesterol in the presence of ATP, since in such preincubation mixtures there was aggregation of vesicles that occurred early on preincubation. However, several points that emerge from the present study argue against the hypothesis of modulation of ACAT by covalent modification, as postulated by others (Suckling *et al.*, 1983a; Gavey *et al.*, 1983). Firstly, assuming that the phosphorylated form of the enzyme is the active species and that NaF inhibits the function of phosphatases, it is difficult to understand that preincubation dependent rates of increase of ACAT activity were lower in the presence of NaF than in its absence. Secondly, assuming that the presence of Mg^{2+} + cytosol during the preincubation results in a higher ratio of dephosphorylated to phosphorylated enzyme, we would have expected to see a decrease of activity below the zero-time value. The possibility that all the enzyme is in the dephosphorylated form is unlikely, since the activity in microsomal fractions from liver homogenates prepared in the presence of 50mM-NaF was considerably lower than that in microsomal fractions obtained by the present conditions (Table 1). Thirdly, in the absence of NaF but in the presence of cytosol + ATP/ Mg^{2+} , activation after 50min preincubation was lower than in the preincubating mixtures containing only cytosol. Fourthly, independent of the liver-homogenization buffer, the resuspension and preincubation of microsomal vesicles in the presence of 30mM-EDTA, resulted

in an increase in activity (Table 1). This would be inconsistent with the action of a protein kinase, since these enzymes are expected to be inhibited under this condition. Nevertheless, the hypothesis that ACAT can be modulated *in vitro* by covalent modification has to be considered tentatively until the purified enzyme is shown to be phosphorylated, and the activity of this purified enzyme in a reconstituted system, under conditions which exclude the influence of substrate availability on activity, is shown to be modulated by this modification.

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