Transfer of exogenous cholesterol to microsomes of hepatocytes investigated with [³H]desmosterol tracer

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The feasibility of using exogenous [³H]desmosterol as a mass metabolic tracer for exogenous non-esterified cholesterol in hepatocytes is investigated with albumin-bound non-esterified cholesterol containing [³H]desmosterol and [¹⁴C]cholesterol tracers. The amounts of uptake and metabolism of exogenous cholesterol monitored by either tracer are the same. In addition, the conversion of [³H]desmosterol into [³H]cholesterol by the Δ^{24} -sterol reductase in the microsomes can be used as an estimate for the mass transfer of exogenous cholesterol to the microsomes. The results obtained indicate that only a small fraction of exogenous cholesterol that was transferred to the microsomes was metabolized into bile acids and sterol esters. The technique of estimating the mass transfer of exogenous cholesterol to the microsomes with [³H]desmosterol may be of importance in investigations dealing with the effect of exogenous plasma cholesterol on changes in the physiological functions of the endoplasmic reticulum in the cells.

The hepatic endoplasmic reticulum ('microsomes') is actively involved in the metabolism of blood cholesterol. It is commonly accepted that plasma non-esterified cholesterol, taken up directly from the blood or obtained from the hydrolysis of plasma-esterified cholesterol by lysosomes, can be transferred to the microsomes for further processing. The transfer of this exogenous non-esterified cholesterol to the microsomes is evident from the catabolism of labelled plasma non-esterified and/or esterified cholesterol to bile acids and the re-esterification of plasma cholesteryl esters (Portman *et al.*, 1980; O'Malley *et al.*, 1981; Schwartz *et al.*, 1981). Whether all, or only a fraction, of cholesterol transferred to the microsomes is converted into metabolites is presently unknown.

In order to assess whether metabolic conversion by the microsomes utilizes all or only a fraction of exogenous plasma-derived non-esterified cholesterol reaching the microsomes, it is necessary to utilize a procedure that can distinguish and quantify that portion of exogenous non-esterified cholesterol that has reached the microsomes, but has not been metabolized by them, from the portion that has not reached the microsomes. The conventionally used radioactive cholesterol tracer for exogenous cholesterol is not suitable for this purpose because radioactive non-esterified cholesterol that has

reached the microsomes and that which has not are chemically indistinguishable.

To overcome this obstacle, radioactive exogenous desmosterol can be substituted for radioactive cholesterol as a mass metabolic tracer for exogenous cholesterol. This possibility is suggested by the following: (1) desmosterol is chemically distinguishable from cholesterol; (2) desmosterol is an immediate precursor of cholesterol and is converted into the latter only by a microsomal enzyme; and (3) desmosterol and cholesterol are metabolized similarly. These characteristics account for the frequent use of desmosterol as a mass metabolic tracer for endogenous cholesterol in previous studies (Pullinger & Gibbons, 1981; Gibbons & Pullinger, 1979; Long *et al.*, 1978; Bates & Rothblat, 1974).

In the present study the feasibility of using exogenous radioactive desmosterol as a mass metabolic tracer to differentiate between exogenous non-esterified cholesterol that has reached the microsomes and that which has not reached the microsomes is investigated in a model system using rat hepatocytes and exogenous non-esterified cholesterol complexed to albumin. The results show that radioactive desmosterol is a valid metabolic tracer capable of differentiating between exogenous cholesterol that has and has not reached the microsomes.

Materials and methods

Radioactive sterols were obtained from New England Nuclear, Boston, MA, U.S.A. [$1,2(n)$ - ^3H]Cholesterol and [26 - ^{14}C]desmosterol were used as internal standards to monitor the recovery of sterols, whereas [4 - ^{14}C]cholesterol and [G - ^3H]desmosterol were used as metabolic tracers. Non-radioactive cholesterol was obtained from Nu-Check Prep, Elusian, MN, U.S.A., and desmosterol was from Steraloids, Wilton, NH, U.S.A., and Sigma Chemical Co., St. Louis, MO, U.S.A. Thin-layer plates were purchased from Whatman Inc., Clifton, NJ, U.S.A. Reagent-grade solvents were obtained from Fisher.

Male Sprague-Dawley rats, obtained from Harlan Industries, Indianapolis, IN, U.S.A., were allowed free access to water and rat chow and were exposed to 12 h (05:00 to 17:00 h) light periods. Rats weighing between 220 and 250 g were killed between 08:00 and 09:00 h and the livers used in the isolation of hepatocytes. Sterile techniques were used in the isolation and incubation of hepatocytes in an isothermal box controlled at 37°C. Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 0.1% glucose with or without calcium, was used as medium for the isolation and incubation of hepatocytes. The livers were first perfused with a single passage of 500 ml of calcium-free medium. This was immediately followed by 25 min of recycling perfusion with 40 ml of calcium-free medium containing 0.05% collagenase (type CLS; Worthington, Freehold, NJ, U.S.A.) and 0.025% trypsin inhibitor (type 1S; Sigma). After perfusion, the liver cells were suspended in 100 ml of calcium-free medium, filtered through a 100 μm^2 nylon mesh and allowed to settle for 5 min at room temperature. The supernatant was removed by aspiration and the cells resuspended in 100 ml of Krebs-Henseleit medium containing calcium. Sedimentation and suspension were repeated twice before cells were diluted with the same medium to a concentration of 50 ml of cell protein/ml of suspension. The suspension (2.5×10^7 cells; 1 ml) was added to a 50 ml polycarbonate centrifuge tube (Nalgene) containing 3 ml of medium with calcium and 1 ml of 15% delipidated bovine serum albumin. Ethanol (50 μl) with or without sterol was added and the mixture was gassed with O_2/CO_2 (19:1) for 30 sec. The tubes were capped tightly and rotated at about 160 rev./min on a platform of rotating brass rods kept in the isothermal box. The mixture was gassed every 30 min and samples of the mixture taken every hour. The viability of hepatocytes, as judged by the exclusion of 0.1% Trypan Blue, was greater than 90% before incubation and 80% at the end of 3 h.

Before use, radioactive and non-radioactive sterols were purified by t.l.c. on LHP-K plates, developed with chloroform, followed by chromato-

graphy on LKC $_{18}$ reverse-phase thin-layer plates, which were developed with acetonitrile/ethanol (4:1, v/v) in an SB/CD chamber (Regis). Desmosterol in biological samples was separated from cholesterol on reverse-phase plates alone. Esterified sterol was separated from non-esterified sterol by t.l.c. on LHP-K plates, which were developed with a mixture of light petroleum (b.p. 37.2–52.5°C)/diethyl ether/acetic acid (84:15:1, by vol.). Total sterol was obtained by saponification (Goh, 1980) and bile acids of cells and medium were isolated as described by Yousef *et al.* (1978). The mass of cholesterol and desmosterol was determined by the method described by Zlatkis & Zake (1969) or by the fluorimetric method of Heider & Boyett (1978). Internal standards for the recovery of sterols were added to biological samples when only a single radioactive tracer was used in the experiment.

Hepatocyte cell protein was determined by the micro-biuret method of Itzhaki & Gill (1964). Microsomes of the hepatocytes were isolated by low-speed centrifugation as described by Lodola *et al.* (1981). Very-low-density lipoprotein was isolated by ultracentrifugation of the cell-free medium at a density of 1.006 g/ml. Radioactivity of the sterols was determined by liquid-scintillation counting and quenching of samples was corrected by the external-standard method.

Results and discussion

In order to establish the use of radioactive desmosterol as a valid mass metabolic tracer of exogenous cholesterol, the metabolism of various amounts of exogenous albumin-bound non-esterified cholesterol in hepatocytes was investigated with concurrent [^3H]desmosterol and [^{14}C]cholesterol tracers.

The metabolism of exogenous cholesterol by the hepatocytes necessarily required the mass uptake of exogenous cholesterol into the cells. Since hepatocytes incubated in the absence of exogenous cholesterol released endogenous cholesterol into the medium (Fig. 1), the net change in the mass of cholesterol in the medium cannot be used for the purpose of estimating the mass uptake of exogenous cholesterol by the cells. This is due to the possible release of endogenous cholesterol by cells incubated with exogenous cholesterol and the lack of a distinguishing characteristic between non-radioactive exogenous cholesterol and non-radioactive endogenous cholesterol released by the cells. Despite this, mass uptake of exogenous cholesterol by the cells is indicated by the reduction of the mass of cholesterol in the incubation medium for hepatocytes incubated with 389 and 649 nmol of exogenous cholesterol (Fig. 1). Since medium cholesterol concentration remained unchanged in experiments

Fig. 1. Changes in the concentration of medium cholesterol with time of incubation

Freshly isolated hepatocytes (50 mg of cell protein) were incubated as described in the Materials and methods section with $0.67 \mu\text{Ci}$ of $[^3\text{H}]\text{desmosterol}$ (sp. radioactivity 575 Ci/mol), $0.4 \mu\text{Ci}$ of $[^{14}\text{C}]\text{cholesterol}$ (sp. radioactivity 59.4 Ci/mol) and 0 (●), 194 (○), 389 (△) and 649 nmol (□) of non-radioactive cholesterol. Samples of cell-free supernatant of the incubation mixture were taken at the indicated times and the mass of total cholesterol determined. Data are means $\pm$ S.E.M. for six or more incubations of hepatocytes obtained from three different livers.

with 194 nmol of exogenous cholesterol added (Fig. 1), the uptake of exogenous cholesterol by the hepatocytes incubated under this condition cannot be ascertained.

Alternatively, mass uptake of exogenous cholesterol by the cells can be estimated by the uptake, that is the disappearance, of radioactive tracer from the incubation medium. The disappearance of $[^3\text{H}]\text{desmosterol}$ tracer from the medium, expressed as mass of exogenous cholesterol taken up by the hepatocytes, is shown in Table 1(A). Identical estimates for the mass uptake of exogenous cholesterol by the cells are obtained when the rate of disappearance of $[^{14}\text{C}]\text{cholesterol}$ tracer, added concurrently with $[^3\text{H}]\text{desmosterol}$, from the medium were used in the calculation (Table 1B).

There exists a likelihood that radioactive tracer by itself may undergo reversible exchange between the cells and the incubation medium. When this occurs, the disappearance of exogenous radioactive tracers may not represent the actual mass uptake of the exogenous cholesterol by the cells. To test whether the movement of exogenous radioactive tracers was

associated with the concurrent movement of non-radioactive exogenous cholesterol mass, in separate experiments, exogenous non-radioactive desmosterol was substituted for the labelled exogenous cholesterol. The mass disappearance of desmosterol from the medium was then measured and the values obtained, expressed as mass uptake by the hepatocytes, are shown in Table 1C. These values are similar to the mass values calculated from the disappearance of radioactive tracers from the incubation medium (Tables 1A and 1B). This demonstrates that the disappearance of exogenous radioactive tracers from the medium is associated with the mass uptake of non-radioactive desmosterol and hence mass uptake of exogenous cholesterol by the cells. In addition, the data in Table 1 indicate that the uptake of exogenous cholesterol by the hepatocytes occurred in all experiments with exogenous cholesterol added and that uptake is near maximum after 1 h of incubation with exogenous cholesterol. Furthermore, the lack of change in the mass of medium cholesterol in experiments with 194 nmol of exogenous cholesterol (Fig. 1) may be due to the concurrent release of endogenous cholesterol by the cells rather than the failure of exogenous cholesterol to be taken up by the cells.

The mass of endogenous cholesterol released by the hepatocytes incubated with exogenous cholesterol can be obtained by the differences between the amounts of exogenous cholesterol added, the amounts of exogenous cholesterol taken up by the cells (Table 1) and the mass of cholesterol remaining in the incubation medium (Fig. 1). Identical amounts can also be calculated from the reduction of the specific radioactivity of added exogenous cholesterol, labelled either with $[^3\text{H}]\text{desmosterol}$ or with $[^{14}\text{C}]\text{cholesterol}$ tracer, by non-radioactive endogenous cholesterol released by the cells. Calculated values for the mass release of endogenous cholesterol by hepatocytes incubated with 194 nmol of exogenous cholesterol are 88 ± 8 , 127 ± 13 , and $137 \pm 12 \text{ nmol/50 mg of cell protein}$ after 1, 2 and 3 h of incubation respectively ($n = 4$). These amounts are similar to the amounts of exogenous cholesterol taken up by the hepatocytes incubated under this condition (Table 1). The similarity in the rate of uptake of exogenous and release of endogenous cholesterol mass accounts for the lack of change in the mass of medium cholesterol of hepatocytes incubated with 194 nmol of exogenous cholesterol (Fig. 1). Thus changes in the mass of medium cholesterol of cells incubated with exogenous cholesterol are a summation of both the uptake of exogenous and release of endogenous cholesterol by the cells. These metabolic processes can be estimated concurrently with either $[^3\text{H}]\text{desmosterol}$ or $[^{14}\text{C}]\text{cholesterol}$ as the tracer for exogenous cholesterol.

Table 1. *Uptake of exogenous cholesterol by the hepatocytes*

The mass values shown were calculated by (amount of exogenous cholesterol added $\times$ {1 - [radioactivity (d.p.m.) of sterol left in the medium/radioactivity (d.p.m.) of sterol added]}) for [^3H]desmosterol (A) and [^{14}C]cholesterol (B) tracers. Values shown for (A) and (B) are obtained from experiments described in the legend to Fig. 1. Values shown for (C) were obtained from separate experiments where non-radioactive non-esterified desmosterol was substituted for the labelled exogenous cholesterol used in obtaining the values shown for (A) and (B). The mass values for (C) were calculated by (amount of desmosterol added - amount of desmosterol left in the medium) after the respective hours of incubation. Values for the uptake are means $\pm$ S.E.M. for three or more incubations of hepatocytes isolated from different livers.

Group	Amount of exogenous sterol added (nmol)	Tracer used in calculation	Time of incubation (h) ...	Uptake of exogenous cholesterol (nmol/50 mg of cell protein)		
				1	2	3
(A)	194	[^3H]Desmosterol		107 $\pm$ 9	104 $\pm$ 4	116 $\pm$ 4
	389			230 $\pm$ 11	219 $\pm$ 11	236 $\pm$ 11
	649			315 $\pm$ 8	323 $\pm$ 20	345 $\pm$ 25
(B)	194	[^{14}C]Cholesterol		108 $\pm$ 10	95 $\pm$ 10	114 $\pm$ 26
	389			230 $\pm$ 10	200 $\pm$ 9	235 $\pm$ 10
	649			319 $\pm$ 5	304 $\pm$ 11	368 $\pm$ 20
(C)	194	Desmosterol		106 $\pm$ 4	103 $\pm$ 9	123 $\pm$ 7
	389			227 $\pm$ 22	248 $\pm$ 10	262 $\pm$ 11
	649			338 $\pm$ 15	369 $\pm$ 16	388 $\pm$ 20

Table 2. *Transfer of exogenous non-esterified cholesterol to microsomes*

The amount of exogenous non-esterified cholesterol that was transferred to the microsomes and remained as non-esterified cholesterol was determined by the amount of total [^3H]cholesterol recovered in the cells and medium. This was calculated by {[radioactivity (d.p.m.) of [^3H]cholesterol/radioactivity (d.p.m.) of [^3H]desmosterol added] $\times$ nmol of non-radioactive exogenous cholesterol added}. Additional experimental details are given in the legend to Fig. 1. Values for the transfer are means $\pm$ S.E.M. for six or more incubations of cells obtained from three different livers.

Exogenous cholesterol added (nmol)	Time of incubation (h) ...	Exogenous cholesterol transferred (nmol/50 mg of cell protein)		
		1	2	3
0		1.0 $\pm$ 0.03	1.4 $\pm$ 0.01	2.0 $\pm$ 0.01
194		23 $\pm$ 2.4	36 $\pm$ 1.8	58 $\pm$ 3.9
389		41 $\pm$ 3.8	74 $\pm$ 4.0	109 $\pm$ 5.7
649		62 $\pm$ 6.2	127 $\pm$ 5.8	171 $\pm$ 10.7

Unlike [^{14}C]cholesterol, however, [^3H]desmosterol can also be used to estimate the total transfer of exogenous cholesterol to the microsomes of the cells. The former cannot be used in this estimation since [^{14}C]cholesterol tracer that is transferred to and/or moves away from the microsomes unmetabolized is chemically in-

Table 3. *Microsomal content of cholesterol*

Hepatic microsomes were isolated after the indicated period of incubation and the mass of total cholesterol determined. Additional experimental details are given in the legend to Fig. 1. Values for microsomal cholesterol content are means $\pm$ S.E.M. for four or more incubations of hepatocytes isolated from three different livers.

Exogenous cholesterol added (nmol)	Time of incubation (h) ...	Microsomal cholesterol content (nmol/50 mg of cell protein)		
		1	2	3
0		109 $\pm$ 5	114 $\pm$ 3	114 $\pm$ 5
194		139 $\pm$ 5	136 $\pm$ 3	142 $\pm$ 3
389		145 $\pm$ 4	149 $\pm$ 4	165 $\pm$ 2
649		149 $\pm$ 2	170 $\pm$ 2	190 $\pm$ 7

distinguishable from [^{14}C]cholesterol tracer that has not been transferred to the microsomes. [^3H]Desmosterol tracer, on the other hand, is converted into [^3H]cholesterol by microsomal Δ^24 -sterol reductase. This chemical difference allows the conversion to be used to distinguish and quantify the mass movement of that portion of exogenous cholesterol that is transferred to and/or moves away from the microsomes unmetabolized from the portion of exogenous cholesterol that has not reached the microsomes. For this study, these amounts, calculated from the radioactivity of [^3H]cholesterol found in the incubation and expressed in mass of exogenous cholesterol transferred to the

Table 4. *Metabolic fate of exogenous non-esterified cholesterol in the hepatocytes*

The amount of exogenous cholesterol that appeared in the indicated samples was calculated by $\{[^{14}\text{C}$ or ^3H radioactivity recovered (d.p.m.)/radioactivity of corresponding isotope added (d.p.m.)] $\times$ amount of exogenous non-radioactive cholesterol added}. Additional experimental details are given in the legend to Fig. 1. Values (nmol/50 mg of cell protein) are means $\pm$ S.E.M. for seven (bile acids and steryl esters) or six (very-low-density lipoproteins) incubations of hepatocytes obtained from five different livers. No difference exists between values obtained from ^3H and ^{14}C tracers as determined by Student's *t* test.

Exogenous cholesterol added (nmol)	Tracer used in calculation	Exogenous cholesterol incorporated (nmol/50 mg of cell protein)		
		Bile acids	Steryl esters	Very-low-density lipoproteins
194	[^{14}C]Cholesterol	3.9 $\pm$ 0.1	2.0 $\pm$ 0.3	2.8 $\pm$ 0.1
	[^3H]Desmosterol	3.9 $\pm$ 0.1	2.2 $\pm$ 0.3	3.3 $\pm$ 0.3
389	[^{14}C]Cholesterol	8.4 $\pm$ 0.4	3.9 $\pm$ 0.3	6.9 $\pm$ 1.2
	[^3H]Desmosterol	7.7 $\pm$ 0.4	4.6 $\pm$ 0.4	7.2 $\pm$ 1.0
649	[^{14}C]Cholesterol	13.5 $\pm$ 0.4	5.9 $\pm$ 0.5	15.3 $\pm$ 1.0
	[^3H]Desmosterol	12.1 $\pm$ 0.8	6.7 $\pm$ 0.3	14.0 $\pm$ 0.3

microsomes, are shown in Table 2. That these values represent the mass transfer of exogenous cholesterol is supported by the mass conversion of desmosterol into cholesterol by the microsomes. For this purpose, in separate experiments, 194, 389 and 649 nmol of non-radioactive desmosterol were added to the hepatocytes in place of tracer-labelled cholesterol. The mass disappearance of desmosterol from the cells and medium was then measured, and taken as the amount of exogenous cholesterol that would have reached the microsomes had exogenous cholesterol been added to the incubation. After 3 h of incubation 60 ± 5 , 82 ± 7 , and 75 ± 4 nmol of desmosterol/50 mg of cell protein ($n = 4$) were converted into cholesterol when incubated with the respective amounts of exogenous desmosterol mentioned above. The results for the experiments with 194 nmol of exogenous desmosterol agree well with the amount transferred to the microsomes as calculated from the conversion of [^3H]desmosterol into [^3H]cholesterol (Table 2). The results obtained with larger amounts of desmosterol do not agree, however. This may be explained by the apparent maximal rate of conversion of desmosterol into cholesterol by the enzyme in the microsomes. This limit would not be reached when trace amounts of [^3H]desmosterol are used for the purpose of estimating the mass transfer of exogenous non-esterified cholesterol to the microsomes. Data in Table 2 suggest that the mass transfer of exogenous cholesterol to the microsomes is dependent on the amount of exogenous cholesterol added. In addition, although uptake of exogenous cholesterol by the cells reached a maximum after 1 h of incubation (Table 1), the transfer of exogenous cholesterol to the microsomes increased linearly during the 3 h of incubation (Table 2).

The mass transfer of exogenous cholesterol to the microsomes, determined by the conversion of [^3H]-

desmosterol tracer into [^3H]cholesterol, is also supported by the increase in the cholesterol content of microsomal fraction isolated from the hepatocytes incubated with exogenous cholesterol (Table 3). The amount of net increase in microsomal cholesterol content, however, is a poor estimate for the total amount of exogenous cholesterol transferred to the microsomes. This is due to the possible contamination of the microsomal fraction with other cellular organelles, variable recovery of microsomes from cell homogenate, and most of all, the movement of exogenous cholesterol that has reached the microsomes as well as endogenous microsomal cholesterol away from the endoplasmic reticulum during cell incubation. These factors severely limit the utility of microsomal cholesterol content to quantify the total mass transfer of exogenous cholesterol to the microsomes. The conversion of [^3H]desmosterol tracer into [^3H]cholesterol, which is unaffected by these factors, is thus a superior method for determining this dynamic metabolic parameter.

A portion of the exogenous cholesterol that was transferred to the microsomes is transformed by the microsomes into bile acids and steryl esters. The amount calculated from the radioactivity recovered in each fraction is shown in Table 4. There is no difference in the amounts obtained with [^3H]desmosterol and [^{14}C]cholesterol tracers. About 15% of the ^3H label in the steryl esters fraction was recovered as [^3H]desmosteryl esters, indicating that a fraction of [^3H]desmosterol tracer was esterified without prior conversion by the microsomes into [^3H]cholesterol. Since exogenous cholesterol can be utilized by the microsomes in the secretion of nascent very-low-density lipoprotein, the amount of exogenous cholesterol recovered in the very-low-density-lipoprotein fraction of the medium was also measured (Table 4). Again the amounts calculated

with [^3H]desmosterol and [^{14}C]cholesterol tracers are similar. As in the steryl esters, a fraction of the ^3H -label in very-low-density lipoprotein was in the form of [^3H]desmosterol. Whether this represents the adsorption of medium exogenous cholesterol or cellular extramicrosomal cholesterol to the lipoproteins is not known.

From these data, it is suggested that [^3H]-desmosterol is as effective a mass metabolic tracer for exogenous cholesterol metabolism as [^{14}C]-cholesterol. The single most important advantage with [^3H]desmosterol is the ability to use [^3H]-cholesterol derived from [^3H]desmosterol tracer to estimate the amount of exogenous cholesterol that is transferred to and unchanged by the microsomes. As shown by this study, this fraction is substantially greater than the amount converted into bile acids, converted into steryl esters and re-utilized by the microsomes for the secretion of very-low-density lipoprotein.

Measuring the magnitude of that fraction of exogenous non-esterified cholesterol that is transferred to and unchanged by the microsomes is important in studying the role of exogenous cholesterol in the regulation of hepatic cholesterol metabolism. Preliminary experiments in this laboratory indicate that the transfer of albumin-bound non-esterified cholesterol to the microsomes, measured with the [^3H]desmosterol technique, can be related to the suppression of cholesterol synthesis by the hepatocytes. Information provided in the present paper will be useful in further work to determine the effect of the transfer of lipoprotein non-esterified and/or esterified cholesterol to hepatic endoplasmic

reticulum on the regulation of hepatic cholesterol synthesis and metabolism.

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