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In vitro Distribution of Added ^{32}P-labeled Lecithin, Sphingomyelin and Lysolecithin in Plasma Lipoproteins and their Exchange with Erythrocyte Phospholipids

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Introduction

In the previous papers[1,2] it was reported that phospholipids in circulating erythrocytes were metabolized through exchange with plasma phospholipids rather than by breakdown or synthesis *in situ* and the exchange of lysolecithin was most active among the phospholipids. Furthermore, it was also found that α- and β-lipoproteins, the main plasma lipoproteins, partook in the exchanges of lecithin and sphingomyelin, while the fraction of density greater than 1.21 participated in the exchange of lysolecithin.

In this paper were presented the results obtained from the following experiments; 1) ^{32}P-labeled lecithin, sphingomyelin and lysolecithin separated from rat plasma were emulsified with non-labeled rat plasma respectively, 2) plasma thus obtained was then incubated with non-labeled rat erythrocytes.

Each of the phospholipids added *in vitro* was found in the plasma lipoprotein fractions with the same distribution as found *in vivo*. It was also found that individual [^{32}P] phospholipid added into plasma was exchanged with the phospholipids in erythrocytes and that the extent of the exchange of lysolecithin was most significant. The fraction heavier than density of 1.21 took part in the exchange of lysolecithin and so did α- and β-lipoproteins in the exchanges of lecithin and sphingomyelin. Lecithin added into plasma was also converted into lysolecithin during the incubation.

Materials and Methods

Plasma and Erythrocytes

Plasma and erythrocytes were collected from 24 h fasting rats (body weight 200–250 g). Blood was drawn by cardiac puncture and collected into centrifuge tubes containing disodium EDTA as anticoagulant. Plasma was separated from cells by centrifugation at 220×g for 15 min and recentrifugation at 10,000×g for 30 min. Erythrocytes were washed three times with 0.9% saline followed by centrifugation at 220×g for 15 min.

^{32}P-labeled plasma and erythrocytes were collected by the same procedure as described above 24 h after intraperitoneal injection of 5 mCi of inorganic [^{32}P] phosphate.

Isolation of ^{32}P-labeled Lecithin, Sphingomyelin and Lysolecithin

After 25 vol. of chloroform-methanol (2 : 1, V/V) were added to ^{32}P-labeled plasma, the mixture was allowed to stand overnight at room temperature with occasional shaking.

After filtration the extracts were washed by Folch's procedure[3] and then evaporated to dryness *in vacuo* at a temperature below 50°C. The residues were redissolved in chloroform. This solution was used for the preparation of ^{32}P-labeled lecithin, sphingomyelin and lysolecithin.

Separation of each phospholipid was carried out first by silicic acid column chromatography[4], then the concentrate of each phospholipid was purified by silicic acid-impregnated paper chromatographic technique by Marinetti and Stotz[5]. Sphingomyelin was further purified by mild alkaline hydrolysis[6] to remove alkali-lablile phospholipids contaminated.

After radioautography each phospholipid was eluted from the paper with chloroform-methanol-water (75 : 25 : 2, V/V)[7] by the descending technique and the purity of each phospholipid was checked by the method of Dawson[6].

Preparation of [^{32}P] *Phospholipid-plasma*

^{32}P-labeled lecithin, sphingomyelin and lysolecithin thus prepared were emulsified with non-labeled rat plasma respectively.

249 μg of [^{32}P] lecithin was taken into a small glass-stoppered flask, then the solvent was evaporated. One mℓ of non-labeled rat plasma containing 543 μg of phospholipids was added into the flask. The mixture was stirred vigorously by a magnetic stirrer for 30 min at 37°C.

120 μg of ^{32}P-labeled sphingomyelin and 176 μg of ^{32}P-labeled lysolecithin were emulsified in the same manner with 1 mℓ of plasma by stirring for 30 min, respectively. Homogenous dispersion of the added phospholipid in plasma was confirmed by estimating the radioactivities of the upper, middle and lower parts of the emulsion. Each plasma thus prepared was designated [^{32}P] lec-plasma, [^{32}P] sph-plasma and [^{32}P] lysolec-plasma. Aliquots of each plasma were subjected to lipid extraction and to lipoprotein fractionation.

Incubation

Each 2.5 mℓ of these [^{32}P] phospholipid-plasma was then incubated respectively with 2.0 mℓ of non-labeled rat erythrocytes containing 3.8 mg of phospholipids in a glass-stoppered flask for 2 h at 37°C. At the same time [^{32}P]-plasma was incubated alone at 37°C for 1 and 2 h. Plasma and erythrocytes separated by above described procedure were subjected to lipid extraction and other determination methods.

Fractionation of Lipoproteins[8~10]

Plasma adjusted to a density of 1.063 with NaCl–NaBr solution of $d=1.35$ was ultracentrifuged at 105,000×g for 24 h. Top 1 mℓ (LDL fraction) was removed from the centrifuge tube using a tube slicer. The infranatant layer was then adjusted to a density of 1.21 with NaBr and subjected to ultracentrifugation at 105,000×g for 48 h. Top 1 mℓ (HDL fraction) was removed with a tube slicer. The infranatant fraction was designated bottom fraction.

Extraction and Analyses of Lipids

Direct extraction of lipids from plasma and erythrocytes was carried out with chloroform-methanol (2 : 1, V/V). After filtration the extract was treated by Folch's procedure[3]. The lipid was extracted from each lipoprotion fraction with ethanol-ether-chlorofrom (6 : 2 : 1, V/V) according to the method of Hillyard et al.[11]. After evaporation

of solvents to dryness *in vacuo* at a temperature below 50°C, the residue was redissolved in chloroform-methanol (2 : 1, V/V) and subjected to washing procedure. Washed extract was evaporated to dryness *in vacuo* at a temperature below 50°C. The lipid residue was redissolved in chloroform and made up to a known volume.

Aliquots of the extract were used for the estimation of radioactivity and lipid-phosphorus and for chromatography.

Radioactivity was measured on planchets by a Geiger-Müller counter and lipid-phosphorus was determined by Bartlett's method[12].

Chromatography

Silicic acid-impregnated paper and thin-layer chromatography were used for the separation and identification of individual phospholipids in each lipid extract. Silicic acid-impregnated paper chromatography was carried out by the method of Marinetti and Stotz[5]. After radioautography each phospholipid was eluted from the paper with chloroform-methanol-water (75 : 25 : 2, V/V)[7] by the descending technique, and the separation was checked by the method of Dawson[6].

Thin-layer plates were prepared with Silica Gel G (Merck, A. G. Darmstadt, Germany) by Stahl's procedure[13]. Chloroform-methanol-water-conc. ammonia (66 : 33 : 5 : 1, V/V) or chloroform-methanol-water (65 : 25 : 4, V/V) was used as developing solvents. Localization of the lipids was detected by 5% phosphomolybdic acid in ethanol and also by radioautography.

Results and Discussion

As shown in Fig. 1, it was found that ^{32}P-labeled lecithin and sphingomyelin emulsified with plasma for 30 min at 37°C were mainly distributed in HDL (α-lipoprotein) and LDL

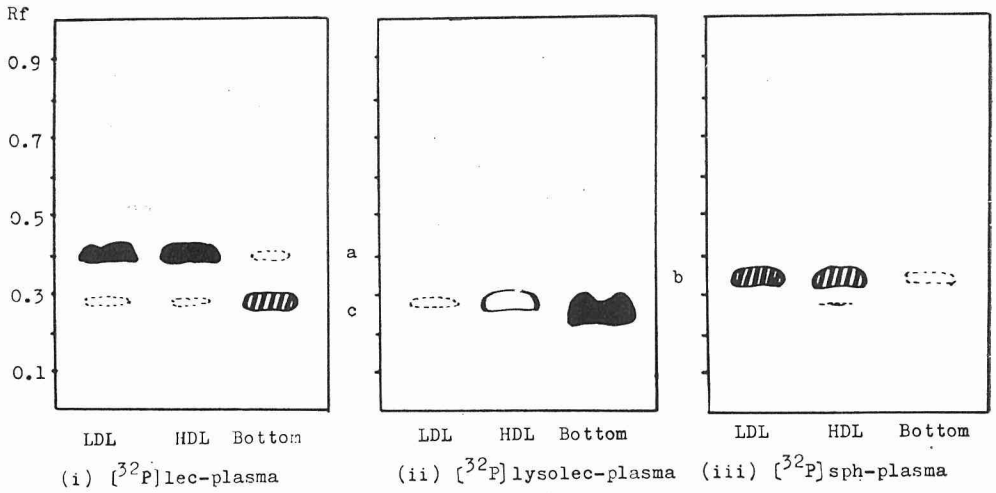


Fig. 1 Radiuoatograms from silicic acid-impregnated paper chromatograms of lipoprotein fractions of [32P] phospholipid-plasma.

a; lecithin, b; sphingomyelin, c; lysolecithin.
Developing solvent; diisobutylketone-glacial acetic acid-water, 40 : 25 : 5 (V/V).

(β-lipoprotein) fractions, while a large part of [32P] lysolecithin in bottom fraction. These distribution patterns were in good agreement with those obtained in plasma *in vivo*[2]. The results obtained also indicate that the phospholipids added *in vitro* were able to combine with corresponding lipoproteins found in native plasma.

It was shown in Fig. 2 and Table 1 that each [32P] phospholipid added into plasma could be also found in erythrocyte phospholipids after the incubation. Among the erythrocyte phospholipids the extent of the radioactivity was most significant in lysolecithin fraction. The total amounts of the lipid phosphorus in plasma and erythrocytes were not of any difference before and after incubation respectively. Therefore, specific activity of each phospholipid in plasma, especially of lysolecithin, was decreased. These results indicated obviously that each phospholipid added into plama *in vitro* was exchanged with the phospholipids in erythrocytes. It was also shown that the extent of the exchange was most active in lysolecithin and not so significant in lecithin and sphingomyelin. The data above described were in agreement with those found in the previous study with [32P] plasma labeled *in vivo* which contained the [32P] phospholipids as components of native lipoprotein[2].

In Table 2 was shown the results obtained by the incubation of 32P-labeled plasma

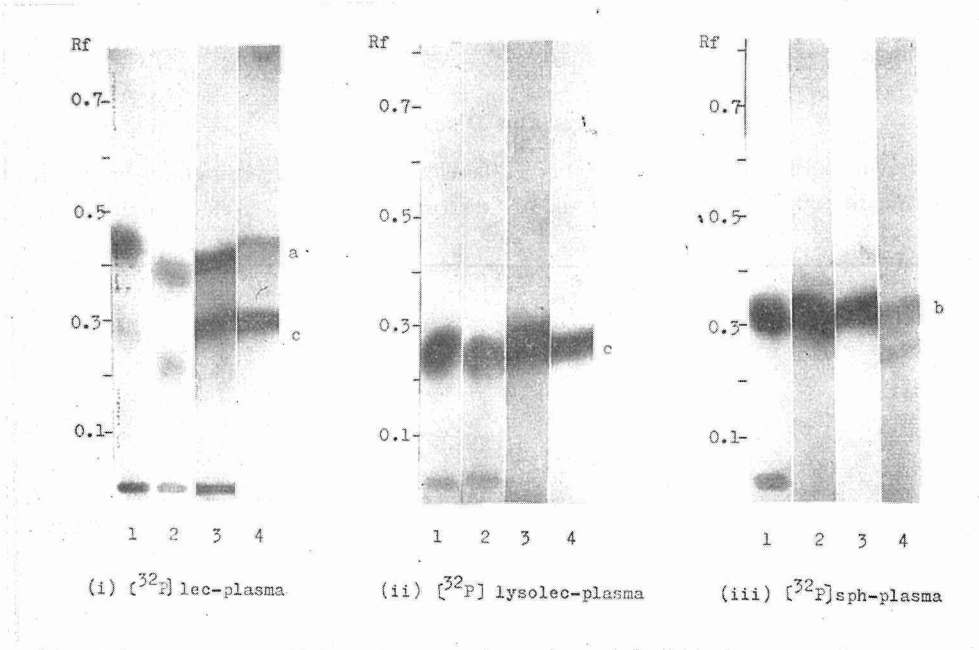


Fig. 2 Radioautograms from silicic acid-impregnated paper chromatograms of plasma and erythrocyte phospholipids before and after incubation.

1; [32P] phospholipid preparation used, 2; [32P] phospholipid-plasma before incubation, 3; plasma after incubation, 4; erythrocytes after incubation.

a; lecithin, b; sphingomyelin, c; lysolecithin. Developing solvent; diisobutylketone-glacial acetic acid-water, 40 : 25 : 5 (V/V). Each [32P] phospholipid-plasma was incubated with erythrocytes at 37°C for 2 h. See text for other details.

Table 1 *Radioactivites of Plasma and Erythrocyte [32P] Phospholipids after Incubation*

Each [32P] phospholipid-plasma was incubated with non-labeled erythrocytes at 37°C for 2 h. See text for other details.

[32P] phospholipid-plasma	Before incubation (cpm×10⁻⁴)	After incubation	
		Plasma (%)	Erythrocytes (%)
i) [32P] lecithin-plasma	7.66	84.4*	9.6*
ii) [32P] lysolecithin-plasma	3.33	68.5*	25.5*
iii) [32P] sphingomyelin-plasma	4.66	86.7*	6.3*

* per cent of radioactivity of each [32P] phospholipid-plasma before incubation.

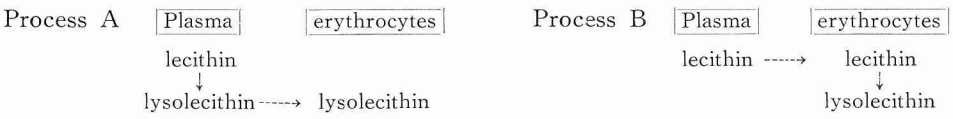
Table 2 *Composition of Plasma Phospholipids During Incubation*

Estimation of individual phospholipid was carried out by silicic acid-impregnated paper chromatography. Incubation temperature: 37°C. See text for other details.

Phospholipids	Incubation time		
	0-h (%)	1-h (%)	2-h (%)
Lecithin	51.0	43.5	37.2
Lysolecithin	30.0	38.0	45.1
Sphingomyelin	13.1	14.8	14.1

alone for 1 and 2 h. This experiment was carried out in order to investigate the change from lecithin to lysolecithin.

As known from Table 2 it is evident that percentage of lecithin to total phospholipids was decreased with lapse of time and that of lysolecithin was increased considerablly, whereas that of sphigomyelin did not show any change. As described above, total amounts of lipid-phosphorus did not change before and after incubation. Accordingly, it could be thought that this change depended upon the remarkable conversion of lecithin into lysolecithin during incubation as pointed out in human plasma by Vogel and Zieve[14]. The conversion did also occur in plasma with the [32P] lecithin added as shown in Fig. 2, (i), namely, lecithin added *in vitro* could be converted into lysolecithin in plasma. On the contrary, the change of lysolecithin to lecithin could not be detected (Fig. 2, ii) under the conditions used. On the other hand, the significant conversion of lecithin into lysolecithin in erythrocytes has not been reported yet. Consequently it could be mentioned that the radioactivity of lysolecithin found in erythrocytes incubated with [32P] lec-plasma was due to process A rather than process B.



From Table 2 it was known that about 27% of plasma lecithin was converted into lysolecithin by 2-h incubation of plasma alone, while from Table 1 it was found that during 2-h incubation with erythrcytes about 26% of plasma lysolecithin moved into

erythrocytes through exchange with erythrocyte lysolecithin. From these results it is evident that about 10% of the added radioactivity found in erythrocytes after the incubation of [32P] lec-plasma with non-labeled erythrocytes (Table 1, i) was due to lysolecithin derived from plasma through active exchange, which was originated from the conversion of lecithin in plasma, rather than to lecithin from plasma by direct exchange. This view was supported by the results shown in Fig. 2, (i).

Furthermore, in the incubation experiment of [32P] lysolec-plasma no radioactive lipid other than lysolecithin could be found in plasma and erythrocytes, although Van Deenen et al.[15] had presented that the change of lysolecithin into lecithin did occur in rabbit erythrocytes. The results obtained indicate that in plasma and also in erythrocytes the synthesis of lecithin from lysolecithin did not take place, or if any, it was not so significant as to be detectable under the condition used.

After the incubation of [32P] sph-plasma with non-labeled erythrocytes the radioactivity found in erythrocytes, which was due to sphingomyelin only, was much weaker than that found with [32P] lysolec-plasma and [32P] lec-plasma.

Summary

When phospholipids, such as lecithin, lysolecithin and sphingomyelin separated from rat plasma, were added into rat plasma *in vitro* respectively, their distribution patterns into plasma lipoproteins were ultracentriufgally same with those obtained *in vivo*. That is, lecithin and sphingomyelin were mainly distributed in HDL and LDL fractions, while lysolecithin was concentrated in the fraction of density greater than 1.21.

Individual phospholipids added into plasma *in vitro* could be exchanged with erythrocyte phospholipids and the extent of exchange was greatest in lysolecithin. The exchanges of lecithin and sphingomyelin were less active, although the former was more active than the latter.

Investigation on participation of plasma lipoprotein in the exchange of phospholipids between plasma and erythrocytes during incubation showed that two major lipoproteins, HDL and LDL, partook principally in the exchange of lecithin and sphingomyelin. On the other hand, the lipoprotein fraction of a density greater than 1.21 participated mainly in the exchange of lysolecithin. These results were in good agreement with those obtained from the studies with native plasma, in which these phospholipids were already present as components of plasma lipoprotein, and it was found, therefore, that the phospholipids added in plasma *in vitro* behaved same as native phospholipids in plasma.

It was clearly noticed that in plasma the conversion of lecithin into lysolecithin could occur during the incubation.

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