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Modulation of 3-hydroxy-3-methylglutaryl-CoA reductase and of acyl-CoA–cholesterol acyltransferase by the transfer of non-esterified cholesterol to rat liver microsomal vesicles

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The incubation of rat liver microsomal fraction with a serum preparation followed by the re-isolation of the microsomal membranes has resulted in an increase in the concentration of non-esterified cholesterol, a considerable decrease in the activity of 3-hydroxy-3-methylglutaryl-CoA reductase and in an increase in the activity of acyl-CoA–cholesterol acyltransferase in the treated microsomal preparation. These effects were related to the concentration of serum in the incubation mixture and to the duration of the incubation. The transfer of non-esterified cholesterol was specific in that the content of protein and the total phospholipids were similar in the original microsomal fraction and the serum-treated microsomal preparation. The incubation of the microsomal fraction with lipoprotein-deficient serum or with no serum resulted in both cases in small changes in the non-esterified cholesterol, the esterified cholesterol and the total phospholipid content in the treated preparations compared with these concentrations in the original microsomal fraction, whereas the activity of acyl-CoA–cholesterol acyltransferase and of 3-hydroxy-3-methylglutaryl-CoA reductase was similar in the lipoprotein-deficient-serum-treated and the buffer-treated microsomal preparations. The activity of 3-hydroxy-3-methylglutaryl-CoA reductase was lower and the activity of acyl-CoA–cholesterol acyltransferase was higher in the lipoprotein-deficient-serum-treated and the buffer-treated microsomal preparations as compared with these activities in the original microsomal fraction. However, the serum-treated microsomal preparation had considerably lower activity of 3-hydroxy-3-methylglutaryl-CoA reductase and considerably higher activity of acyl-CoA–cholesterol acyltransferase than these activities in buffer-treated and in lipoprotein-deficient-serum-treated microsomal preparations.

The rate-limiting step for the biosynthesis of cholesterol is the reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid (Rodwell *et al.*, 1973; Dietschy & Brown, 1974) catalysed by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34). In agreement with this, the inhibition of hepatic cholesterogenesis by dietary cholesterol is brought about by a parallel decrease in the activity of hydroxymethylglutaryl-CoA reductase in rat liver microsomal fraction (Linn, 1967). The molecular mechanism by which the activity of the enzyme is altered by cholesterol is not understood. A direct modulation of activity by cholesterol (Siperstein & Guest, 1960) has been thought of as unlikely since an inverse relation between non-esterified cholesterol in the microsomal fraction and the activity of the enzyme was not apparent (Gould, 1977). Moreover, attempts to inhibit the rate of cholesterol biosynthesis or to modulate the activity of hydroxymethylglutaryl-CoA reductase in subcellular fractions by the addition of cholesterol in various forms has not been successful (Linn, 1967; Kandutsch & Packie, 1970; Shapiro & Rodwell, 1971; Brown *et al.*, 1974).

We have previously shown that hydroxymethylglutaryl-CoA reductase is confined to endoplasmic-reticular membranes and that these membranes contain only a small proportion of the total non-esterified cholesterol in the microsomal fraction (Mitropoulos *et al.*, 1978*c*). These observations have provided an explanation for the lack of correlation between non-esterified cholesterol and the activity of

hydroxymethylglutaryl-CoA reductase in the microsomal fraction. Moreover, we have provided indirect evidence showing that hydroxymethylglutaryl-CoA reductase can be influenced *in vivo* by the size of a pool of non-esterified cholesterol in the membrane in the immediate environment of the enzyme. Such evidence has been consistent with the concept of direct modulation, showing that in rat liver the decrease in the activity of the enzyme in the microsomal fraction from rats in a number of experimental conditions is associated with an increase in the size of this pool of cholesterol (Mitropoulos & Venkatesan, 1977; Mitropoulos *et al.*, 1978*a*, 1980). In contrast, the conditions associated with an increase in the activity of the enzyme are also associated with a decrease in the size of the pool (Mitropoulos & Venkatesan, 1977; Mitropoulos *et al.*, 1978*b*, 1979).

To obtain direct evidence that hydroxymethylglutaryl-CoA reductase can be modulated by an increase in the concentration of cholesterol in endoplasmic-reticular membrane we have, in the present study, transferred non-esterified cholesterol to the vesicles of liver microsomal fraction and studied the effect of this treatment on the activity of the enzyme. Since the activity of acyl-CoA–cholesterol acyltransferase (EC 2.3.1.26) can also be influenced by the concentration of non-esterified cholesterol in endoplasmic-reticular membrane (Balasubramaniam *et al.*, 1978*a*; Mitropoulos *et al.*, 1978*a*, 1979) and can be altered by the transfer of cholesterol to microsomal vesicles (Hashimoto & Dayton, 1979) we have measured the activity of this enzyme after the same treatment of the rat liver microsomal fraction.

Materials and methods

The sources of radiolabelled compounds and other materials used in the present work have been reported elsewhere (Mitropoulos *et al.*, 1978*a*,*c*). Serum was prepared from human blood drawn in 0.1% EDTA from normal donors. The plasma obtained after separation of the erythrocytes was incubated at 56°C for 30min and the heat-inactivated serum (Shinitzky, 1978) was separated from the precipitated material by centrifugation. The clear supernatant was freeze-dried and stored at −20°C. The dried powder was reconstituted by dissolving in water before use. Lipoprotein-deficient serum was prepared on removal of the various classes of lipoproteins, by sequential ultracentrifugation of the serum from the same donor at the appropriate densities (Havel *et al.*, 1955) using solid KBr for density adjustments (Radding & Steinberg, 1960). Thus, very-low-density lipoproteins were floated and removed after adjusting the density at 1.019g/ml, low-density lipoproteins at 1.063g/ml and high-density lipoproteins at 1.215g/ml. The lipoprotein-deficient serum was dialysed as described previously (Brown *et al.*, 1973) and either freeze-dried and reconstituted at the time of the experiment or kept in the liquid form at−20°C.

Preparation of the microsomal fraction and its treatment

Male Wistar rats weighing 180–250g were used for all experiments. The rats were kept under conditions of controlled lighting and feeding as described previously (Mitropoulos *et al.*, 1973). The rats were killed at about 07:00h on the day of the experiment and the livers were removed at once to prepare the microsomal fraction as described previously (Mitropoulos *et al.*, 1980). The microsomal fraction contained, in each ml, the microsomes recovered from 0.25g fresh wt. of liver (4–6mg of protein).

The microsomal fraction was incubated with serum or lipoprotein-deficient serum at the concentrations indicated or with no serum (buffer-treated) for the periods indicated at 37°C. All incubations contained the same amount of microsomal protein and at the end of the incubation period the mixture was placed on ice. The incubation mixture was centrifuged at 104000*g* for 60min, the supernatant was removed and the microsomal pellet was resuspended in the same buffer to give the volume of the original microsomal fraction used.

Assay of enzyme activities

The activity of hydroxymethylglutaryl-CoA reductase was measured in portions of the microsomal fraction or treated microsomal preparation in the presence of 90μM-DL-hydroxymethyl[3-^{14}C]glutaryl-CoA (sp. radioactivity 6Ci/mol) in a total incubation volume of 0.5ml. The incubation mixture, incubation conditions and the determination of the rate of product formation were as described previously (Mitropoulos & Balasubramaniam, 1976; Mitropoulos & Venkatesan, 1977). The activity of acyl-CoA–cholesterol acyltransferase was assayed in portions of the microsomal fraction or treated microsomal preparation at the concentration of endogenous cholesterol. The portion (0.15–0.25mg of microsomal protein) was added to a mixture containing 0.1M-potassium phosphate buffer, pH7.4, 2mM-dithiothreitol, 1.2mg of human serum albumin (free of fatty acids) and 50μM-[1-^{14}C]-oleoyl-CoA (sp. radioactivity 9Ci/mol). After incubation for 6min at 37°C the reaction was stopped. Extraction, separation of the cholesteryl ester fraction and determination of the rate of product formation were as described previously (Balasubramaniam *et al.*, 1978*a*).

Determination of the components of the microsomal fraction, of the treated microsomal preparations and of the supernatant from this treatment

Cholesteryl esters and non-esterified cholesterol (Balasubramaniam *et al.*, 1978*b*), total phospholipids (Bartlett, 1959) and protein (Lowry *et al.*, 1951) in serum, lipoprotein-deficient serum, the microsomal fraction, treated microsomal preparations and the supernatant obtained from such treatment were determined by methods described previously.

Results

Portions of rat liver microsomal fraction (3.3 mg of protein/ml) were incubated with serum preparation (3.2 mg of serum protein/mg of microsomal protein) at 37°C for various periods of time. Portions of the microsomal fraction at the same concentration were also incubated without serum for the same periods of time. At the end of the incubation period the mixture was centrifuged to obtain the buffer-treated or the serum-treated microsomal preparations and the corresponding supernatant fractions. The phospholipid content of the buffer-treated and the serum-treated microsomal preparations were almost identical, did not change with time and were similar to that of the original microsomal fraction. The protein concentrations showed a similar pattern to that observed for phospholipids, except in the case of buffer-treated microsomal preparations. In this case, protein content was 38% lower than that of the original microsomal fraction. The non-esterified cholesterol and the cholesteryl ester content of the buffer-treated microsomal preparations have remained constant throughout the incubation at values slightly lower than those in the non-treated microsomal fraction (Fig. 1*a*). The cholesteryl ester content of the serum-treated microsomal preparation remained constant throughout the incubation at values 2–3-fold higher than the content of the original microsomal fraction. The content of non-esterified cholesterol in the serum-treated preparations increased progressively with the incubation time to values considerably higher than the non-treated microsomal fraction. Thus in serum-treated microsomal preparation incubated for 3 h the content of non-esterified cholesterol was 64% higher than in the corresponding buffer-treated microsomal preparation (Fig. 1*a*). The ratio of non-esterified to esterified cholesterol in the supernatant of the serum-incubated samples decreased progressively with the incubation time, whereas the cholesteryl ester content in this fraction remained constant. The protein, phospholipid, cholesteryl esters and non-esterified cholesterol lost from the buffer-treated preparations

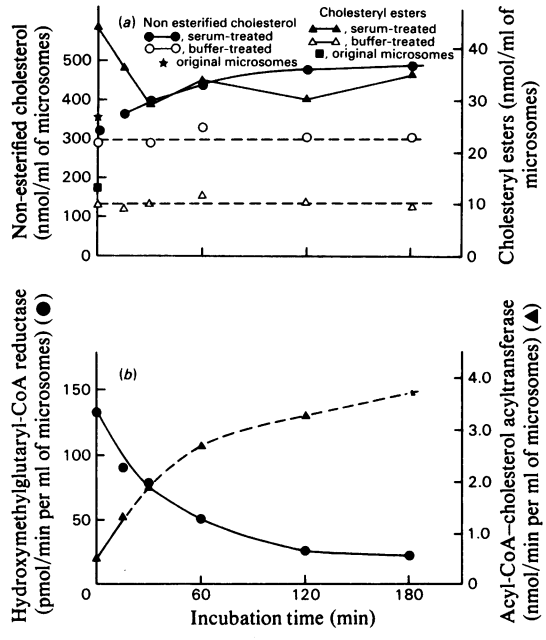

Fig. 1. *The composition of serum-treated and buffer-treated microsomal preparations and the activities of hydroxymethylglutaryl-CoA reductase and of acyl-CoA–cholesterol acyltransferase in preparations after treatment with serum for various time periods*

(*a*) The concentration of non-esterified cholesterol and of cholesteryl esters. (*b*) The activity of hydroxymethylglutaryl-CoA reductase and of acyl-CoA–cholesterol acyltransferase. All values given are means for at least two determinations or assays performed. The experimental details are given in the text.

were recovered in the corresponding supernatant fraction. Thus in four independent experiments the average non-esterified cholesterol content in the buffer-treated microsomal preparation was $83.3 \pm 4.4\%$ (mean $\pm$ S.D.) of that in the original microsomal fraction and $8.6 \pm 2.6\%$ (mean $\pm$ S.D.) of the non-esterified cholesterol of the latter fraction was recovered in the supernatant of the buffer-treated incubation. The supernatant fraction from serum-treated microsomal preparation contained the balance of serum components that were not transferred to the microsomal membranes. The activity of hydroxymethylglutaryl-CoA reductase decreased progressively and the activity of acyl-CoA–cholesterol acyltransferase increased progressively with incubation time in the serum-treated microsomal preparation (Fig. 1*b*). An inverse correlation between the logarithm of activity of hydroxymethylglutaryl-CoA reductase and the concentration of non-esterified cholesterol in the serum-treated microsomal preparation has been shown (Fig. 2). There

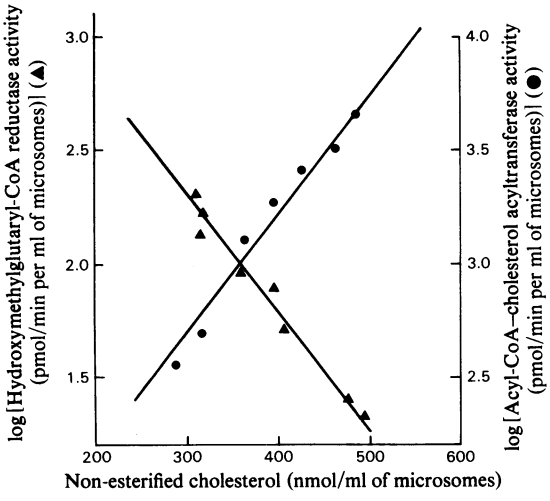

Fig. 2. *The relation between the concentration of non-esterified cholesterol in serum-treated microsomal preparations and the activity of hydroxymethylglutaryl-CoA reductase or that of acyl-CoA–cholesterol acyltransferase*

The microsomal fraction was incubated at 37°C for various periods of time in the presence of serum (serum protein, 10.7mg/ml; non-esterified cholesterol, 317nmol/ml; cholesteryl esters, 508nmol/ml; and phospholipids, 300μg/ml of the incubation mixture). At the end of the incubation, the treated microsomal pellet was obtained and resuspended to the volume of the original microsomal fraction. Assays of the enzymes and determinations of non-esterified cholesterol in the serum-treated preparation were as described in the Materials and methods section. For the composition and the activities of the enzymes in the original microsomal fraction and the serum-treated preparations, see Fig. 1. The lines drawn are the best fit for the points obtained. The correlation coefficient for the relation of hydroxymethylglutaryl-CoA reductase was −0.979 and that for acyl-CoA–cholesterol acyltransferase was 0.956.

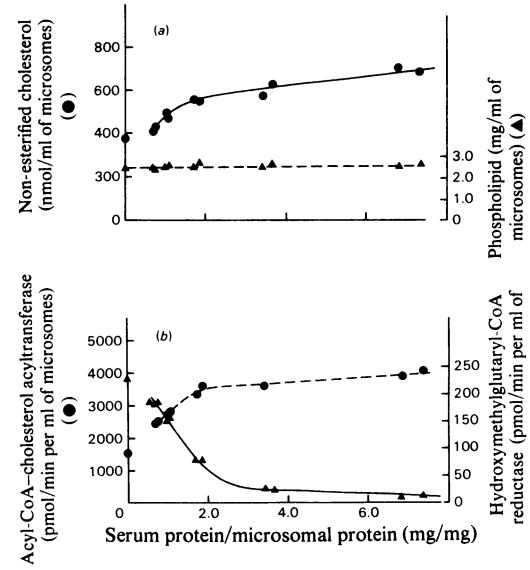

Fig. 3. *Serum-concentration-dependent changes in the activity of hydroxymethylglutaryl-CoA reductase of acyl-CoA–cholesterol acyltransferase and in the concentration of non-esterified cholesterol and of the total phospholipids in the treated preparations*

Portions of the microsomal fraction were incubated at 37°C for 2h in the presence of the indicated concentration of serum (serum protein, 83.4mg/ml; non-esterified cholesterol, 1400nmol/ml; cholesteryl esters, 3275nmol/ml; total phospholipids, 2.27mg/ml). (*a*) The concentration of non-esterified cholesterol and of phospholipids. (*b*) The activity of hydroxymethylglutaryl-CoA reductase and of acyl-CoA–cholesterol acyltransferase. Experimental details are given in the text.

was also a linear correlation between the logarithm of activity of acyl-CoA–cholesterol acyltransferase and the concentration of non-esterified cholesterol in the same preparations (Fig. 2). There was no detectable acyl-CoA–cholesterol acyltransferase and only a small fraction of the hydroxymethylglutaryl-CoA reductase was detected in the supernatant from the buffer-incubated microsomal fraction.

A similar experiment involved the incubation of the microsomal fraction (4.3mg of microsomal protein/ml) with various concentrations of serum. All samples were incubated for 2h at 37°C and at the end of this period the mixture was centrifuged to obtain the treated microsomal pellet and the supernatant. The microsomal pellets obtained were resuspended to give the volume of the original microsomal fraction added to the incubation mixture. The treated microsomal preparations and the supernatant fractions were assayed for non-esterified cholesterol, phospholipid and protein. The serum-treated preparations were also assayed for activities of hydroxymethylglutaryl-CoA reductase and acyl-CoA–cholesterol acyltransferase. The phospholipid content of the treated microsomal preparation remained similar in all cases (Fig. 3*a*). However, the non-esterified cholesterol content increased progressively with the increasing concentration of serum from a value of 378nmol/ml for the preparation incubated with no serum (buffer-treated) to 795nmol/ml for the preparation incubated with the highest concentration of serum (Fig. 3*a*). The activity of acyl-CoA–cholesterol acyltransferase increased and the activity of hydroxymethylglu-

taryl-CoA reductase decreased and these changes were dependent on the serum concentration in the incubation mixture (Fig. 3*b*). Again there was an inverse correlation between the logarithm of activity of hydroxymethylglutaryl-CoA reductase and the concentration of non-esterified cholesterol in the serum-treated preparation and a linear relation in the case of acyl-CoA–cholesterol acyltransferase (Fig. 4).

To exclude the possibility that a factor other than the lipoproteins in the serum was responsible for the changes in the activities of the two enzymes, another control preparation was introduced: the preparation obtained after the incubation of the microsomal fraction with lipoprotein-deficient serum. In this experiment portions of four independent microsomal preparations were incubated for 1h at 37°C with serum, with lipoprotein-deficient serum or with buffer. The treated microsomal preparations obtained were assayed for acyl-CoA–cholesterol acyltransferase and for hydroxymethylglutaryl-CoA reductase activities. The composition of the various microsomal preparations were also determined and compared with the values from the corresponding original microsomal fraction. The results are shown in Table 1. The recovery of protein and of total phospholipid was similar in the serum-treated and in the lipoprotein-deficient-serum-treated preparations. The total phospholipid, the non-esterified cholesterol and the cholesteryl ester recoveries were not significantly different in buffer-treated and lipo-

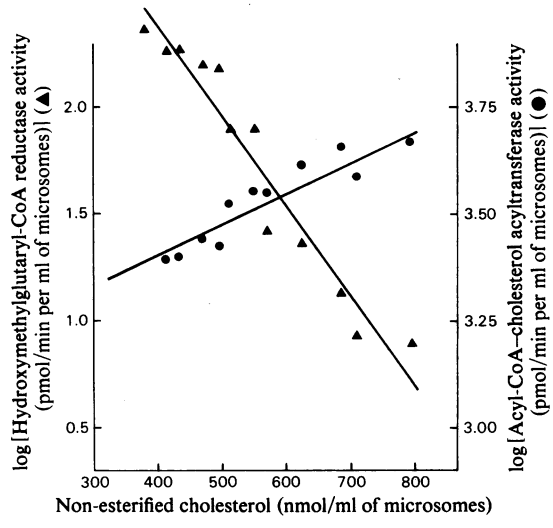

Fig. 4. *The relation between the concentration of non-esterified cholesterol and the activity of hydroxymethylglutaryl-CoA reductase or that of acyl-CoA–cholesterol acyltransferase in serum-treated microsomal preparations*

The microsomal fraction was incubated for 2h at 37°C in the presence of various concentrations of serum (see the legend to Fig. 3). The lines drawn are the best fit for the points obtained. The correlation coefficient for the relation of hydroxymethylglutaryl-CoA reductase was −0.968 and that for acyl-CoA–cholesterol acyltransferase was 0.932.

Table 1. *The effect of incubation of the rat liver microsomal fraction with serum, lipoprotein-deficient serum or buffer on the composition of the microsomal preparation and on the activity of hydroxymethylglutaryl-CoA reductase and of acyl-CoA–cholesterol acyltransferase*

Four groups of rats each containing three rats were killed at 07:00h. The livers in each group were pooled to prepare the microsomal fraction and portions were incubated for 1h at 37°C (3.2mg of protein/ml of mixture) with buffer, serum (protein, 19.2mg; non-esterified cholesterol, 373nmol; cholesteryl esters, 772nmol; and phospholipid, 0.435mg/ml of incubation mixture) or lipoprotein-deficient serum (protein, 20.2mg; non-esterified cholesterol, 4.3nmol; cholesteryl esters, 15nmol; and phospholipid, 0.063mg/ml of incubation mixture). At the end of this period all preparations were centrifuged at 104000*g* for 1h and the pellets were resuspended to give the volume of the original microsomal portion. The original microsomal preparations contained: protein, 5.33 ± 0.24mg/ml; non-esterified cholesterol, 399 ± 10nmol/ml; cholesteryl esters, 15.5 ± 3.2nmol/ml; phospholipid, 2.05 ± 0.09mg/ml; hydroxymethylglutaryl-CoA reductase activity, 976 ± 577pmol/min per ml; and acyl-CoA–cholesterol acyltransferase activity, 558 ± 169pmol/min per ml (all values are the means ± S.D. of the values of the four microsomal preparations). Values given are means ± S.D. obtained from the comparison of the treated preparations with the corresponding non-treated preparations.

	Percentage of content or activity in non-treated microsomal preparation					
			Cholesterol			
Treatment	Protein	Total phospholipid	Non-esterified	Esters	Hydroxymethyl-glutaryl-CoA reductase	Acyl-CoA–cholesterol acyltransferase
Buffer	68.7 ± 3.0	90.6 ± 4.3	95.0 ± 4.2	86.4 ± 20.7	56.5 ± 4.1	244 ± 21
Serum	87.7 ± 1.2	87.4 ± 4.2	140.0 ± 4.4	369 ± 109	7.4 ± 1.0	601 ± 121
Lipoprotein-deficient serum	87.3 ± 4.8	89.1 ± 2.9	105.5 ± 2.5	123 ± 19	80.7 ± 11.8	263 ± 38

protein-deficient-serum-treated preparations. However, the protein recovered in the buffer-treated preparations was significantly lower than that in serum- or lipoprotein-deficient-serum-treated preparations. The concentration of non-esterified cholesterol was significantly higher in serum-treated preparations as compared with this concentration in either the buffer-treated or the lipoprotein-deficient-serum-treated preparations. The activity of hydroxymethylglutaryl-CoA reductase was considerably lower and the activity of acyl-CoA–cholesterol acyltransferase was considerably higher in the serum-treated than in the corresponding original microsomal fraction or in the preparations treated in the absence of lipid (Table 1).

Discussion

The present results demonstrate specific transfer of non-esterified cholesterol from the serum preparation to vesicles of the rat liver microsomal fraction and that this transfer is associated with changes in the activity of hydroxymethylglutaryl-CoA reductase and of acyl-CoA–cholesterol acyltransferase. The nature of such a cholesterol-donor *in vivo* or the mechanism of transfer are currently unknown, although several possibilities exist (Bell, 1978). In the present work, for convenience, we have used a preparation of serum as the vehicle for rapid transfer of non-esterified cholesterol to the microsomal vesicles containing hydroxymethylglutaryl-CoA reductase (Mitropoulos *et al.*, 1978*c*) and to those that contain acyl-CoA–cholesterol acyltransferase (Balasubramaniam *et al.*, 1978*b*). Modulation of these two enzymes by the cholesterol transfer *in vitro* suggests that, regardless of the actual mechanism of transfer, a similar modulation may exist *in vivo*.

Studies on alteration of the non-esterified cholesterol concentration in membranes have mainly been carried out on intact cells (Vanderkooi *et al.*, 1974; Cooper *et al.*, 1975; Borochou & Shinitzky, 1976; Shinitzky & Rivnay, 1977). Such studies involve the use of liposomes containing high cholesterol/phospholipid ratios to achieve the enrichment of membrane cholesterol or the use of liposomes containing only phospholipids to achieve membrane depletion in cholesterol. The cells to be treated are incubated with mixtures of liposomes and serum preparations for periods of 12 to 36h. Such studies have demonstrated that the observed changes in cholesterol/phospholipid ratios were exclusively due to the changes in the concentration of non-esterified cholesterol. The amount of cholesterol transferred depended on the cholesterol/phospholipid in the donor and this ratio in the acceptor membrane (Vanderkooi *et al.*, 1974; Shinitzky & Inbar, 1974; Cooper *et al.*, 1975; Shattil *et al.*, 1975; Shinitzky, 1978). Serum or various lipoprotein fractions have also been used to transfer non-esterified cholesterol to rabbit liver microsomal fraction (Hashimoto & Dayton, 1979) or the microsomal fraction from arterial tissue (Hashimoto & Dayton, 1977) and it has been shown that the ratio of cholesterol to phospholipid in the donor is important for the amount of cholesterol transferred to the membranes.

The microsomal fraction from rat liver is not a homogeneous population of membranes. It contains vesicles derived from the endoplasmic-reticular membranes with low and with high ribosomal coating, from plasma membranes and from smooth membranes from Golgi elements (Amar-Costesec *et al.*, 1974). Since various vesicles contain different concentrations of cholesterol (Mitropoulos *et al.*, 1978*c*), the ratio of cholesterol to phospholipid would be expected to vary between the various populations of membranes in the microsomal fraction. It would therefore be expected that various experimental conditions *in vitro* will result in varying transfer of non-esterified cholesterol to the microsomal fraction and in a differing transfer among the various membrane populations. We assume that under the present conditions of treatment with the serum preparation all vesicle populations have been enriched with non-esterified cholesterol. Consistent with this 5′-nucleotidase, an enzyme confined to plasma membranes, was also modulated by the transfer of cholesterol to the microsomal fraction (S. Venkatesan, unpublished work). Therefore in both experiments involving the time-dependent and the serum-concentration-dependent transfer of cholesterol a linear relation is expected between the concentration of non-esterified cholesterol in the microsomal fraction and that in a particular population of vesicles. Consistent with this in each of these experiments a semi-logarithmic relation has been shown between the concentration of non-esterified cholesterol in the microsomal preparation and the activity of hydroxymethylglutaryl-CoA reductase, of acyl-CoA–cholesterol acyltransferase or of 5′-nucleotidase (such a relation was inverse in the case of hydroxymethylglutaryl-CoA reductase and of 5′-nucleotidase).

The relatively large increase in the cholesteryl ester content of the serum-treated microsomal preparations is probably due to the small contamination of the treated microsomal fraction with serum. Consistent with this the time-dependent serum treatment has resulted in a similar concentration of cholesteryl esters in all treated microsomal preparations but in a progressive increase in the concentration of non-esterified cholesterol and change of enzyme activities. The treatment of the microsomal fraction with buffer resulted in the release to the supernatant fraction of a considerable portion of the microsomal protein and of a rela-

tively small fraction of the phospholipid, the non-esterified and the esterified cholesterol (Venkatesan *et al.*, 1980). Such changes in the compositions of the microsomal fraction were independent of the incubation period. However, there was an incubation-time-dependent increase in the activity of acyl-CoA–cholesterol acyltransferase and a decrease in that of hydroxymethylglutaryl-CoA reductase in the buffer-treated and the lipoprotein-deficient-serum-treated microsomal preparations. Whatever the mechanism underlying such changes and whatever is the ideal control to the serum-treated preparation the latter has shown a significant increase in the concentration of non-esterified cholesterol and considerable changes in the activity of the enzymes as compared with the control preparations or with the original microsomal fraction.

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