Biochem. J. (1983) **215**, 191–199
Printed in Great Britain

On the mechanism of the modulation *in vitro* of acyl-CoA:cholesterol acyltransferase by progesterone

Stella SYNOURI-VRETTAKOU and K. A. MITROPOULOS
Medical Research Council Lipid Metabolism Unit, Hammersmith Hospital, DuCane Road, London W12 0HS, U.K.

(*Received 23 May 1983/Accepted 7 July 1983*)

1. The assay of acyl-CoA:cholesterol acyltransferase (ACAT) in the presence of progesterone resulted in a lower enzyme activity and this inhibition was dependent on the concentration of steroid in the assay mixture. 2. The incubation at 37°C of rat liver microsomal fraction followed by the re-isolation of treated microsomal vesicles and the assay of ACAT resulted in a pre-incubation-time-dependent increase in the activity of the enzyme. This rate of increase was inhibited by the presence of progesterone in the pre-incubation mixture. 3. The incubation of the microsomal fraction in the presence of cholesterol/phosphatidylcholine liposomes, followed by the re-isolation of the treated microsomal vesicles and assay of ACAT, resulted in time-dependent and liposomal cholesterol-concentration-dependent transfer of cholesterol to microsomal vesicles and in an increase in the activity of ACAT. The presence of progesterone during pre-incubation had no effect on the rate of transfer of liposomal cholesterol to the microsomal vesicles. However, progesterone decreased the rate of change in ACAT activity. This effect can be attributed to progesterone associated with treated microsomal vesicles and present during the enzyme assay. Consistent with this, the presence of progesterone has no effect on the size of the non-esterified cholesterol pool that acts as substrate for ACAT. 4. The size of the ACAT substrate pool was modulated *in vitro* or *in vivo* and ACAT activity was assayed in the presence of various concentrations of progesterone. The data suggest that the interaction of the steroid with ACAT is at a site other than the catalytic site and that changes in the size of the substrate pool are associated with an increase in ACAT activity, but do not result in changes in the conformation of the enzyme or in co-operative transitions of the enzyme.

Administration of dietary cholesterol is associated with increased activity of ACAT in liver microsomal fraction (Balasubramaniam *et al.*, 1978*a*; Drevon, 1978; Erickson *et al.*, 1980; Mathur *et al.*, 1981) and a manyfold increase in the concentration of cholesteryl esters (Harry *et al.*, 1973; Mitropoulos & Venkatesan, 1977). Moreover, the injection into rats of various intestinal or plasma lipoproteins is associated with a high increase in the concentration of cholesteryl esters, whereas non-esterified cholesterol increases only moderately (Nervi & Dietschy, 1975; Andersen *et al.*, 1979). The fact that non-esterified cholesterol can be transferred to microsomal fraction from various lipoproteins or serum preparations (Hashimoto & Dayton, 1979; Mitropoulos *et al.*, 1981) or from cholesterol/phospholipid liposomes (Mathur *et al.*, 1981; Poorthuis & Wirtz, 1982; Suckling *et al.*, 1982; Synouri-Vrettakou & Mitropoulos, 1983) and that this transfer is associated with increased activity suggests that at the cellular level non-esterified cholesterol can control ACAT activity.

The effects of certain oxygenated derivatives of cholesterol, added to cells in culture, on the rate of cholesterol esterification has focused considerable attention on the mechanism involved. Thus some steroids, including 25-hydroxycholesterol, added to cells in culture or isolated cells increase the rate of cholesterol esterification (Schroepfer, 1981; Drevon *et al.*, 1980), whereas addition of progesterone to these cells results in a decrease in the rate of cholesterol esterification (Goldstein *et al.*, 1978).

Abbreviation used: ACAT, acyl-CoA:cholesterol acyltransferase.

The fact that the presence of progesterone in the ACAT assay system for rat liver microsomal fraction (Erickson *et al.*, 1980; Lichtenstein & Brecher, 1980) and human placental microsomal fraction (Simpson & Burkhart, 1980*a*) results in considerable loss of enzyme activity suggests that progesterone in various cells in culture reduces the rate of cholesterol esterification by direct interaction with ACAT. The inhibition of the rate of cholesterol esterification by progesterone in various cells in culture is concentration-dependent (Goldstein *et al.*, 1978; Simpson & Burkhart, 1980*b*) and, although this steroid cannot be esterified, it has been suggested (Erickson *et al.*, 1980) that its effect on ACAT is due either to competition with substrate for the catalytic site or to an unspecific effect on the membrane that influences the interaction of substrate with the enzyme.

The present paper attempts to understand the mechanism involved in the effect of progesterone on the activity of ACAT in rat liver microsomal fraction. For this we have investigated (i) the effect of progesterone on the rate of transfer of cholesterol from cholesterol/phosphatidylcholine liposomes to the microsomal fraction and (ii) the effects of the steroid on the rate of transfer of liposomal and/or microsomal cholesterol to the ACAT substrate pool. We have also (iii) modulated the size of the non-esterified cholesterol pool available to ACAT, either *in vitro* or *in vivo* and under these conditions studied the effects of progesterone on ACAT activity.

Materials and methods

[1α,2α-$^3H_2$]Cholesterol, [1α,2α(*n*)-^{3}H]progesterone and [1-^{14}C]oleoyl-CoA were obtained from Amersham International (Amersham, Bucks., U.K.). [1α,2α-$^3H_2$]Cholesteryl oleate (sp. radioactivity 47 Ci/mmol) was prepared by coupling [1α,2α-^{3}H]cholesterol with oleoyl-chloride and separating the product by thin-layer chromatography. Oleoyl-CoA, dithiothreitol, human serum albumin (essentially free of fatty acids), egg-yolk phosphatidylcholine, progesterone and cholesteryl oleate were from Sigma (London) Chemical Co., Poole, Dorset, U.K. No impurities could be detected in the phosphatidylcholine by t.l.c. using standard solvent systems. [^{3}H]Cholesterol and [^{3}H]-progesterone were purified before use by t.l.c. on Kieselgel H with diethyl ether and dichloromethane/acetone (4:1, v/v) as developing solvent systems respectively. Cholesterol was from a commercial source and was recrystallized three times before use.

Preparation of liposomes

Phosphatidylcholine/cholesterol (1:1, mol/mol) single-bilayer liposomes were prepared as described previously (Batzri & Korn, 1973). The dilute suspension was washed free of ethanol by ultrafiltration on an Amicon (Woking, Surrey, U.K.) ultrafiltration device using an XM-100 A membrane with rapid stirring under N_2 pressure (103 kPa).

Preparation of the microsomal fraction and its treatment

Male Wistar rats weighing 180–220 g were used for all experiments. The animals were kept under controlled lighting and feeding in a room maintained at 23°C, for at least 2 weeks before the experiment. The composition of the standard diet and the preparation of the diet supplemented with 1.5% cholesterol have been reported previously (Mitropoulos *et al.*, 1973). Cholesterol-fed rats were presented with cholesterol-containing pellets for 12 h before the experiment. The rats were killed by cervical dislocation at about 07:00 h on the day of the experiment. The livers were removed immediately, chilled on ice and perfused with ice-cold 0.25 M-sucrose to remove contaminating blood. The liver homogenate and therefrom the microsomal fraction containing 3–5 mg of protein/ml was obtained as described previously (Venkatesan *et al.*, 1980).

The microsomal fraction was pre-incubated at 37°C with cholesterol/phosphatidylcholine (1:1, mol/mol) liposomes at the concentrations indicated or with no liposomes (buffer-treated) for various time periods. Progesterone was added to the ACAT assay mixture or to the pre-incubation mixtures as an ethanolic solution, in a volume of ethanol not exceeding 1.5% of the total volume of the incubation mixture. Samples not treated with progesterone contained an equal volume of ethanol. All pre-incubations contained the same amount of microsomal protein and at the end of the pre-incubation period the mixture was cooled in ice. Unless otherwise stated, the mixture was centrifuged at 104000 ***g*** for 60 min, the supernatant was removed and the microsomal pellet was resuspended in 0.25 M-sucrose/3 mM-imidazole buffer, pH 7.4, to give the volume of the original microsomal fraction used.

ACAT assay

The activity of ACAT was assayed in portions of the microsomal fraction or treated microsomal preparation at the concentration of cholesterol available to the enzyme. A portion of the preparation (150–250 μg of microsomal protein) was added to a mixture containing 0.1 M-potassium phosphate buffer, pH 7.4, 2 mM-dithiothreitol, 1.2 mg of human serum albumin (free of fatty acids) in a final volume of 0.18 ml. Where indicated, progesterone was added to the mixture as an ethanolic solution. The esterification of cholesterol was initi-

ated by the addition of 10nmol of [1-^{14}C]oleoyl-CoA (sp. radioactivity 5Ci/mol) in 0.02ml of sodium acetate buffer making the final volume 0.2ml. After incubation at 37°C for 6min, the reaction was stopped by adding 4.0ml of chloroform/methanol (2:1, v/v). Extraction, separation of the cholesteryl ester fraction and determination of the rate of product formation were as described previously (Balasubramaniam *et al.*, 1978*a*). [^{3}H]-Cholesteryl oleate (sp. radioactivity 0.12Ci/mol) was used as an internal standard to correct for losses during the extraction and separation of the cholesteryl esters formed. In incubations where [^{3}H]cholesterol-labelled microsomal preparations were used, non-radioactive cholesteryl ester was used as a carrier, so that the specific radioactivity of the product formed could be calculated. In all such cases, recovery was better than 90% and was corrected from the average recovery in a number of assays in non-labelled microsomes that received internal standard and were treated in an identical manner as the other assays. In all experiments described, zero-time controls and incubations containing all reagents except microsomes (blanks) were used as controls and in these assays no esterification was observed.

Other assays

Total lipids were extracted by the method of Folch *et al.* (1957). Phospholipids in the total lipid extracts were assayed by the method of Bartlett (1959) and protein was measured by the method of Lowry *et al.* (1951). Cholesterol was quantified by g.l.c. (Mitropoulos *et al.*, 1978).

Results

The presence of progesterone in the standard ACAT assay mixture decreased the rate of synthesis of cholesteryl oleate. On assays of ACAT in the presence of [^{3}H]progesterone there was no [^{3}H]radioactivity incorporated into the cholesteryl ester fraction. The effect of progesterone on ACAT activity is steroid concentration-dependent (Fig. 1). At a given concentration of progesterone the degree of inhibition decreased with increasing amounts of microsomal protein added to the assay mixture (results not shown). The relation between the reciprocal of ACAT activity and inhibitor concentration (Dixon, 1953) was not linear, suggesting that this inhibition is unlikely to involve interaction of progesterone with the catalytic side. However, when the data in Fig. 1(*a*) were plotted by the method of Hill (1913) (Fig. 1*b*), a highly significant correlation was obtained. The fact that the Hill coefficient (calculated from the slope of Fig. 1*b*) is higher than 1.0 (Levitzki, 1978) is consistent with the possibility that progesterone inhibits ACAT by interaction with a site other than the catalytic site.

We have previously shown that the incubation of the microsomal fraction results in a temperature- and time-dependent increase in the activity of ACAT assayed either in the re-isolated microsomal vesicles or directly in portions of the pre-incubation mixture (Synouri-Vrettakou & Mitropoulos, 1983). The effect of this pre-incubation at 37°C in the absence and in the presence of progesterone is shown in Fig. 2. The presence of progesterone results in a decrease in ACAT activity at zero time of pre-incubation as well as in the rate of increase of cholesterol

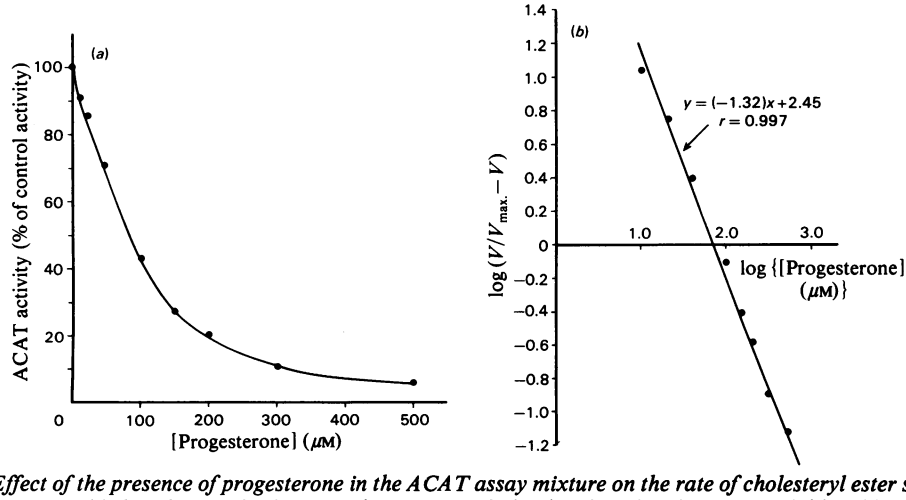

Fig. 1. *Effect of the presence of progesterone in the ACAT assay mixture on the rate of cholesteryl ester synthesis*
Progesterone was added to the standard assay mixture as a solution in ethanol and assay was initiated by addition of [^{14}C]oleoyl-CoA. (*a*) Activity is expressed as a percentage of that in the absence of progesterone. (*b*) The same results are plotted by the method of Hill. $V_{max.}$ is activity in the absence of progesterone and V is the activity in the presence of the inhibitor. The Hill coefficient is the slope of the linear relation and this is 1.32.

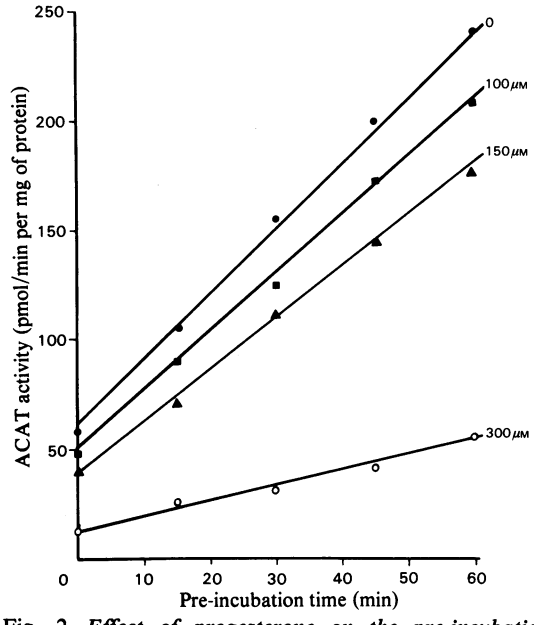

Fig. 2. *Effect of progesterone on the pre-incubation time-dependent increase in ACAT activity*

The microsomal fraction (2.1 mg of protein/ml of mixture) was pre-incubated at 37°C in the absence and in the presence of various concentrations of progesterone. At the times shown portions were removed to assay ACAT activity. The concentration of progesterone present during the pre-incubation is shown beside each line.

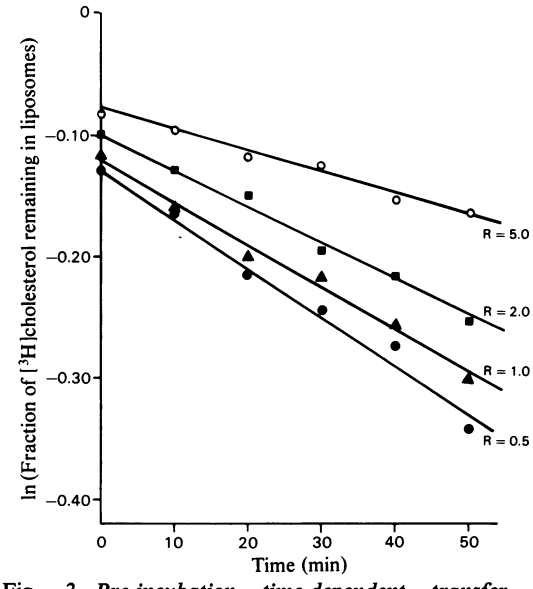

Fig. 3. *Pre-incubation time-dependent transfer of cholesterol from [3H]cholesterol/phosphatidylcholine liposomes to microsomal vesicles*

The microsomal fraction (2.4 mg of protein/ml of mixture) was pre-incubated at 37°C in the presence of various concentrations of [^{3}H]cholesterol/phosphatidylcholine liposomes (sp. radioactivity of cholesterol 12.9 d.p.m./pmol). Portions of the mixture were removed at the times shown to separate the treated microsomal from the liposomal vesicles and to determine the radioactivity associated with the microsomal fraction. Each line is the best fit for the points determined (correlation coefficient was better than 0.99) and the ratio of liposomal to microsomal cholesterol at the beginning of pre-incubation (R) is given beside each line.

esterification due to pre-incubation. These effects were progesterone concentration-dependent.

Effect of progesterone on the transfer of liposomal cholesterol to the microsomal vesicles

The microsomal fraction was pre-incubated in the absence or in the presence of progesterone and various concentrations of [^{3}H]cholesterol/lecithin (1:1, mol/mol) liposomes at 37°C. At various time intervals, portions of the pre-incubation mixture were removed and the treated microsomal vesicles were separated from the liposomal vesicles. Under these conditions, the phospholipid content of the treated microsomal vesicles was identical with that of the original microsomal preparation and the specific radioactivity of cholesterol in the re-isolated liposomal vesicles was not significantly different from that in the original liposomal preparation. Thus, it can be assumed that transfer of liposomal cholesterol to the microsomal vesicles is specific and unidirectional. Therefore, the mass of liposomal cholesterol transferred can be calculated from the radioactivity incorporated into the treated microsomal vesicles and the specific radioactivity of liposomal cholesterol (Synouri-Vrettakou & Mitropoulos, 1983).

Plots of the logarithm of the fraction of cholesterol remaining in liposomes against pre-incubation time are linear (Fig. 3), suggesting that transfer of liposomal cholesterol follows first-order kinetics with respect to donor concentration. The rate constant is the slope of the line in Fig. 3 and the half-time of the transfer $[t/2 = \ln(2/k)$ can be calculated. This rate constant decreased progressively with increasing concentration of liposomal cholesterol but did not change significantly when the pre-incubation mixture contained progesterone (Table 1).

Distribution of progesterone between microsomal and liposomal vesicles

The microsomal fraction was pre-incubated in the presence or in the absence of various concentrations

Table 1. *Effect of liposomal cholesterol concentration on rate of transfer of cholesterol to rat liver microsomal vesicles in the absence and in the presence of progesterone*

Experimental conditions are given in the legend to Fig. 3. Pre-incubations were in the absence or in the presence of 200 μM-progesterone. The first-order rate constant (k) is the slope of the relation shown in Fig. 3 and $t/2 = \ln(2/k)$ was calculated from this slope.

Ratio of liposomal to microsomal cholesterol	10^{-3} × Rate constant (min^{-1})		Half time (h)	
	No progesterone	+ Progesterone	No progesterone	+ Progesterone
0.5	4.03	3.46	2.87	3.34
1.0	3.51	3.68	3.29	3.14
2.0	2.95	3.10	3.91	3.72
5.0	1.70	2.19	6.78	5.28
10.0		1.18		9.79

of liposomes and in the presence of various concentrations of [^{3}H]progesterone (20–300 μM). At various time intervals the microsomal vesicles were re-isolated and radioactivity was measured in this fraction and the supernatant. A constant proportion of progesterone added to the pre-incubation mixture (24%) was associated with the treated microsomal vesicles and this was not influenced either by the concentration of liposomal cholesterol or the pre-incubation time.

Effect of progesterone on the transfer of cholesterol to the ACAT substrate pool

To investigate the effect of progesterone on the transfer of liposomal cholesterol to the ACAT substrate pool, the microsomal fraction was pre-incubated with [^{3}H]cholesterol/phosphatidylcholine liposomes either in the absence or in the presence of progesterone. These pre-incubations resulted in a time-dependent increase in the activity of ACAT and in the rate of synthesis of [^{3}H]cholesteryl esters formed on assay of ACAT (Fig. 4). In the presence of progesterone, the time-dependent increase in the rate of synthesis of total esters and of [^{3}H]-cholesteryl esters is lower than the corresponding values in the absence of the steroid. Table 2 also shows the effect of progesterone on the rate of increase of [^{3}H]cholesteryl oleate and of total cholesteryl esters due to pre-incubation in the presence of various concentrations of liposomal cholesterol. In the presence as well as in the absence of the steroid, these rates progressively increased with increasing concentration of liposomal cholesterol. At all concentrations of liposomal cholesterol the ratio of the increase in total cholesteryl oleate in the presence of progesterone to that of the increase in its absence was similar (for five observations: 0.58 ± 0.01; mean ± S.D.). Similarly, the ratio of the rate of increase in [^{3}H]-cholesteryl oleate in the presence of progesterone to that observed in the absence of the steroid was independent of the concentration of liposomal cholesterol (0.60 ± 0.03; mean ± S.D.).

Effect of progesterone on ACAT activity after modulation of the substrate pool in vivo

Membrane-bound allosteric enzymes have been used as sensitive probes to investigate membrane compositional changes (Farias *et al.*, 1975; Farias, 1980). Thus changes in the fatty acyl composition of membrane phospholipids or the cholesterol concentration have been correlated with changes in the co-operativity of membrane-bound allosteric enzymes. On the assumption that inhibition of ACAT by progesterone involves allosteric interaction, we investigated the relationship between the size of the ACAT substrate pool and the inhibition of the enzyme.

Administration of cholesterol-supplemented diet results in an increase in ACAT activity and an increase in the concentration of non-esterified cholesterol in liver microsomal fraction (Balasubramaniam *et al.*, 1978*a*; Erickson *et al.*, 1980; Mathur *et al.*, 1981). To understand further the effect of progesterone on ACAT activity, the liver microsomal fraction was prepared from rats fed the standard diet and from rats fed overnight 1.5% cholesterol-supplemented diet. Portions of these preparations were pre-incubated at 37°C for 50 min either with buffer or with two concentrations of [^{3}H]cholesterol/phosphatidylcholine liposomes. At the end of the pre-incubation period, the treated microsomal vesicles were re-isolated and ACAT was assayed in the presence of various concentrations of progesterone. In the absence of progesterone, ACAT activity was higher in the non-treated preparation from rats fed cholesterol as compared with that from rats fed the standard diet (Table 3). Treatment in the absence of liposomes resulted in increased activity and there was a further increase in the liposome-treated preparations. The latter pre-incubations resulted in transfer of liposomal cholesterol to

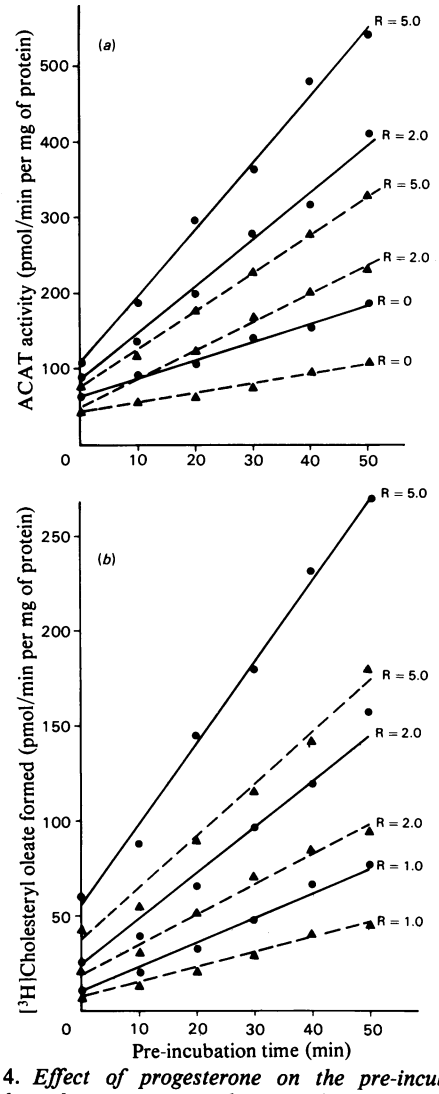

Fig. 4. *Effect of progesterone on the pre-incubation time-dependent increase in the rate of synthesis of total cholesteryl oleate and of [^{3}H]cholesteryl oleate produced on ACAT assay in liposome-treated preparations*

Experimental details are given in the legend to Fig. 3 except that pre-incubations were either in the presence of 200μM-progesterone or in its absence. ACAT activity was directly assayed in the portions removed from the pre-incubation mixture at the times indicated. The [^{3}H]cholesteryl oleate synthesized on ACAT assay was determined from the ratioactivity incorporated into cholesteryl esters and the specific radioactivity of [^{3}H]cholesterol in the liposomes. (*a*) Pre-incubation time-dependent increase in ACAT activity. (*b*) Time-dependent increase in the rate of synthesis of [^{3}H]cholesteryl oleate. Continuous lines show the relations in the absence of progesterone; broken lines are for pre-incubations in the presence of the inhibitor. The ratio of liposomal to microsomal cholesterol present in the pre-incubation (R) is indicated beside each line.

the re-isolated microsomal vesicles and the mass of liposomal cholesterol transferred was similar for the two microsomal preparations (results not shown). However, the specific radioactivity of non-esterified cholesterol was lower in the liposome-treated preparations from rats fed cholesterol (Table 3). The specific radioactivity of [^{3}H]cholesteryl oleate that was synthesized on assay of ACAT was similar in the absence or in the presence of various concentrations of progesterone. The reciprocal of ACAT activity plotted against the concentration of progesterone present in the ACAT assay mixture failed in all cases to give a linear relation. However, Hill plots showed in all titrations a highly significant correlation. The Hill coefficient for the inhibition of ACAT activity was similar to that for inhibition of [^{3}H]cholesteryl oleate synthesis in the same preparation (Table 3). Moreover, the Hill coefficients were similar in the original microsomal fraction from the two experimental conditions and the buffer- or liposome-treated microsomal vesicles.

Discussion

The incorporation of [^{14}C]oleoyl-CoA into cholesteryl oleate by human fibroblasts in culture (Goldstein *et al.*, 1978) or cultured chorio-carcinoma cells (Simpson & Burkhart, 1980*b*) is markedly reduced when progesterone is present in the culture medium. Unlike other oxysteroids, such as 25-hydroxycholesterol, 7-oxocholesterol and 6-oxocholesterol, which affect both ACAT and hydroxymethylglutaryl-CoA reductase activities in the integrated cell, the presence of progesterone in human fibroblasts in culture has no effect on hydroxymethylglutaryl-CoA reductase (Goldstein *et al.*, 1978). The addition of progesterone to rat-liver microsomal fraction (Lichtenstein & Brecher, 1980; Erickson *et al.*, 1980) or human placental microsomal fraction (Simpson & Burkhart, 1980*a*) is also associated with a considerable decrease in ACAT activity. The present data also show that the presence of progesterone in the standard ACAT assay mixture for rat liver microsomal fraction results in a decrease in the rate of cholesteryl oleate synthesis and that the extent of this decrease is related to the concentration of the steroid.

The size of the pool of cholesterol that acts as substrate for ACAT is the non-esterified cholesterol in the environment of the enzyme (Venkatesan *et al.*, 1980) or that confined in vesicles of the microsomal fraction derived from rough-endoplasmic-reticular membranes (Balasubramaniam *et al.*, 1978*b*). The present results are consistent with the following mechanism for the increase in the size of the ACAT substrate pool. The time-dependent increase in the activity of ACAT in buffer-treated preparations can

Table 2. *Effects of progesterone on the rate of increase in ACAT activity and the rate of [^{3}H]cholesteryl oleate synthesis in microsomal preparations pre-incubated in the absence or in the presence of [^{3}H]cholesterol/phosphatidylcholine liposomes*

Rates of increase owing to pre-incubation are from the slopes of the linear relation (see Fig. 4). Other experimental details are given in the text and in the legend to Fig. 4.

	Pre-incubation time-dependent increase in the synthesis (min^{-1})					
	Total cholesteryl oleate		[^{3}H]Cholesteryl oleate			
Ratio of liposomal to microsomal cholesterol	+ progesterone (*a*)	− progesterone (*b*)	+ progesterone (*c*)	− progesterone (*d*)	(*a*)/(*b*)	(*c*)/(*d*)
0	6.4	10.8	—	—	0.59	—
0.5	8.8	15.4	2.0	3.4	0.57	0.59
1.0	11.1	20.0	3.7	6.3	0.56	0.59
2.0	17.8	30.4	7.3	12.9	0.59	0.57
5.0	24.6	42.3	13.4	20.8	0.58	0.64

Table 3. *Hill coefficient for inhibition of ACAT activity by progesterone in various liver microsomal preparations*

The liver microsomal fraction was prepared from rats fed the standard diet or from rats fed cholesterol-supplemented diet. Portions of these preparations were pre-incubated at 37°C for 50 min either in the absence (buffer-treated) or in the presence of [^{3}H]cholesterol/phosphatidylcholine liposomes (sp. radioactivity 11.8 d.p.m./pmol of cholesterol) at the ratios of liposomal to microsomal cholesterol (R) shown. At the end of pre-incubation the treated microsomal vesicles were re-isolated. ACAT was assayed in all preparations in the absence and in the presence of seven concentrations of progesterone (20–300 μM). The specific radioactivity of non-esterified cholesterol was calculated from the radioactivity associated with re-isolated microsomal vesicles and the non-esterified cholesterol content. The specific radioactivity of cholesteryl oleate was calculated from the ^{3}H radioactivity associated with cholesteryl oleate on assay of ACAT and values are means ± S.D. of eight observations conducted in the absence or in the presence of various concentrations of progesterone. Hill coefficient of the inhibition of ACAT activity by progesterone is the slope of the linear relation shown in Fig. 1(*b*). This relation was in all cases highly significant.

		Specific radioactivity (d.p.m./pmol)		Hill coefficient	
Microsomal fraction and its treatment	ACAT activity (pmol/min per mg of protein)	Non-esterified cholesterol	Cholesteryl oleate synthesized	Total cholesteryl oleate	[^{3}H]Cholesteryl oleate
From rats fed standard diet					
No treatment	109			1.35	
Buffer-treated	438			1.15	
Liposome-treated					
R = 0.85	604	2.66	2.62 ± 0.06	1.37	1.27
R = 3.00	869	5.25	4.36 ± 0.1	1.35	1.34
From rats fed cholesterol-supplemented diet					
No treatment	211			1.30	
Buffer-treated	627			1.36	
Liposome-treated					
R = 0.85	762	2.16	1.94 ± 0.03	1.08	1.12
R = 3.00	930	4.57	3.64 ± 0.06	1.44	1.42

be attributed to transfer of cholesterol from other pools in the microsomal fraction to the ACAT substrate pool, which thus results in an expansion of its size (Synouri-Vrettakou & Mitropoulos, 1983). During the pre-incubation of the microsomal fraction with cholesterol/phospholipid liposomes, cholesterol is transfered from the liposomal to the microsomal vesicles and therefrom to the ACAT substrate pool to increase further the concentration of substrate available to the enzyme. In the buffer- or liposome-treated preparations the presence of progesterone considerably decreased the time-dependent increase in ACAT activity. Nevertheless, on the basis of the following evidence, progesterone does not seem to interfere with the transfer of cholesterol from liposomal and/or microsomal vesicles to the ACAT substrate pool.

First, progesterone has no effect on the time- and donor concentration-dependent transfer of cholesterol from the liposomal to the microsomal vesicles. Thus, at all ratios of liposomal to microsomal cholesterol tested there was no significant difference in the first-order rate constants and the half-times of the unidirectional cholesterol transfer between pre-

incubations in the absence and in the presence of progesterone (Table 1). Secondly, the specific radioactivity of cholesteryl ester formed on assay of ACAT was independent of the presence of the steroid during the pre-incubation or during the assay. Since the specific radioactivity of the product reflects that of the substrate, the presence of progesterone either during the pre-incubation or during the assay does not influence the size of the substrate pool available to ACAT. Thirdly, in pre-incubations carried out in the presence of progesterone, the distribution of the steroid between the re-isolated microsomal vesicles and the supernatant is independent of the concentration of liposomes and the pre-incubation time. Therefore, the lower rate of increase in cholesteryl ester synthesis observed when progesterone was present during the pre-incubation is due to this distribution and the concentration of progesterone present during the enzyme assay. Consistent with this is the observation that in various pre-incubations, the ratio of the rate of increase in total cholesteryl oleate of [^{3}H]cholesteryl oleate observed in the presence of progesterone to that noted in its absence was constant (Table 2).

The concentration of non-esterified cholesterol was higher in the microsomal fraction from rats fed the cholesterol-supplemented diet compared with rats fed the standard diet. Since equal amounts of [^{3}H]cholesterol from liposomes were transferred to both of the microsomal preparations, the lower specific radioactivity of non-esterified cholesterol in the cholesterol-fed group can be attributed to the increased cholesterol concentration found in this preparation. Similarly, the specific radioactivity of the product of ACAT was significantly lower in the liposome-treated preparations from rats fed cholesterol compared with the corresponding preparations from rats fed the standard diet (Table 3). This difference can be attributed to the larger size of the ACAT substrate pool in rats fed the cholesterol-supplemented diet, which would be consistent with the increased activity of ACAT found in the non-treated preparations from rats in this condition compared with that from rats fed the standard diet. However, it should be borne in mind that the microsomal fraction contains, in addition, a second pool of non-esterified cholesterol that can donate cholesterol to the ACAT substrate pool during the pre-incubation (Synouri-Vrettakou & Mitropoulos, 1983). Hence, the observed differences in the specific radioactivity of the product of ACAT may arise either from an increase in the size of this pool in the microsomal fraction from rats fed cholesterol or from a decrease in the rate of transfer of cholesterol from this pool to the ACAT pool during pre-incubation.

Plots of the reciprocal of ACAT activity assayed in the presence of progesterone against the concentration of the inhibitor gave no linear relationship, suggesting that this inhibition is unlikely to involve interaction of the steroid with the catalytic site. In contrast, when the data were plotted by the method of Hill, a good correlation was obtained in all cases and values for the Hill coefficient were always higher than 1.0. These results are consistent with the possibility that progesterone interacts with ACAT at a site other than the catalytic site. Assuming that inhibition of ACAT by progesterone is allosteric, we explored the relationship between the size of the ACAT substrate pool and the Hill coefficient obtained on titration of the enzyme with progesterone. The size of the pool was modulated *in vitro* by the pre-incubation of the microsomal fraction with buffer or cholesterol/phosphatidylcholine liposomes and *in vivo* by feeding cholesterol-supplemented diet. Similarities in the Hill coefficients obtained in all cases suggested that changes in the concentration of cholesterol available to ACAT are not associated with changes in the conformation of the enzyme or with co-operative transitions. This is consistent with previous observations showing that administration of polyunsaturated fat to rats results in changes in the activity of ACAT and changes in the fatty acid composition of liver microsomal fraction but no change in the characteristics of Arrhenius plots of the enzyme in the microsomal fraction (Spector *et al.*, 1980).

The inhibition of ACAT activity by progesterone is unlikely to have physiological significance in the regulation of cholesteryl ester in the liver, especially in view of the high concentration of steroid needed to produce effects. Nevertheless, this effect may be relevant to the cholesterol homeostasis in steroidogenic tissues. For example, it has been shown that in choriocarcinoma cells, aminoglutethimide, a substance that inhibits conversion of cholesterol into progesterone, increased cholesteryl ester synthesis (Simpson & Burkhart, 1980*b*). Moreover, in such tissues the overall cellular concentration of progesterone may be lower than that in endoplasmic-reticular membranes. Thus it has been shown that the progesterone content of fresh human placenta is 7μg/g of tissue, whereas the concentration in the environment of ACAT may be 30–40μM, a concentration sufficient to achieve considerable inhibition of ACAT activity (Simpson & Burkhart, 1980*a*). It has been suggested that the concentration of progesterone in the environment of ACAT may be considerably higher than the overall cellular concentration, since both ACAT as well as 3β-hydroxy-steroid dehydrogenase and Δ^5–Δ^4-isomerase, which catalyse the conversion of pregnenolone into progesterone, are all located in endoplasmic-reticular membranes (Simpson & Burkhart, 1980*b*).

References

Andersen, J. M., Turley, S. D. & Dietschy, J. M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 165–169

Balasubramaniam, S., Mitropoulos, K. A. & Venkatesan, S. (1978*a*) *Eur. J. Biochem.* **90**, 377–383

Balasubramaniam, S., Venkatesan, S., Mitropoulos, K. A. & Peters, T. J. (1978*b*) *Biochem. J.* **174**, 863–872

Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468

Batzri, S. & Korn, E. D. (1973) *Biochim. Biophys. Acta* **298**, 1015–1019

Dixon, M. (1953) *Biochem. J.* **55**, 170–171

Drevon, C. (1978) *Atherosclerosis* **30**, 123–136

Drevon, C. A., Weinstein, D. B. & Steinberg, D. (1980) *J. Biol. Chem.* **255**, 9128–9137

Erickson, S. K., Shrewsbury, M. A., Brooks, C. & Meyer, D. J. (1980) *J. Lipid Res.* **21**, 930–941

Farias, R. N. (1980) *Adv. Lipid Res.* **17**, 251–282

Farias, R. N., Bloj, B., Morero, R. D., Siñeriz, F. & Trucco, R. E. (1975) *Biochim. Biophys. Acta* **415**, 231–251

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509

Goldstein, J. L., Faust, J. R., Dygos, J. H. & Chorvat, R. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1877–1881

Harry, D. S., Dini, M. & McIntyre, N. (1973) *Biochim. Biophys. Acta* **296**, 209–220

Hashimoto, S. & Dayton, S. (1979) *Biochim. Biophys. Acta* **573**, 354–360

Hill, A. V. (1913) *Biochem. J.* **7**, 471–480

Levitzki, A. (1978) *Molecular Biology, Biochemistry and Biophysics*, vol. 28, Springer-Verlag, Berlin

Lichtenstein, A. H. & Brecher, P. (1980) *J. Biol. Chem.* **255**, 9098–9104

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mathur, S. N., Armstrong, M. L., Alber, C. A. & Spector, A. A. (1981) *J. Lipid Res.* **22**, 659–667

Mitropoulos, K. A. & Venkatesan, S. (1977) *Biochim. Biophys. Acta* **489**, 126–142

Mitropoulos, K. A., Balasubramaniam, S. & Myant, N. B. (1973) *Biochim. Biophys. Acta* **326**, 428–438

Mitropoulos, K. A., Venkatesan, S., Balasubramaniam, S. & Peters, T. J. (1978) *Eur. J. Biochem.* **82**, 419–429

Mitropoulos, K. A., Venkatesan, S., Reeves, B. E. A. & Balasubramaniam, S. (1981) *Biochem. J.* **194**, 265–271

Nervi, F. O. & Dietschy, J. M. (1975) *J. Biol. Chem.* **250**, 8704–8711

Poorthuis, B. J. H. M. & Wirtz, K. W. A. (1982) *Biochim. Biophys. Acta* **710**, 99–105

Schroepfer, G. S. (1981) *Annu. Rev. Biochem.* **50**, 585–621

Simpson, E. R. & Burkhart, M. F. (1980*a*) *Arch. Biochem. Biophys.* **200**, 79–85

Simpson, E. R. & Burkhart, M. F. (1980*b*) *Arch. Biochem. Biophys.* **200**, 86–92

Spector, A. A., Kaduce, T. L. & Dane, R. W. (1980) *J. Lipid Res.* **21**, 169–179

Suckling, K. E., Boyd, G. S. & Smith, C. G. (1982) *Biochim. Biophys. Acta* **710**, 154–163

Synouri-Vrettakou, S. & Mitropoulos, K. A. (1983) *Eur. J. Biochem.* **133**, 299–307

Venkatesan, S., Mitropoulos, K. A., Balasubramaniam, S. & Peters, T. J. (1980) *Eur. J. Cell Biol.* **21**, 167–174