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The Submicrosomal Localization of Acyl-Coenzyme A–Cholesterol Acyltransferase and its Substrate, and of Cholesteryl Esters in Rat Liver

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To determine the submicrosomal distribution of acyl-CoA–cholesterol acyltransferase and of cholesteryl esters, the microsomal fraction and the digitonin-treated microsomal preparation of rat liver were subjected to analytical centrifugation on sucrose density gradients. With untreated microsomal fractions the distribution profile and the median density of acyl-CoA–cholesterol acyltransferase were very similar to those of RNA. This is in contrast with hydroxymethylglutaryl-CoA reductase and cholesterol 7α-hydroxylase, which are confined to endoplasmic reticulum membranes with low ribosomal coating. In digitonin-treated microsomal preparations activity of acyl-CoA–cholesterol acyltransferase was not detectable. The labelling of untreated microsomal fractions with trace amounts of [^{14}C]cholesterol followed by subfractionation of the labelled microsomal fraction showed that the specific radioactivity of cholesteryl esters obtained *in vitro* by the various subfractions was similar with all subfractions but different from the specific radioactivity of the 7α-hydroxycholesterol obtained *in vitro* by the same subfraction. These results demonstrate the existence of two pools of cholesterol confined to membranes from the endoplasmic reticulum, one acting as substrate for cholesterol 7α-hydroxylase and the other acting as substrate for acyl-CoA–cholesterol acyltransferase. The major part of cholesteryl esters present in both untreated and digitonin-treated microsomal fractions was distributed at densities similar to those of membranes from the smooth endoplasmic reticulum and at densities lower than those of smooth membranes from Golgi apparatus. The ratio of the concentrations of non-esterified to esterified cholesterol in the subfractions from both untreated and digitonin-treated microsomal fractions was highest at the maximum distribution of plasma membranes.

Hepatic cholesterol, including that reaching the liver from the plasma and that biosynthesized *in situ*, is converted into bile acids or is secreted into the plasma as lipoproteins in the non-esterified or esterified form. The esterification of hepatic cholesterol is catalysed by the enzyme acyl-CoA–cholesterol acyltransferase (EC 2.3.1.26), an enzyme confined to the microsomal fraction of liver homogenates (Goodman *et al.*, 1964; Chesterton, 1968; Stokke & Norum, 1970). In the conversion of cholesterol into bile acids, the 7α-hydroxylation of cholesterol catalysed by the enzyme cholesterol 7α-hydroxylase (EC 1.14.13.17) controls the overall rate of bile acid biosynthesis (Danielsson *et al.*, 1967; Shefer *et al.*, 1970; Mitropoulos *et al.*, 1973). The rate-limiting step for the biosynthesis of cholesterol is considered to be the reduction of 3-hydroxy-3-methylglutaryl CoA to mevalonic acid (Dietschy & Brown, 1974; Rodwell *et al.*, 1973) catalysed by the enzyme hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34). Both cholesterol 7α-hydroxylase and hydroxymethylglutaryl-CoA reductase are confined to membranes of the endoplasmic reticulum isolated with the microsomal fraction of liver homogenates (Mitropoulos *et al.*, 1978*a*).

The main component of the microsomal fraction of rat liver is derived from the endoplasmic reticulum, fragments of which form self-sealing vesicles during the homogenization of the tissue (Palade & Siekevitz, 1956). The microsomal fraction contains in addition a number of minor components, including most of the Golgi apparatus, more than half the plasma membrane and membrane fragments from other subcellular organelles (Amar-Costesec *et al.*, 1974), although the degree of such contamination may depend on the method used to obtain the microsomal fraction (DePierre & Dallner, 1976). The membranes that contaminate those originating from the endoplasmic reticulum contain the major part of the non-esterified cholesterol in the microsomal fraction

(Amar-Costesec *et al.*, 1974; Mitropoulos *et al.*, 1978*a*). We have now established (Mitropoulos *et al.*, 1978*a*) that hydroxymethylglutaryl-CoA reductase, cholesterol 7α-hydroxylase and the non-esterified cholesterol that acts as substrate for the hydroxylase are confined to membranes originating from the endoplasmic reticulum with low density of ribosomes. Moreover, we have recently provided evidence that the size of the pool of free cholesterol in the environment of these two enzymes is important for their physiological regulation (Mitropoulos *et al.*, 1978*b*).

In the present paper the submicrosomal distribution of acyl-CoA–cholesterol acyltransferase, of the endogenous substrate pool of cholesterol for this enzyme and of cholesteryl esters are determined. In addition, the relationship between the substrate pools for cholesterol 7α-hydroxylase and for acyl-CoA–cholesterol acyltransferase are explored.

Materials and Methods

Materials

[1-^{14}C]Oleic acid, [9,10(*n*)-^{3}H]oleic acid, [4-^{14}C]-cholesterol and [1α,2α-$^{3}H_2$]cholesterol were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). The sources of other radiolabelled compounds and other materials used in the present work have been reported elsewhere (Mitropoulos *et al.*, 1978*a*).

Animals and their treatment

Male Wistar rats weighing 180–220g were used for all experiments. For at least 2 weeks before each experiment the rats were kept under conditions of controlled lighting and feeding (Mitropoulos *et al.*, 1973) in a room maintained at 23°C.

Preparation of the microsomal fraction, treatment with digitonin and labelling with [4-^{14}C]cholesterol

In all experiments the rats were killed at about 07:00h on the day of the experiment, by cervical dislocation, and the livers were removed immediately. The preparation of the microsomal fraction from these livers, the treatment of the microsomal fraction with digitonin and the labelling of microsomal preparations with [4-^{14}C]cholesterol were carried out essentially as described elsewhere (Mitropoulos *et al.*, 1978*a*). The [^{14}C]cholesterol-labelled microsomal preparation contained, per ml, 2.5μCi of [^{14}C]-cholesterol, 0.425mg of Tween-80 or 125μl of acetone, and the microsomal fraction from 1g fresh weight of liver.

Subfractionation of microsomal preparations

The microsomal preparations were subfractionated by isopycnic centrifugation on continuous sucrose density gradients in a small-volume zonal rotor type E40 (Beaufay, 1966) as described elsewhere (Mitropoulos *et al.*, 1978*a*). After centrifugation, some 15 fractions were collected and assayed for protein, RNA, cholesterol, specific radioactivity of cholesterol and enzyme activities.

Enzyme assays

Acyl-CoA–cholesterol acyltransferase was assayed in microsomal preparations or the submicrosomal fractions at the endogenous cholesterol concentration. A portion of the microsomal preparation or the submicrosomal fraction was added to a solution containing 0.1M-potassium phosphate, pH7.4, 2mM-dithiothreitol, 1.2mg of human serum albumin (free of fatty acids), 2mM-ATP, 4mM-$MgCl_2$, 4mM-CoA and 0.1mM-[1-^{14}C]oleic acid (10μCi/μmol). After incubation for 10min at 37°C, the reaction was stopped by the addition of 20vol. of chloroform/methanol (2:1, v/v) and a known amount of [1α,2α-$^{3}H_2$]cholesteryl oleate as an internal standard. The cholesteryl esters were isolated by t.l.c. (Balasubramaniam *et al.*, 1977), and the activity of acyl-CoA–cholesterol acyltransferase was calculated from the rate of incorporation of radioactivity into the cholesteryl [1-^{14}C]oleate fraction and the specific radioactivity of the [1-^{14}C]oleic acid used. In incubations of the [^{14}C]cholesterol-labelled microsomal preparations or the subfractions obtained from such preparations, [9,10(n)-^{3}H]oleic acid was used as substrate. This permitted the calculation of the activity and of the specific radioactivity of [4-^{14}C]-cholesteryl esters formed during the incubation. Microsomal preparations incubated with 0.1mM-oleic acid gave an enzyme activity that was linear with respect to time and to protein concentration (in the range used).

Cholesterol 7α-hydroxylase was assayed in 0.4ml of the [4-^{14}C]cholesterol-labelled microsomal preparations or in 0.6ml of the submicrosomal fractions at the endogenous cholesterol concentration. The incubation conditions, the extraction of the mixture after incubation, the separation and isolation of the radioactive 7α-hydroxycholesterol, the measurement of the mass of 7α-hydroxycholesterol produced during the incubation and its specific radioactivity have been described previously (Mitropoulos & Balasubramaniam, 1972). Hydroxymethylglutaryl-CoA reductase in the microsomal preparations or in the submicrosomal fractions was assayed essentially as described elsewhere (Mitropoulos & Balasubramaniam, 1976). NADPH–cytochrome *c* oxidoreductase, cytochrome *P*-450, 5′-nucleotidase and galactosyltransferase were assayed as reported elsewhere (Mitropoulos *et al.*, 1978*a*).

Analytical determinations

The determination of non-esterified and of esterified cholesterol in the microsomal preparations and

the submicrosomal fractions was carried out as described previously (Mitropoulos *et al.*, 1978*a*; Mitropoulos & Venkatesan, 1977). [1α,2α-$^3H_2$]-Cholesteryl oleate (20000d.p.m.; 43mCi/μmol) was used as internal standard to correct for the recovery of cholesteryl esters. The ^{14}C specific radioactivity of cholesterol in the subcellular fractions was calculated from the mass of non-esterified cholesterol in the fraction and the ^{14}C radioactivity eluted with the non-esterified cholesterol band corrected for losses during extraction, chromatography and elution, by the use of the [^3H]cholesterol internal standard.

RNA was determined as described by LePecq & Paoletti (1966). Protein was determined by the method of Lowry *et al.* (1951) or in the density-gradient fractions by the method of Hiraoka & Glick (1963) with bovine serum albumin as standard.

Results

Specific radioactivity of 7α-hydroxycholesterol and of cholesteryl esters formed in vitro by the microsomal preparation prelabelled with [4-^{14}C]cholesterol

When the microsomal fraction was labelled with trace amounts of [^{14}C]cholesterol added as a suspension in Tween 80 and then incubated to assay the activity of acyl-CoA–cholesterol acyltransferase and the specific radioactivity of [^{14}C]cholesteryl esters formed, the specific radioactivity of cholesteryl esters was lower than the specific radioactivity of non-esterified cholesterol in the incubation mixture. Consistent with previous observations (Mitropoulos & Balasubramaniam, 1972; Balasubramaniam *et al.*, 1973) the specific radioactivity of the product of cholesterol 7α-hydroxylase, under the same conditions, was significantly higher than the specific radioactivity of non-esterified cholesterol in the microsomal fraction. Thus the specific radioactivity of the product of cholesterol 7α-hydroxylase was much higher than that of the product of acyl-CoA–cholesterol acyltransferase. When the microsomal fraction was labelled with trace amounts of [4-^{14}C]-cholesterol added as a solution in a small volume of acetone, the specific radioactivity of cholesteryl ester formed during the assay of acyl-CoA–cholesterol acyltransferase was 6–7-fold higher than that of non-esterified cholesterol in the incubation mixture (Table 1). The specific radioactivity of the 7α-hydroxycholesterol formed from endogenous cholesterol, labelled under the same conditions, was lower than the specific radioactivity of the non-esterified cholesterol in the microsomal fraction. Thus the specific radioactivity of the product of acyl-CoA–cholesterol acyltransferase was in this case 8–9-fold higher than the specific radioactivity of the product of cholesterol 7α-hydroxylase. Since the specific radioactivity of the product of a reaction should reflect the specific radioactivity of the precursor, these experiments suggest that there is compartmentation of non-esterified cholesterol in the microsomal fraction and indicate that the pool of endogenous cholesterol that acts as substrate for cholesterol 7α-hydroxylase is different from the pool that acts as substrate for acyl-CoA–cholesterol acyltransferase.

Distribution of acyl-CoA–cholesterol acyltransferase, of cholesterol 7α-hydroxylase and of non-esterified cholesterol in submicrosomal fractions

Fig. 1 shows the frequency/density histograms of typical experiments in which the microsomal fraction was subjected to analytical fractionation on sucrose density gradients. Table 2 shows the median densities of the various microsomal components or component activities in a series of experiments with untreated or digitonin-treated microsomal preparations. In the untreated microsomal fractions, acyl-CoA–cholesterol acyltransferase activity showed a similar distribution to that of RNA and had a very similar median density. The average median density of

Table 1. *Influence of the method of labelling of microsomal fractions with trace amounts of [4-^{14}C]cholesterol in vitro on the specific radioactivity of the product of cholesterol 7α-hydroxylase and of acyl-CoA–cholesterol acyltransferase*

[4-^{14}C]Cholesterol (57Ci/mol) as a suspension in Tween 80 (0.170mg/μCi of [^{14}C]cholesterol) or as a solution in acetone (50μl/1μCi of [^{14}C]cholesterol) was added to the incubation mixture (0.156μCi/mg of protein per 0.78ml of incubation mixture) at 4°C. The mixture was incubated to assay the activity of cholesterol 7α-hydroxylase and the specific radioactivity of 7α-hydroxy[^{14}C]cholesterol formed or the activity of acyl-CoA–cholesterol acyltransferase and the specific radioactivity of [^{14}C]cholesteryl oleate formed. Other experimental details are given in the text.

		Activity (pmol/min per mg of protein)		Specific radioactivity of		
[4-^{14}C]Cholesterol added in:	Non-esterified cholesterol (nmol/mg protein)	Cholesterol 7α-hydroxylase	Acyl-CoA–cholesterol acyltransferase	^{14}C-labelled non-esterified cholesterol (d.p.m./nmol)	7α-Hydroxy-[^{14}C]cholesterol (d.p.m./nmol)	[^{14}C]Cholesteryl oleate (d.p.m./nmol)
Tween 80	67.1	31.0	60	5380 ± 440	7980 ± 350	4500 ± 280
Acetone	67.1	29.8	63	5380 ± 440	3900 ± 70	35460 ± 420

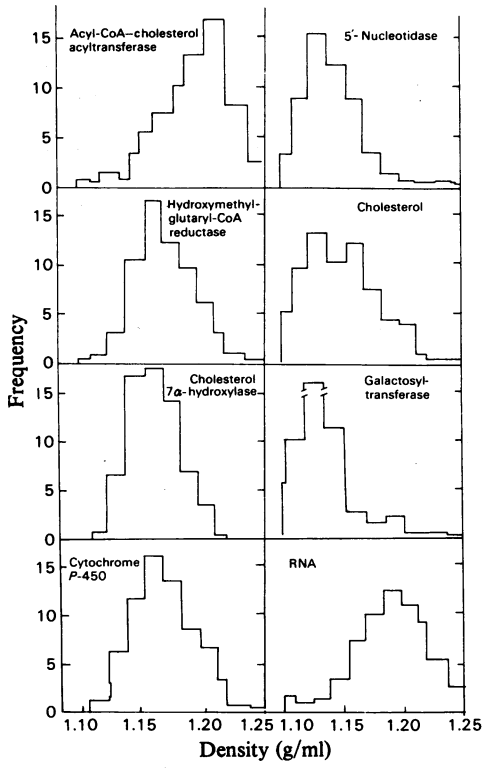

Fig. 1. *Subfractionation of microsomal preparations by isopycnic centrifugation on sucrose gradients*
Frequency/density histograms of various activities or components of the microsomal fraction are shown. Frequency is defined as the portion of total recovered activity present in the individual submicrosomal fraction divided by the density span covered. The percentage recovered activity or component from the gradient was in all cases near to 100.

cholesterol 7α-hydroxylase and of hydroxymethylglutaryl-CoA reductase was similar to those obtained for NADPH–cytochrome *c* oxidoreductase and cytochrome *P*-450, and these median densities were significantly lower than that of acyl-CoA–cholesterol acyltransferase.

The treatment of the microsomal fraction with digitonin inactivated acyl-CoA–cholesterol acyltransferase, and there was no significant activity of this enzyme either in the digitonin-treated microsomal preparation or in the supernatant obtained from the treatment of the microsomal fraction with digitonin. Consistent with previous results (Mitropoulos *et al.*, 1978*a*), digitonin-treated microsomal fractions subfractionated by isopycnic centrifugation gave density profiles and median densities for various microsomal components or component enzyme activities that were considerably different from those obtained with untreated microsomal fractions (Table 2).

Submicrosomal localization of the substrate pools for acyl-CoA–cholesterol acyltransferase and for cholesterol 7α-hydroxylase

To compare the labelling of the pool of cholesterol that acts as substrate for acyl-CoA–cholesterol acyltransferase with that available to cholesterol 7α-hydroxylase, the labelled microsomal fraction was subfractionated by isopycnic centrifugation on a continuous sucrose gradient. Portions of fractions were incubated to assay the activity of acyl-CoA–cholesterol acyltransferase, of cholesterol 7α-hydroxylase and the specific radioactivity of cholesteryl esters and of 7α-hydroxycholesterol formed during such incubations. Fig. 2(*a*) shows the distribution profile of acyl-CoA–cholesterol acyltransferase and the specific radioactivity of [^{14}C]cholesteryl esters formed with the various subfractions. The specific radioactivity of

Table 2. *Median densities of activities and components of digitonin-treated and untreated microsomal preparations equilibrated in sucrose water gradients*

Activity or component	Untreated microsomal fractions		Digitonin-treated microsomal fractions	
	No. of expts.	Median density (g/ml)	No. of expts.	Median density (g/ml)
Protein	7	1.165 ± 0.006	5	1.161 ± 0.002
RNA	7	1.193 ± 0.003	6	1.170 ± 0.010
Cholesterol (non-esterified)	7	1.145 ± 0.005	6	1.177 ± 0.004
5'-Nucleotidase	6	1.143 ± 0.006	5	1.185 ± 0.001
Galactosyltransferase	3	1.128 ± 0.002	1	1.160
NADPH–cytochrome *c* oxidoreductase	7	1.167 ± 0.006	6	1.156 ± 0.004
Cytochrome *P*-450	3	1.167 ± 0.002	3	1.157 ± 0.001
Acyl-CoA–cholesterol acyltransferase	3	1.198 ± 0.003	—	—
Cholesterol 7α-hydroxylase	4	1.167 ± 0.005	3	1.165 ± 0.002
Hydroxymethylgutaryl-CoA reductase	5	1.173 ± 0.007	4	1.164 ± 0.005

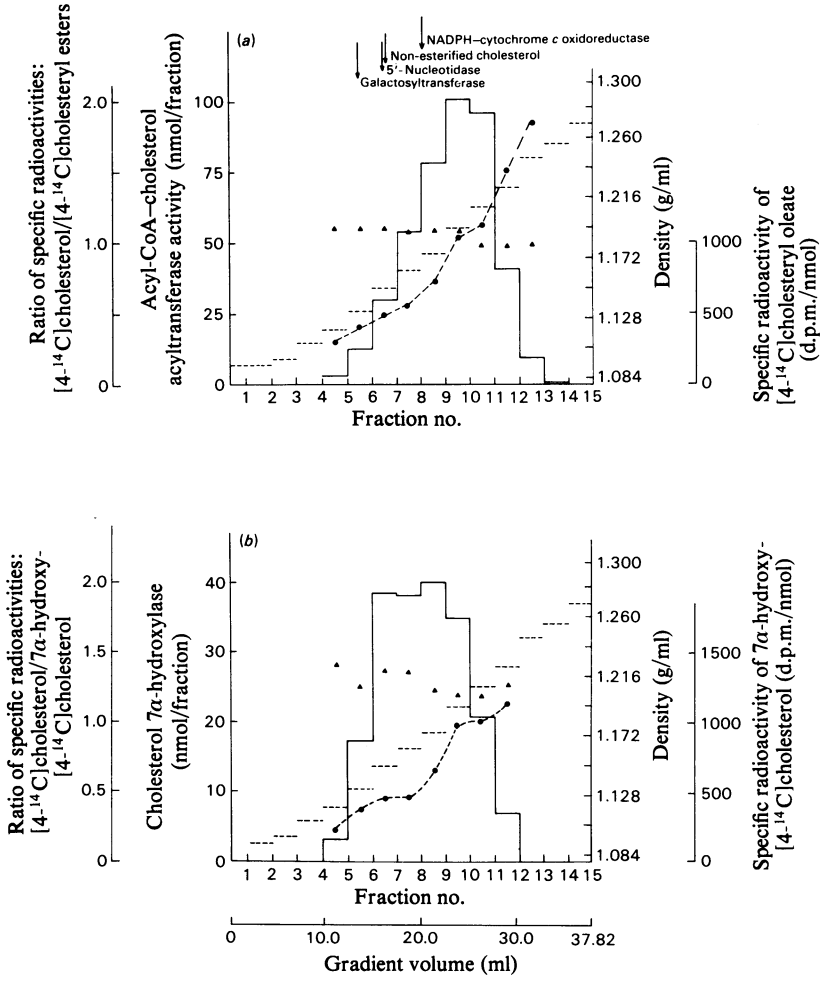

Fig. 2. *Distribution of the activity of acyl-CoA–cholesterol acyltransferase, of cholesterol 7α-hydroxylase and of the specific radioactivities of the products of these enzymes formed by fractions obtained on isopycnic centrifugation of untreated microsomal fractions labelled with [^{14}C]cholesterol*

Broken horizontal lines (----) show the density of the gradient at each fraction, and the arrows indicate the median volume of the activity or component shown. (*a*) Portions of the fractions obtained were incubated to assay the activity of acyl-CoA–cholesterol acyltransferase (——) and the specific radioactivity of [4-^{14}C]cholesteryl esters formed during the incubation (▲). The ratio of the specific radioactivities of non-esterified cholesterol to the product of the incubation (●) was calculated for each fraction. The activity of acyl-CoA–cholesterol acyltransferase recovered from the gradient was 99% of the original microsomal preparation. (*b*) Portions of the fractions were incubated to assay the activity of cholesterol 7α-hydroxylase (——) and the specific radioactivity of 7α-hydroxycholesterol formed (▲). The ratio of the specific radioactivity of cholesterol to 7α-hydroxycholesterol (●) was calculated for each fraction. The activity of cholesterol 7α-hydroxylase recovered from the gradient was 93% of the original microsomal preparation.

cholesteryl esters formed during the assay was similar in all fractions that contained significant amounts of enzyme.

In fraction 13 the specific radioactivity of the non-esterified cholesterol was twice that of the cholesteryl esters formed during the assay of acyl-CoA–cholesterol acyltransferase (ratio of specific radioactivities cholesterol/cholesteryl esters is 2), but with decreasing density this ratio fell progressively to a value of 0.15 in fraction 5. Fig. 2(*b*) shows the distribution profile of cholesterol 7α-hydroxylase and the specific radioactivity of 7α-hydroxy[^{14}C]chole-

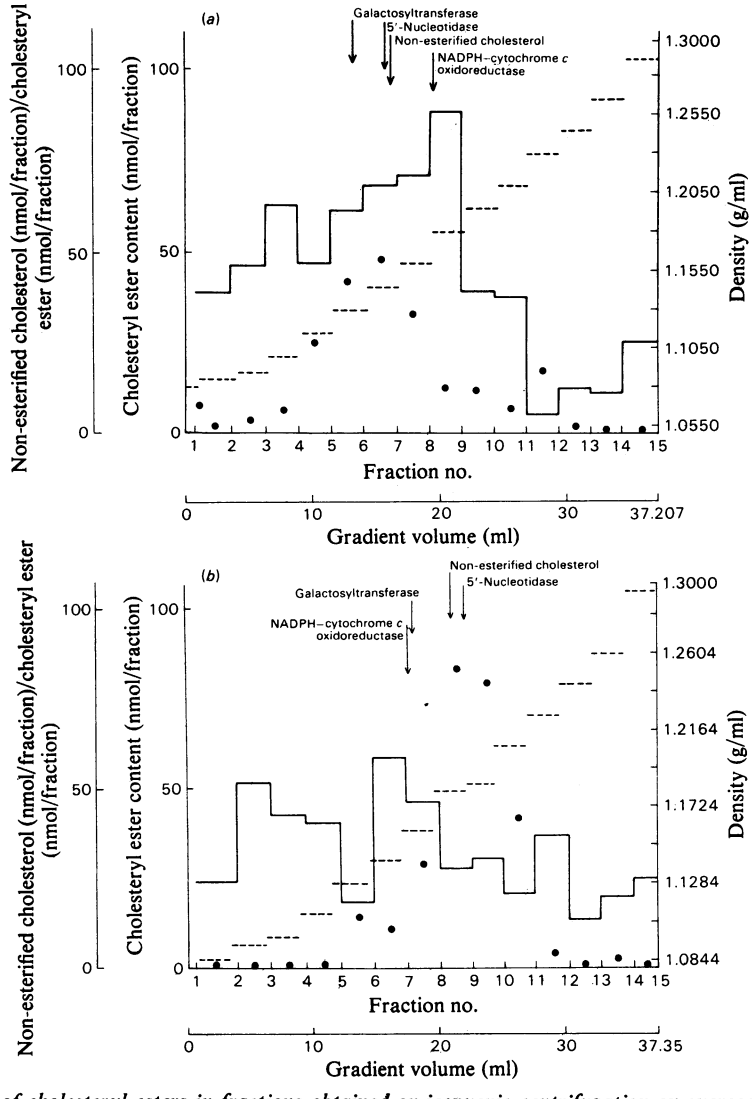

Fig. 3. *Distribution of cholesteryl esters in fractions obtained on isopycnic centrifugation on sucrose gradient of untreated and of digitonin-treated microsomal preparations*

The microsomal fraction was obtained, and a portion was treated with digitonin. The digitonin-treated and the untreated microsomal preparation were subjected to isopycnic centrifugation to obtain 15 fractions in each case. A portion from each fraction was analysed for non-esterified cholesterol and for cholesteryl ester content (——). The ratio of non-esterified cholesterol to cholesteryl ester content (●) was calculated for each fraction. The broken horizontal lines show the density of the gradient at each fraction, and the arrows show the median volume of the activity or component marked. (*a*) Fractionation of untreated microsomal fractions. In this experiment the amount of cholesteryl esters recovered from the gradient was 114% of that in the original microsomal fraction and the recovery of non-esterified cholesterol from the gradient was 108%. (*b*) Fractionation of digitonin-treated microsomal fractions. The recovery of cholesteryl esters was 108%, and that of non-esterified cholesterol, 91%.

sterol formed with the various fractions of the same experiment. This enzyme is distributed in the gradient in lower densities as compared with the distribution of acyl-CoA–cholesterol acyltransferase shown in Fig. 2(*a*). The specific radioactivity of the 7α-hydroxycholesterol was similar in all fractions that contained significant amounts of the enzyme. The mean specific radioactivity of 7α-hydroxycholesterol obtained with

the various fractions (1250 ± 79 d.p.m./nmol) was significantly different ($P < 0.001$) from the mean specific radioactivity of the product of acyl-CoA–cholesterol acyltransferase (1018 ± 69 d.p.m./nmol) shown in Fig. 2(*a*). In agreement with previous results (Mitropoulos *et al.*, 1978*a*), the ratio of the specific radioactivities of non-esterified cholesterol to 7α-hydroxycholesterol decreased progressively with decreasing density as the contamination of the subfractions with cholesterol confined to plasma membranes and smooth membranes from Golgi apparatus increased (Fig. 2*b*).

Submicrosomal localization of cholesteryl esters

Fig. 3 shows the distribution of cholesteryl esters in the various subfractions obtained with untreated and digitonin-treated microsomal fractions. In this experiment, to avoid experimental and biological variations in the cholesteryl ester content between two microsomal preparations when comparing the ester distribution in untreated and digitonin-treated microsomes, a portion from the same microsomal preparation was treated with digitonin and the untreated and digitonin-treated microsomal fractions were subjected to subfractionation on the same day. With untreated microsomal fractions there was a detectable amount of cholesteryl esters in all 15 fractions (Fig. 3*a*). The major part of the cholesteryl esters was distributed between fractions 1–11, and there were peaks of distribution at fractions 4 and 9. The peak of fraction 4 was at a considerably lower density than the median density of galactosyltransferase, and the peak at fraction 9 was at a density similar to that of NADPH–cytochrome *c* oxidoreductase, cytochrome *P*-450, ethylmorphine demethylase, hydroxymethylglutaryl-CoA reductase and protein. The ratio of the concentrations of non-esterified to esterified cholesterol was low in the early fractions, but it increased progressively to reach a high value at gradient volume similar to the median volume of the enzyme 5′-nucleotidase and then decreased to low values after fraction 8. Fig. 3(*b*) shows the distribution of cholesteryl esters in the subfractions obtained with digitonin-treated microsomal fractions. All fractions contained detectable amounts of cholesteryl esters, and there were peaks of distribution in fractions 3, 7 and 12. The first peak was at a density considerably lower than the median density of NADPH–cytochrome *c* oxidoreductase. The peak of distribution of cholesteryl esters in fraction 7 was at a gradient density similar to the median density of NADPH–cytochrome *c* oxidoreductase, of ethylmorphine demethylase and of cytochrome *P*-450. The treatment of the microsomal fraction with digitonin results in solubilization of some of the non-esterified and esterified cholesterol recovered in the supernatant fraction obtained after such treatment (Mitropoulos & Venkatesan, 1977). A comparison of the distribution of cholesteryl esters in the experiment with untreated microsomal fractions (Fig. 3*a*) with that in the experiment with digitonin-treated microsomal fractions (Fig. 3*b*) suggests that the major part of the cholesteryl esters solubilized on treatment with digitonin is confined to membranes obtained originally with fractions 8–11 (Fig. 3*a*). In the subfractions obtained with digitonin-treated microsomal fractions, the ratio of non-esterified to esterified cholesterol was low in the early fractions but increased sharply after fraction 6 to reach a high value in fractions 9–10 and then declined progressively to reach very low values after fraction 11. The gradient volume for the maximum value of this ratio was similar to the median volume distribution for 5′-nucleotidase.

Discussion

Isopycnic equilibration studies of rat liver microsomal fractions (Beaufay *et al.*, 1974), together with the differential effect of treatment of microsomal preparations with digitonin, EDTA or PP_i (Amar-Costesec *et al.*, 1974) have led to the differentiation of outer mitochondrial membranes, plasma membranes and Golgi-complex membranes that contaminate membranes originating from the endoplasmic reticulum of varying ribosomal granulations. We have previously used isopycnic fractionation techniques to obtain information on the submicrosomal localization of enzymes relating to cholesterol metabolism, namely hydroxymethylglutaryl-CoA reductase and cholesterol 7α-hydroxylase, and to study the microsomal compartmentation of non-esterified cholesterol (Mitropoulos *et al.*, 1978*a*). In the present paper we have used a similar approach to obtain information on the submicrosomal distribution of cholesteryl esters, on the localization of acyl-CoA–cholesterol acyltransferase and the pool of cholesterol that acts as substrate for the esterifying enzyme.

The distribution profile and the median density of acyl-CoA–cholesterol acyltransferase from untreated microsomal fractions were very similar to those of RNA. Since RNA is a component of the rough endoplasmic reticulum, the present results demonstrate that acyl-CoA–cholesterol acyltransferase has a similar localization. Assuming that acyl-CoA–cholesterol acyltransferase is not a cytosolic enzyme that was adsorbed to the ribosomes as are fumarase and aldolase (Amar-Costesec *et al.*, 1974) or lactate dehydrogenase (Tilleray & Peters, 1976), as far as we are aware this is the first example of an enzyme having a distribution similar to that of RNA. This suggests that either the product of acyl-CoA–cholesterol acyltransferase is synthesized and assembled on to

apoprotein during the synthesis of apoprotein B or that rough-endoplasmic-reticulum membranes are different from those of the smooth endoplasmic reticulum. The latter possibility would imply that it is unlikely that there is only one kind of endoplasmic reticulum membrane with varying degrees of RNA coating. The median density of acyl-CoA–cholesterol acyltransferase was significantly higher than that of NADPH–cytochrome *c* oxidoreductase, cytochrome *P*-450, cholesterol 7α-hydroxylase and hydroxymethylglutaryl-CoA reductase, activities or components that have already been assigned to the less granulated membranes of the endoplasmic reticulum (DePierre & Dallner, 1975; Mitropoulos *et al.*, 1978*a*). The median density of acyl-CoA–cholesterol acyltransferase was also higher than that of 5′-nucleotidase, an enzyme assigned to plasma membranes (Solyom & Trams, 1972), or than that of galactosyltransferase, an enzyme of Golgi smooth membranes (Schachter *et al.*, 1970). Assmann *et al.* (1974), using a discontinuous sucrose-gradient technique, obtained data suggesting that the major part of the cholesterol esterifying activity is confined to a fraction rich in Golgi membranes. However, the conditions used for the assay of cholesterol-esterifying activity suggest that Assmann *et al.* (1974) were measuring the reverse reaction of cholesterol esterase catalysed by a lysosomal enzyme that presumably was contaminating their preparation. We have previously suggested (Mitropoulos *et al.*, 1978*a*) that the tensioactive properties of digitonin on certain membrane populations cannot be ignored. The present study shows that digitonin inactivates acyl-CoA–cholesterol acyltransferase. However, the present results do not provide evidence whether the effect of digitonin on acyl-CoA–cholesterol acyltransferase is due to the solubilization of the enzyme, of the non-esterified cholesterol in the environment of the enzyme that acts as its substrate, or both. There was no acyl-CoA–cholesterol acyltransferase activity in the supernatant obtained from the treatment of the microsomal fraction with digitonin, nor could activity of the enzyme be restored by the addition of exogenous cholesterol to the digitonin-treated microsomal preparation.

Previous work from our laboratory (Mitropoulos & Balasubramaniam, 1972; Balasubramaniam *et al.*, 1973; Balasubramaniam *et al.*, 1975) and from other laboratories (Björkhem & Danielsson, 1975; Van Cantfort & Gielen, 1975) on the labelling of microsomal cholesterol *in vivo* and *in vitro*, has suggested the multiplicity of cholesterol pools in microsomal preparations. In agreement with this suggestion, the present results demonstrate that the pools of cholesterol that act as substrate for acyl-CoA–cholesterol acyltransferase and for cholesterol 7α-hydroxylase are labelled differently on the addition of trace amounts of [^{14}C]cholesterol as a suspension with Tween 80 or as a solution in acetone. Moreover, the labelling of untreated microsomes with trace amounts of [^{14}C]cholesterol, followed by subfractionation of the microsomal fraction, showed that the specific radioactivity of the 7α-hydroxycholesterol obtained *in vitro* by the various subfractions was similar in all subfractions but significantly different from the specific radioactivity of cholesteryl esters obtained *in vitro* by the same subfraction. These results demonstrate that there are at least two distinct pools of cholesterol in the endoplasmic reticulum, the pool that acts as substrate for acyl-CoA–cholesterol acyltransferase and the pool for cholesterol 7α-hydroxylase, and that each of these pools of cholesterol is uniformly labelled on the addition of [^{14}C]-cholesterol under the conditions described. Since the specific radioactivity of cholesteryl esters obtained during the incubation was similar with all subfractions obtained with untreated microsomal fractions, a comparison of the ratio of specific radioactivities of non-esterified cholesterol and the product of acyl-CoA–cholesterol acyltransferase reflects the relative labelling of the pool of cholesterol that acts as substrate for acyl-CoA–cholesterol acyltransferase to the labelling of other pools of cholesterol that contaminate the particular fraction. This ratio of non-esterified cholesterol to cholesteryl ester specific radioactivities becomes progressively lower as the contamination of the subfraction with pools of cholesterol in plasma membranes and smooth membranes from Golgi increases. Similar results are derived from the comparison of the ratio of specific radioactivities of cholesterol to 7α-hydroxycholesterol obtained for the various subfractions, and suggest (Mitropoulos *et al.*, 1978*a*) that [^{14}C]cholesterol added to untreated microsomal fractions did not label the plasma membrane and the Golgi-membrane cholesterol pools as heavily as it labelled other pools of microsomal cholesterol.

Several lines of evidence support the concept that the function of acyl-CoA–cholesterol acyltransferase in liver is associated with the synthesis of lipoproteins and the transport of cholesterol from the liver to the plasma. Thus the cholesteryl ester composition of plasma very-low-density lipoprotein has been shown to be identical with the ester composition of the microsomal fraction (Gidez *et al.*, 1967), to which the major proportion of the cellular acyl-CoA–cholesterol acyltransferase activity is confined (Stokke & Norum, 1970) and in which the synthesis of the apoprotein of very-low-density lipoprotein occurs (Havel, 1975). Moreover, it has been shown that the precursor of plasma very-low-density lipoprotein, the nascent very-low-density lipoprotein, is found in the secretory vesicles of the Golgi apparatus, and its composition is identical with that of plasma very-low-density lipoprotein (Hamilton *et al.*, 1967). The present results, showing that acyl-CoA–cholesterol acyl-

transferase is localized in membranes originating from the endoplasmic reticulum with high RNA coating, are consistent with the above concept and suggest that sites of apoprotein B and cholesteryl ester synthesis are located close to each other for the assembly of very-low-density lipoprotein molecules. Electron-microscopic studies have suggested that, in isolated livers, strongly osmiophilic 30–80nm particles that approximate to the size range of plasma very-low-density lipoproteins appear within minutes after perfusion with non-esterified fatty acids, first in the smooth endoplasmic reticulum (Jones *et al.*, 1967) and Golgi apparatus (Hamilton *et al.*, 1967), but within 15min are found in the space of Disse (Jones *et al.*, 1967; Hamilton *et al.*, 1967). From the space of Disse such particles proceed directly to the circulation (Schumaker & Adams, 1969). The distribution of cholesteryl esters between the subfractions of microsomal fractions shown in the present study is consistent with such sequence of events for the assembly, progression and excretion of lipoprotein particles. Thus with both untreated and digitonin-treated microsomal fractions, the major part of the cholesteryl esters were distributed at densities similar to those of membranes from the endoplasmic reticulum with low RNA coating and at densities lower than those of smooth membranes from Golgi. This last population of membranes may represent secretory vesicles rich in lipoprotein particles.

The ratio of non-esterified to esterified cholesterol in subfractions from untreated microsomal fractions was highest in the subfractions of maximal distribution of 5′-nucleotidase (a marker enzyme for plasma membranes) and of galactosyltransferase (a marker enzyme for smooth membranes from Golgi apparatus) and this is consistent with previous evidence (Amar-Costesec *et al.*, 1974; Mitropoulos *et al.*, 1978*a*) that plasma membranes and smooth membranes from Golgi contain the major part of non-esterified cholesterol present in the microsomal fraction. Consequently, the treatment of the microsomal fraction with digitonin in the present experiments shifted the populations of plasma membranes and smooth membranes from Golgi to higher densities, but the ratio of non-esterified cholesterol to cholesteryl esters was again highest at the maximum distribution of plasma membranes. This is consistent with previous findings suggesting that plasma membranes contain relatively little esterified cholesterol (Thinès-Sempoux, 1972).

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