In Vitro Exchange of β -Sitosterol between Erythrocytes and Plasma of Rats

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Received June 17, 1980

The erythrocytes and plasma of rats were not labeled equally with sterols even after feeding plant sterols for 2 months.

When erythrocytes and plasma were labeled *in vivo* with radioactive sterols, the *in vitro* exchange of cholesterol between cells and plasma was considerably greater than that of β -sitosterol. The dependence of the transfer on plasma lecithin : cholesterol acyltransferase was much less with β -sitosterol.

More labeled β -sitosterol existed in high density lipoprotein and less in very-low density and low density lipoproteins than cholesterol, when plasma was labeled *in vivo*. A similar distribution pattern was observed when plasma was incubated with labeled erythrocytes. These results suggest that an extra ethyl group in the side chain of the molecule substantially influences the metabolic behavior of the sterols.

Although cholesterol is always the pre-dominant sterol, a few closely related sterols such as plant sterols are also detected in mammalian cells.^{1,2)} These plant sterols are without doubt dietary origin. Regular diets for mammals contain plentiful amounts of plant sterols,³⁾ but, because of the limited absorption from the intestine, the magnitude of their incorporation into the body tissues is usually markedly low.^{4~6)}

There appears species difference in the rate of absorption of plant sterols; in rats the mucosal ability to distinguish plant sterols from the animal sterol is apparently inferior to human.^{1,2)} Previously, we have shown that the absorbed plant sterols were not equally distributed in serum lipoproteins and liver sub-cellular organelles in rats.⁵⁾ The significance of this observation is not clear at the moment. However, it may be relevant to the demonstration that in rats plant sterols, in particular β -sitosterol, are more readily excreted into the intestinal lumen *via* the liver than cholesterol.^{7,8)}

In relation to the post-absorptive effect of plant sterols on cholesterol metabolism,^{9,10)} it seems reasonable to pursue their behavior in

the body. The study on the sterol exchange is estimated at least to be suited well with this purpose. Despite extensive studies on cholesterol exchange between the erythrocytes and plasma, only limited information is available as to the role of side chain of the sterol molecule.^{11,12)} Edwards and Green¹³⁾ found that the plant sterols were readily incorporated into the erythrocyte membrane, but to a lesser extent than cholesterol.

In the present study, the exchange *in vitro* of β -sitosterol between the erythrocytes and plasma from rats was compared with that of cholesterol by utilizing blood specimens labeled intravenously with radioactive sterols.

MATERIALS AND METHODS

Male white rats bred locally (Kyudo Co., Kumamoto) from a Wistar strain were used throughout.

Exp. 1. Rats weighing about 50 g were fed *ad libitum* the purified diet with or without supplementary β -sitosterol for 2 months. The composition of the diet was in percent:¹⁴⁾ casein 20, stripped soy-bean oil 5, mineral mixture 4, vitamin mixture 1, choline chloride 0.15, cellulose powder 4 and sucrose to 100. The stripped oil contained less than 0.08% of plant sterols. β -Sitosterol

(ICN Pharmaceuticals Inc., Cleveland) was repeatedly recrystallized from ethyl acetate until over 98% purity as sterols was achieved as measured by gas-liquid chromatography.^{5,15)} The recrystallized sterol which was consisted of 93% β -sitosterol and 7% campesterol was added to the diet at the 0.5% level at the expense of sucrose. Blood was collected in heparin (approximately 400 units/dl) by decapitation after overnight-fast. The erythrocytes were isolated by centrifugation ($1,500 \times g$, 15 min); the plasma and buffy coat were removed by aspiration. The cells were then washed twice by resuspension in ice-cold isotonic saline and incubated by gentle shaking at a hematocrit of 30% in plasma at 37°C for 18 hr in the presence of benzyl penicillin (100 units/ml) and streptomycin sulfate (100 μ g/ml). The cells were separated from the plasma and washed three times in the saline prior to extraction of lipids. Lipids were extracted from the cells and plasma by the technique of Nelson¹⁶⁾ and Floch *et al.*¹⁷⁾ respectively. Cholesterol was determined according to Sperry and Webb.¹⁸⁾ Gas-liquid chromatographic analysis of the sterols was performed as previously reported.⁵⁾

Exp. II. 4-¹⁴C-Cholesterol (specific activity 57 mCi/mmol) and 4-¹⁴C- β -sitosterol (specific activity 54 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. Sonicated emulsion of these isotopes was injected intravenously, at the level of 2 μ Ci/0.2 ml saline containing 0.2 mg purified egg lecithin, to the jugular vein of rats fed a commercial stock diet (Oriental

Yeast Co., Tokyo, Type NMF) and weighing 280 g.¹⁹⁾ The animals were killed 2 days after the dose, and plasma and red blood cells were obtained as described above, except that the cells were washed in Tyrode's buffer, pH 7.4. Red blood cells were incubated at a hematocrit of 33% in plasma at 37°C for 18 hr in the presence of antibiotics. Plasma lipoproteins were separated by ultracentrifugation.^{19,20)} An aliquot of the lipid extracts was used for the radioactivity measurement (Inter technique liquid scintillation spectrometer SL-32).¹⁹⁾ Corrections for quenching were made by an external standard.

Exp. III. The labeled sterols (2~3 μ Ci) were injected intravenously to rats weighing 370~450 g as in the preceding experiment and blood was collected 2 days later. The erythrocytes were washed as in the Exp. II. Incubation at a hematocrit of 33% was continued at 37°C for 16 hr. Only streptomycin sulfate was added. Two types of plasma were used; fresh plasma and heated plasma (56°C for 50 min in order to inactivate lecithin : cholesterol acyltransferase, LCAT).

RESULTS

Incorporation of dietary plant sterols (Exp. I)

In order to compare the extent of incorporation of dietary plant sterols in plasma and the erythrocytes, one group (control) of

TABLE I. THE DISTRIBUTION OF PLANT STEROLS IN PLASMA AND RED BLOOD CELLS (EXP. I)

Incubation systems ^a	Sterol distribution (%) ^b					
	Plasma			Red blood cells		
	Cholesterol	Campesterol	Sitosterol	Cholesterol	Campesterol	Sitosterol
Control red blood cells				99.0 $\pm$ 0.0	0.2 $\pm$ 0.0	0.2 $\pm$ 0.0
Sitosterol plasma	91.0 $\pm$ 0.3	1.0 $\pm$ 0.1	7.1 $\pm$ 0.2			
Control red blood cells incubated in sitosterol plasma	91.6 $\pm$ 0.1	1.4 $\pm$ 0.1	7.0 $\pm$ 0.1	98.9 $\pm$ 0.1	0.3 $\pm$ 0.0	0.8 $\pm$ 0.1
Control plasma	99.6 $\pm$ 0.0	0.2 $\pm$ 0.0	0.2 $\pm$ 0.0			
Sitosterol red blood cells				93.3 $\pm$ 0.1	2.5 $\pm$ 0.2	4.2 $\pm$ 0.1
Sitosterol red blood cells incubated in control plasma	99.0 $\pm$ 0.0	0.5 $\pm$ 0.0	0.5 $\pm$ 0.0	94.6 $\pm$ 0.2	1.3 $\pm$ 0.0	4.1 $\pm$ 0.2

^a Plasma and the red blood cells were obtained from rats given diets free of (control group) or containing 0.5% plant sterols (sitosterol group) for 2 months. Incubation was carried out at a hematocrit of 30% at 37°C for 18 hr.

^b Values are the means of triplicate determinations $\pm$ SE (each consisted of pooled blood samples from 3 to 4 rats).

weanling rats was fed a diet containing essentially no plant sterols and the other received a β -sitosterol-supplemented diet for 2 months. The results are presented in Table I.

The cells and plasma from the control rats contained virtually no plant sterols. In contrast, considerable amounts of plant sterols were found in rats given the sitosterol preparation. However, the percentage of plant sterols was obviously lower in the cells (6.4%) than in plasma (9.0%). Comparisons of sterol compositions of the diet and plasma or the cells indicated that campesterol is absorbed much more readily than β -sitosterol; the ratio of C_{28} to C_{29} sterols was 0.075 in the former and 0.27 or 0.52 in the latter two respectively. Relatively more campesterol tended to be incorporated into the cells than into plasma.

When control-erythrocytes were incubated with sitosterol-plasma, there was a small but detectable increase in percentage of plant sterols in the cells. The similar movement was also demonstrated when the sitosterol-cells

were incubated in the control-plasma.

Sterol exchange between plasma and erythrocytes (Exp. II & III)

The transfer of the sterols between plasma and the erythrocytes was determined by utilizing the blood specimens labeled *in vivo*. Since blood was collected 2 days after the intravenous injection, labeled sterols should be equilibrated at least with sterols within the rapid exchangeable pool. This was confirmed by comparing the mass and radioactivity distribution of sterols in plasma lipoproteins (see also Table III).

As Table II shows, approximately 15% of cholesterol was transferred from plasma to the cells during incubation for 16 to 18 hr. The corresponding value for β -sitosterol was around 10%. The extent of the transfer of cholesterol and β -sitosterol from the cells to plasma was considerably greater as compared with that in the reverse direction and the values corresponded to 39~41% and

TABLE II. TRANSFER OF LABELED STEROLS BETWEEN PLASMA AND RED BLOOD CELLS (EXP. II & III)

Exp. No.	Incubation systems ^a	Sterols transferred ^b	
		Radioactivity (%)	Mass (μ g/system) ^c
II	[¹⁴ C]Cholesterol plasma→Red blood cells	15.4	320
	[¹⁴ C] β -Sitosterol plasma→Red blood cells	10.1	8
	[¹⁴ C]Cholesterol red blood cells→Plasma	38.5	510
	[¹⁴ C] β -Sitosterol red blood cells→Plasma	17.7	9
III	[¹⁴ C]Cholesterol plasma→Red blood cells	15.8	230
	[¹⁴ C] β -Sitosterol plasma→Red blood cells	10.9	14
	[¹⁴ C]Cholesterol red blood cells		
	→Plasma	40.8	670
	→Heated plasma	25.2	410
	[¹⁴ C] β -Sitosterol red blood cells		
	→Plasma	28.9	33
	→Heated plasma	23.0	26

^a Plasma and the red blood cells were labeled with cholesterol or β -sitosterol by intravenous injection of these sterols. Rats fed commercial pellets served as blood donors. Labeled plasma or the cells were incubated with non-labeled counterparts. Incubation was carried out at a hematocrit of 33% at 37°C for 18 hr (Exp. II) or 16 hr (Exp. III). Radioactive sterols were transferred in the direction of the arrow.

^b Values are the means of duplicate incubations.

^c Rate of labeled sterols transferred $\times$ sterols originally present.

19~28% respectively. The same trend was observed when the mass transferred was calculated. Anyhow, in both cases, the transfer appeared to be much greater with cholesterol than with β -sitosterol.

As the results of Exp. III show, the transfer of cholesterol from red blood cells to plasma seemed at least LCAT-dependent; when heated plasma was applied, the transfer decreased by about 40% as compared to the case of fresh plasma. The similar trend was observed with β -sitosterol, but the extent of the dependence on the LCAT activity was much less, the decrease due to heating being by 20%.

Distribution of plant sterols in plasma lipoproteins (Exp. II)

Table III describes the distribution of labeled sterols in plasma lipoproteins. Incubation of labeled plasma with non-labeled erythrocytes did not alter the radioactivity distribution among lipoproteins, although there were marked differences in the distribution between two sterols. Compared with cholesterol, more β -sitosterol was presented in the high density lipoprotein (HDL) fraction

and less in the very-low (VLDL) and low density lipoprotein (LDL) fractions. Only negligible proportion of radioactive β -sitosterol was detected in VLDL.

When the labeled erythrocytes were incubated in fresh non-labeled plasma, the distribution pattern of the sterols transferred to plasma lipoproteins resembled that observable in plasma labeled *in vivo*. Again, the distribution of β -sitosterol was lower in VLDL and LDL and higher in HDL as compared with that of cholesterol. However, in contrast to the case of *in vivo* labeling, appreciable amounts, approximately 10%, of β -sitosterol were detected in VLDL.

DISCUSSION

Although the exchange *in vitro* of sterols between plasma and the erythrocytes has been established in a number of studies with human blood, information about rats is rather scarce.^{11,12)} The ability of plant sterols to enter erythrocyte membranes is obviously lower than that of cholesterol and appears species-specific.¹³⁾ Plant sterols were more

TABLE III. DISTRIBUTION OF LABELED STEROLS IN PLASMA LIPOPROTEINS (EXP. III)

Incubation systems ^a	Radioactivity distribution (%) ^b			
	VLDL	LDL	HDL	VHDL
[¹⁴ C]Cholesterol plasma				
Non-incubated	4.9	17.4	77.5	0.2
Incubated with red blood cells	5.1	16.4	77.6	0.9
[¹⁴ C] β -Sitosterol plasma				
Non-incubated	0.7	10.6	88.5	0.2
Incubated with red blood cells	1.4	8.8	89.3	0.5
[¹⁴ C]Cholesterol red blood cells				
Incubated with plasma	16.2	19.7	63.4	0.7
[¹⁴ C] β -Sitosterol red blood cells				
Incubated with plasma	11.4	15.7	72.0	0.9

^a See footnote of Table I.

^b Plasma lipoproteins were fractionated by ultracentrifugation into VLDL (very-low density lipoprotein, $d < 1.006$ g/ml), LDL (low density lipoprotein, $1.006 < d < 1.063$), HDL (high density lipoprotein, $1.063 < d < 1.21$) and VHDL (very-high density lipoprotein, $d > 1.21$). Values are the means of duplicate analyses.

readily incorporated into the rat erythrocyte membranes than into those of the human and ox. The results of Table I indicate that the exchange of the plant sterol may not complete even *in vivo*. Thus, the percentage of plant sterols in plasma from rats fed β -sitosterol for 2 months was higher than that in the cells. The proportions of campesterol and β -sitosterol varied with the total incorporation.

When labeled sterols were used as an index for the exchange phenomenon, the movement of cholesterol from both directions apparently exceeded over that of β -sitosterol and the difference was particularly marked in the transfer from the red blood cells to plasma. This observation may explain the uneven labeling *in vivo* of the cells and plasma with plant sterols. The exchange of cholesterol from the cells to plasma appeared to be at least LCAT-dependent.^{11,21} Thus, the magnitude of the cholesterol transfer was considerably low when the cells were incubated in heated plasma. The transfer of plant sterols from the cells to plasma was also LCAT-dependent, but to a lesser extent than in the case of cholesterol. This difference is probably connected with the substrate specificity of LCAT to naturally occurring sterols; the rate of esterification of plant sterols is markedly low as compared with cholesterol.²²

As previously reported,⁵ the distribution of plant sterols in plasma was evidently heterogeneous. The concentration as well as percentage of plant sterols increased with the increase in the density of lipoproteins. Also, there were distinct differences in the ratios of campesterol to β -sitosterol among lipoproteins, it was the highest in VLDL and the lowest in HDL. The distribution of intravenously injected β -sitosterol in plasma lipoproteins agreed well with the results obtained by the chemical analysis,⁵ indicating that exogenous sterols were already equilibrated with endogenous ones. The similar distribution pattern was also observed when plasma was incubated with the red blood cells containing radioactive β -sitosterol; again, more radioactivity presented in HDL than in

the case of cholesterol.

Plant sterols have been demonstrated to exert the hypocholesterolemic effect when they were given intraperitoneally⁹ or intravenously.¹⁰ The predominance of plant sterols in HDL may relate to this effect. Only negligible proportions of radioactive β -sitosterol were carried by VLDL when labeled *in vivo*. In contrast, when plasma was labeled *in vitro* by the cells containing ^{14}C - β -sitosterol, approximately 10% of the label was associated with VLDL, the value being comparable with that of cholesterol. This may support the view that the transfer of membranous β -sitosterol is largely a physical phenomenon alike the case of cholesterol.^{11,12} Although the significance of the heterogeneous labeling of plasma lipoproteins with plant sterols is not known, it is likely that the small difference in the structure of the side chain of the sterol molecule, *i.e.*, an extra methyl or ethyl group, substantially influences the metabolic behavior of the sterols even *in vitro*. The present study also supports the view that the ability of the intestinal mucosa to discriminate different dietary sterols is perhaps the major contributing factor for the low absorbability of plant sterols.^{1,2,13}

Acknowledgment. Ajinomoto Co. kindly provided stripped soy-bean oil.

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