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DETERMINATION OF MOLECULAR SPECIES OF LECITHIN FROM ERYTHROCYTES AND PLASMA

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The molecular species of lecithin from erythrocyte and plasma of man and rabbit were determined after conversion of the lecithins into diglycerides by means of hydrolysis with phospholipase C. The resultant diglycerides were separated by thin-layer chromatography on silica impregnated with silver nitrate into 6 or 7 fractions differing with respect to their degree of unsaturation. The positional distribution of the fatty acids in these fractions was determined by hydrolysis with pancreatic lipase and was found to be in agreement with the positional distribution of the fatty acids in the lecithin as ascertained by means of phospholipase A hydrolysis. Using these techniques about 20 molecular species accounting for about 90% of the total lecithin, could be evaluated in the erythrocyte and plasma of man and rabbit.

It became clear that qualitatively the molecular species of lecithin in the red cell and the plasma are similar. Quantitatively, however, there were some striking differences to be noted: in man the amount of (dipalmitoyl)- and (di-oleoyl)-lecithin was higher in the corpuscles when compared with plasma. On the other hand (1-palmitoyl-2-linoleoyl)- and (1-palmitoyl-2-arachidonoyl)-lecithin were more abundant in plasma.

In rabbit similar differences were found in the make-up of the molecular species of lecithin between the erythrocyte membrane and the surrounding plasma.

Introduction

Dietary studies demonstrated that phospholipids in the membrane of circulating erythrocytes are in a dynamic state[1–3]). Exchange of phospholipid molecules between the red cell membrane and serum lipoproteins[4]) presumably is one process which contributes to the dietary induced alterations in the fatty acid composition of the red cell phospholipids. The replacement of intact phospholipid molecules in the membrane by those originating from the serum appears to have its limitations. On the other hand Oliveira and Vaughan[5]) and van Deenen *et al.*[6]) demonstrated that red cells are capable to incorporate fatty acids into their phosphoglycerides by means of an acylation of monoacyl-phosphoglycerides e.g. lysolecithin.

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Further studies of these investigators[7-9]) and those of Robertson and Lands[10]) and Mundner *et al.*[11]) confirmed this somewhat unexpected finding. The significance of lysolecithin as a metabolite for the erythrocyte was endorsed by the observations of Heemskerk and van Deenen[12]), Mulder and van Deenen[13,14]), Mundner *et al.*[11]) and Klibansky and de Vries[15]) that these cells like many other tissues are equipped with lysophospholipase activity. On the other hand these four research groups were unable to detect any appreciable phospholipase A activity in the red blood cell. In this respect it is of particular significance that Paysant and Polonovski[16]) and later Mulder and van Deenen[17]) and Sakagami[18]) found that lysolecithin is very rapidly taken up by the erythrocyte from the serum, where it is bound to albumin. It was proposed that the enzymatic renewal of phospholipids in the erythrocyte membrane is linked with the exchange processes[17]). The dynamic events of the phospholipids of the erythrocyte membrane are rather complex since appreciable differences exist between different classes of phospholipids between red cells of different animal species[1]).

For a given type of phospholipid e.g. lecithin, one can envisage that certain molecules remain bound to the membrane during the entire life span of the erythrocytes whereas others are expelled and replaced by either one of the processes discussed above. The average time of binding of a lecithin molecule to the membrane probably depends on the side where it is bound to the protein core of the membrane. It may be speculated that to a certain extent such differences in location and dynamic state of the various lecithin molecules are to be traced back to variations in their chemical structure. In any case it seems appropriate to approach in future work the lecithins of the membrane as individual molecules rather than as one group. Possibilities to recognize the lecithin molecules differing in their fatty acid composition are given by chromatographic techniques using silica impregnated with silver nitrate[19-21]). As a basis for further metabolic studies the molecular species of lecithin from serum and erythrocytes have been determined for man and rabbit.

Experimental

Six male rabbits, maintained at chow pellets (N.V. Gog, Doesburg, The Netherlands) were fasted overnight before sacrifice. The blood of each animal was collected in a glucose-citrate solution in order to prevent clotting. After pooling, the blood was centrifuged immediately at 3000 rev/min. and the plasma was carefully aspirated. After removal of the buffy coat, the erythrocytes were washed three times with an equal volume of a physiological saline solution. Human erythrocytes and plasma were obtained in a similar way

from blood, donated by three male subjects, fasted overnight. The lipids of both red cells and plasma were immediately extracted according to the procedure of Reed[22]). The lipids were dissolved in pure chloroform and stored under nitrogen atmosphere at −20 °C.

Isolation of the lecithin samples

The total lipids were fractionated using column-chromatography following the procedure of Hanahan[23]). The elution pattern was controlled by testing several aliquots of the effluent by means of thin-layer chromatography on silica gel coated microslides. The combined lecithin fractions were further purified by thin-layer chromatography on 1 mm thick silica-layered plates (20 × 20 cm) using chloroform–methanol–concentrated ammonia–water (70:30:2:3, v/v/v/v) as a developer. After colouring with a 0.01% solution of Rhodamine 6 G in water, the lecithin spots were scraped from the glass-plate and extracted with several portions of methanol–chloroform (9:1, v/v). All experiments were performed in a CO_2 atmosphere. In this way samples of chromatographically very pure lecithins were obtained as demonstrated by comparison with synthetic substances.

Enzymatic hydrolysis processes

The hydrolysis of the lecithins with phospholipase A from *Crotalus adamanteus* venom (EC 3.1.1.4) and with phospholipase C from *Bacillus cereus* (EC 3.1.4.3) was carried out as described in detail previously[20, 21]). The fatty acid distribution of the fractionated diglycerides was determined with the aid of pancreatic lipase (EC 3.1.1.3)[20, 21]).

Subfractionation on silver nitrate impregnated silica

The silver nitrate impregnated silica plates which were used for the fractionation of the 1.2-diglycerides obtained from lecithin, were prepared as follows: 20 g of silica gel G (Merck) were mixed up with 45 ml of a 6% aqueous solution of $AgNO_3$. The resultant slurry was applied to the glass plates with the aid of a Desaga applicator. After activating overnight, the plates are ready for use. The developing system for the subfractionation of 1.2-diglycerides on the plates was: chloroform–absolute ethanol (96:4, v/v) (fig. 1). After development, the plates were sprayed with a 0.01% solution of Rhodamine 6 G and the diglycerides subfractions scraped from the plates. The diglycerides were extracted from the silica by repeatedly extracting with chloroform–methanol (9:1, v/v). The ratio of the fractions obtained was determined by adding to each fraction as an internal standard heptadecanoic acid which fatty acid does not occur in measurable amounts in the investigated lecithin samples.

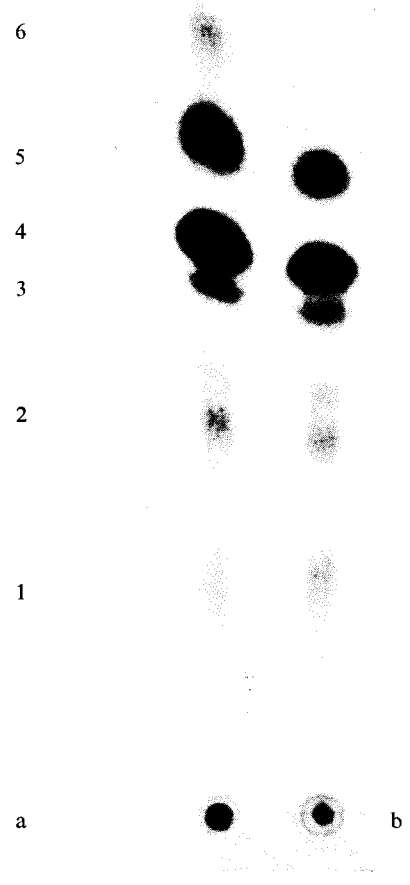

Fig. 1. Subfractionation of diglycerides obtained by phospholipase C hydrolysis of rabbit erythrocyte (a) and plasma (b) lecithin on $AgNO_3$ impregnated silica. The plates were prepared as described in the experimental part. The developing system was chloroform–absolute ethanol (96:4, v/v). The plates were stained by spraying with an aqueous 20% (w/v) solution of $(NH_4)_2SO_4$ and subsequently heating in an oven at 150 °C for 1 hour. The fatty acid composition and distribution of the fatty acids among the 1- and 2-position of these 6 subfractions are given in table 4 and 5.

Gas-liquid chromatography

The preparation of the methylesters was achieved similarly as described before[20,21]). The methylesters were analysed at an F&M instrument equipped with flame-ionisation detection and a 3% EGSS-X (Applied Science) column. The operating temperature of the column was 182 °C. The standard fatty acids were obtained respectively from Fluka A.G., Switzerland (palmitic and stearic acid), Light&Co, England (linoleic acid), and from the Hormel Institute, U.S.A. (oleic acid and arachidonic acid).

Results and discussion

The overall fatty acid composition of both human and rabbit plasma and erythrocytes is recorded in table 1. In this table is also given the positional distribution of the fatty acids among both ester positions as determined by hydrolysis with snake venom phospholipase A. It should be noted that the lecithins isolated from human and rabbit plasma contain a lower palmitic and oleic acid percentage when compared with the lecithins from human and

TABLE 1

Positional distribution of the fatty acids in erythrocyte and plasma lecithin from man and rabbit

	Rabbit plasma			Rabbit erythrocyte		
	overall	1-pos.	2-pos.	overall	1-pos.	2-pos.
14:0	+	+	—	+	+	+
16:0	27.9	50.6	7.7	33.6	57.0	15.2
16:1	+	—	1.1	+	—	+
18:0	18.4	38.9	+	16.9	30.4	4.1
18:1	14.2	9.0	19.2	15.3	8.7	20.9
18:2	33.3	2.1	62.1	27.4	4.0	48.5
18:3	4.3	—	6.0	2.5	—	4.6
20:3	+	—	+	1.5	—	2.4
20:4	1.9	—	4.0	2.8	—	4.2
	Human plasma			Human erythrocyte		
	overall	1-pos.	2-pos.	overall	1-pos.	2-pos.
14:0	+	+	—	0.2	0.5	—
16:0	30.9	59.4	7.1	33.7	58.3	8.3
16:1	+	2.5	0.4	2.4	1.2	3.5
18:0	16.0	31.0	+	13.5	26.7	0.4
18:1	12.3	7.1	13.7	18.4	12.0	25.2
18:2	29.5	+	58.8	23.1	1.3	43.8
18:3	0.5	—	1.3	0.3	—	0.7
20:3	2.2	—	3.5	2.1	—	4.5
20:4	8.8	—	15.2	6.3	—	13.4

rabbit erythrocytes. On the other hand a higher level of linoleic acid can be noted in plasma when compared with the corpuscles. Some differences are to be noted in the fatty acid composition of the lecithins between both mammalian species. In general these results are in close agreement with those reported by other authors[24–26]). According to expectation the 1-position of the lecithins is occupied mainly by saturated fatty acids. It is to be noted, however, that there is a considerable amount of palmitic acid at the

2-position, especially in the erythrocytes of both animals. This is to be attributed to quantitative differences in molecular species between plasma and erythrocytes as will be demonstrated.

Subfractionation of diglycerides derived from human erythrocytes lecithin

As can be inferred from table 1, natural lecithins are a heterogeneous family of molecules only differing with respect to their alifatic side-chains. The best resolution of the individual molecular species of lecithin was obtained by fractionation of the diglycerides rather than by fractionation of lecithin itself. The diglycerides derived from human erythrocytes lecithin could be separated into seven fractions. The separation is based on differences in total number of double bonds and the distribution of the double bonds among the 1- and 2-position of the diglycerides. The fatty acid constituents of these seven fractions together with their ratios, are compiled in table 2. Fraction 1 was found to contain species with four double bonds, palmitic acid, stearic acid and arachidonic acid being the main fatty acids. By digestion with pancreatic lipase, which enzyme acts preferentially on the fatty acids of the 1-position[27,28]), it was shown that the 2-position was

TABLE 2

Fatty acid composition of the fractionated diglycerides derived from human erythrocyte lecithin and the positional distribution of the fatty acids in each fraction of diglycerides

Subfraction	1	2	3	4	5	6	7
Weight percentages	10.2	6.1	10.4	33.7	5.2	20.4	14.0
Fatty acid composition							
14:0	+	+	—	0.2	+	+	2.1
16:0	32.3	33.1	4.3	30.3	+	36.6	76.5
16:1	+	—	+	+	4.8	5.1	—
18:0	16.6	14.3	1.1	17.6	+	13.7	14.4
18:1	1.4	1.1	46.6	+	95.2	44.6	7.0
18:2	1.5	1.3	48.0	51.9	—	—	—
18:3	—	6.4	+	—	—	—	—
20:3	—	43.8	—	—	—	—	—
20:4	48.2	—	—	—	—	—	—
Fatty acid composition of the 2-position*							
14:0	—	—	—	—	—	—	+
16:0	+	+	—	3.1	—	7.6	84.6
16:1	—	—	+	—	1.8	9.1	—
18:0	+	+	—	—	—	1.3	1.8
18:1	—	—	25.7	1.7	98.2	82.0	13.3
18:2	—	+	74.3	95.2	—	—	—
18:3	—	15.2	—	—	—	—	—
20:3	—	84.8	—	—	—	—	—
20:4	100.0	—	—	—	—	—	—

* Determined by lipase hydrolysis.

occupied exclusively by arachidonic acid. From this we can derive that the molecular species occurring in this fraction are: 1-palmitoyl-2-arachidonoyl- and 1-stearoyl-2-arachidonoyl-lecithin denoted as 16:0/20:4-PC and 18:0/20:4-PC respectively. The amounts in which these two species are present in the lecithin can be calculated in the following way: in fraction 1 the 1-position is occupied to an extent of 64.6% by palmitic acid. Thus 64.6% of this fraction represents the species 16:0/20:4-PC. Because fraction 1 can be accounted for 10.2% of the total lecithin sample, it has to be concluded that $64.6 \times \frac{10.2}{100}\%$ of the total lecithin represents 16:0/20:4-PC. In a similar way the amount of 18:0/20:4-PC was found to be 3.4%. The quantitative data of all molecular species are compiled in table 6. Fraction 2 appears to consist of molecules having three double bonds, the unsaturated fatty acids 18:3 and 20:3 being linked nearly exclusively at the 2-position. It can be concluded that both 16:0/20:3-PC and 18:0/20:3-PC were present, but it is not possible to be certain of the simultaneous occurrence of both 16:0/18:3-PC and 18:0/18:3-PC. Because of this uncertainty it is possible only to calculate the maximal and minimal values of the molecular species in this fraction. However, because of the relative small amounts of 18:3, the ranges between maximum and minimum values are rather small. Fraction 3 also contained molecules with three double bonds, though it was clearly separated from fraction 2. This separation appears to be brought about by a different distribution of the double bonds among the two ester positions in the diglycerides. It is interesting that this fraction is nearly exclusively consisting of unsaturated fatty acids, although the oleic acid is more concentrated at the 1-position and linoleic acid at the 2-position. Assuming legitimately the presence of three double bonds in this fraction, the species deducible from these data were 18:1/18:2-PC and 18:2/18:1-PC. Fraction 4 (2 double bonds), quantitatively the most important spot, can be concluded to consist of 16:0/18:2-PC and 18:0/18:2-PC whereas it can be speculated that also a small amount of 18:2/16:0-PC was present (table 6). The fatty acids present in the minor fraction 5 appeared to be 16:1 and 18:1 giving molecular species with a total number of two double bonds. The separation between spot 4 and 5 is caused by the fact that in fraction 5 each ester position of the diglycerides contains 1 double bond, whereas in fraction 4 both double bonds are located in the same fatty acid chain. From the data of fraction 5 it is evident that the major molecular species occurring are: 18:1/18:1-PC and 16:1/18:1-PC. Furthermore one or both of 18:1/16:1-PC and 16:1/16:1-PC could be present as minor species. Applying a similar discussion on the results obtained on fraction 6 (one double bond) we get as major species 16:0/18:1-PC, 18:0/18:1-PC and 18:1/16:0-PC. In addition one or both of 16:0/16:1 and 18:0/16:1 in only extremely small amounts can be present.

In the last spot (0 double bonds) occurred nearly exclusively disaturated diglycerides, *viz.* 16:0/16:0-PC, 18:0/16:0-PC and 14:0/16:0-PC.

Molecular species of lecithin isolated from human plasma

Separation of the diglycerides derived from human plasma lecithin gave 6 fractions. Qualitatively, the same molecular species as detected in erythrocytes were found to be present in plasma. However, there are several important quantitative differences to be noted. As could be expected already from the results mentioned in table 1, fraction 1 and 4, representing molecular species in which arachidonic acid respectively linoleic acid are combined with a saturated fatty acid, are quantitatively more important in human plasma lecithin. Conversely, the amount of dioleoyl respectively disaturated lecithin is higher in human erythrocytes. Actually, the fraction representing dioleoyl diglycerides was found to be smaller than 1% of the overall diglycerides. This amount was too small for further analysis and therefore the fatty acid composition of this fraction is not recorded in table 3.

TABLE 3

Fatty acid composition of the fractionated diglycerides derived from human plasma lecithin and the positional distribution of the fatty acids in each fraction of diglycerides

Subfraction	1	2	3	4	6	7
Weight percentages	14.7	5.8	11.1	44.3	20.1	4.0
Fatty acid composition						
14:0	+	+	–	1.4	1.7	3.2
16:0	31.3	33.2	1.1	34.7	36.6	79.3
16:1	–	–	1.0	+	4.1	–
18:0	18.7	16.8	0.3	14.0	12.9	10.8
18:1	+	+	49.4	1.5	44.4	6.7
18:2	–	+	48.2	48.4	–	–
18:3	–	5.1	+	–	–	–
20:3	–	45.0	–	–	–	–
20:4	50.1	–	–	–	–	–
Fatty acid composition of the 2-position*						
14:0	–	–	–	–	–	+
16:0	+	+	–	3.1	5.1	86.7
16:1	–	–	+	–	7.2	–
18:0	–	+	–	+	–	3.1
18:1	+	–	43.7	+	87.7	10.2
18:2	–	–	56.3	96.9	–	–
18:3	–	9.1	–	–	–	–
20:3	–	90.9	–	–	–	–
20:4	100.0	–	–	–	–	–

* Determined by lipase hydrolysis.

Molecular species of lecithin isolated from rabbit erythrocytes

Fractionation of diglycerides obtained from rabbit erythrocytes lecithins, on silica impregnated with silver nitrate yielded 6 spots (fig. 1). The fatty acid composition of these subfractions is recorded in table 4. Fraction 1, representing diglycerides with a total number of 4 double bonds, was found to contain as principal fatty acids 16:0, 18:0 and 20:4. As demonstrated by hydrolysis with pancreatic lipase, the arachidonic acid was located almost exclusively at the 2-position. Therefore, it is possible to calculate the following molecular species of lecithin: 16:0/20:4-PC and 18:0/20:4-PC (table 6). In addition it seems likely that there is present also a small amount of 18:2/18:2-PC lecithin. Fraction 2 (3 double bonds) was found to contain 16:0 and 18:0 at the 1-position of the diglycerides, and 18:3 and 20:3 at the 2-position. In some experiments this fraction was, more or less, resolved into two spots (see fig. 1), but these spots were always analysed together. Using the data obtained on this fraction it is only possible to calculate the sum of 16:0/18:3-PC and 18:0/18:3-PC, respectively 16:0/20:3-PC and 18:0/20:3-PC. Like in the separation of the two foregoing samples, there was obtained

TABLE 4

Fatty acid composition of the fractionated diglycerides derived from rabbit erythrocyte lecithin and the positional distribution of the fatty acids in each fraction of diglycerides

Subfraction	1	2	3	4	5	6
Weight percentages	4.1	6.9	9.6	42.3	23.6	13.4
Fatty acid composition						
14:0	—	—	—	+	—	+
16:0	24.4	29.8	3.3	27.6	42.3	87.0
16:1	+	—	—	—	2.2	—
18:0	17.6	20.3	3.4	21.0	8.2	11.2
18:1	5.6	+	46.7	3.9	47.5	1.8
18:2	4.8	+	45.0	47.5	—	—
18:3	—	30.2	1.7	—	—	—
20:3	—	19.7	—	—	—	—
20:4	47.5	+	—	—	—	—
Fatty acid composition of the 2-position *						
14:0	—	—	—	—	—	+
16:0	+	+	—	3.6	4.1	96.9
16:1	—	—	—	—	4.5	—
18:0	+	+	—	+	+	+
18:1	+	—	14.6	4.2	91.4	3.1
18:2	5.1	—	82.4	92.2	—	—
18:3	—	59.4	3.0	—	—	—
20:3	—	40.6	—	—		—
20:4	94.9	+	—	—	—	—

* Determined by lipase hydrolysis.

TABLE 5

Fatty acid composition of the fractionated diglycerides derived from rabbit plasma lecithin and the positional distribution of the fatty acids in each fraction of diglycerides

Subfraction	1	2	3	4	5	6
Weight percentages	4.8	7.0	12.6	49.3	21.8	4.5
Fatty acid composition						
14:0	+	—	—	+	+	2.8
16:0	24.9	20.4	1.5	24.7	36.2	72.2
16:1	—	—	2.5	—	2.1	—
18:0	21.3	31.3	1.9	24.9	14.2	19.2
18:1	4.9	—	43.1	3.5	47.5	5.8
18:2	—	—	51.0	46.9	—	—
18:3	4.1	45.2	—	—	—	—
20:3	—	4.1	—	—	—	—
20:4	44.9	—	—	—	—	—
Fatty acid composition of the 2-position *						
14:0	—	—	—	—	—	—
16:0	+	+	—	4.2	6.4	90.3
16:1	—	—	—	—	4.0	+
18:0	+	+	—	0.5	1.3	+
18:1	—	—	18.2	5.3	88.3	9.7
18:2	—	—	81.8	90.1	—	—
18:3	7.2	89.6	—	—	—	—
20:3	—	10.3	—	—	—	—
20:4	92.8	—	—	—	—	—

* Determined by lipase hydrolysis.

TABLE 6

Molecular species of erythrocyte and plasma lecithin from man and rabbit

		Man		Rabbit	
1-position	2-position	plasma	erythrocyte	plasma	erythrocyte
16:0	16:0	2.9	9.5	2.5	10.3
18:0	16:0	0.7	3.7	1.7	3.0
16:0	18:0	+	+	—	—
16:0	16:1	<1.4	<1.8	<1.0	<1.0
16:0	18:1	13.0 ± 0.7	12.5 ± 0.9	13.0 ± 0.5	18.5 ± 0.5
18:0	16:1	<1.4	<1.8	<1	<1
18:0	18:1	4.5 ± 0.7	4.4 ± 0.9	5.5 ± 0.5	3.4 ± 0.5
18:1	16:0	—	1.5	1.4	1.0
16:1	18:1	—	+	—	—
18:1	18:1	+	4.8	—	+
16:0	18:2	29.3	19.4	22.2	21.8
18:2	16:0	—	1.0	1.3	1.2
18:0	18:2	12.4	11.3	24.3	17.8
18:1	18:2	6.1	7.0	8.5	7.5
18:2	18:1	4.4	2.3	2.5	0.7
16:0	18:3	<0.5	<1.0	2.5 ± 0.3	2.8
18:0	18:3	<0.5	<1.0	4.0 ± 0.3	
16:0	20:3	3.5 ± 0.3	3.5 ± 0.4	<0.6	4.1
18:0	20:3	1.7 ± 0.3	1.3 ± 0.4	<0.6	
16:0	20:4	9.2	6.6	2.4	2.0
18:0	20:4	5.5	3.4	2.0	1.4

a second fraction having three double bonds. The species present in this fraction can be deduced to be mainly 18:1/18:2-PC and 18:2/18:1-PC. Fraction 4 (2 double bonds) being quantitatively the most important one, revealed after digestion with pancreatic lipase the presence of 16:0/18:2-PC and 18:0/18:2-PC. Similarly, 16:0/18:1-PC, 18:0/18:1-PC, and 18:1/16:0-PC were found to be present in fraction 6 (1 double bond), whereas there may be speculated on the presence of 16:0/16:1-PC and 18:0/16:1-PC as minor species. Finally the analysis of fraction 6 (0 double bonds), resulted in the formulation of the species 16:0/16:0-PC and 18:0/16:0-PC.

Molecular species of rabbit plasma lecithin

The separation of the diglycerides obtained from rabbit plasma lecithin, resulted also in 6 fractions, from which the fatty acid composition together with the positional distribution of the fatty acids among the 1- and 2-position, is given in table 5. Comparing the results obtained from rabbit erythrocytes and plasma diglycerides (table 4 and 5, respectively), it is to be seen that there are practically no qualitative differences. However, fraction 4 representing the species 16:0/18:2 and 18:0/18:2 mainly, is quantitatively more important in rabbit plasma, whereas fraction 6 accounting for the species 16:0/16:0 and 18:0/16:0 is more abundant in rabbit erythrocytes. This pattern is strictly in accordance with the distribution found in human erythrocytes and plasma.

Conclusions

About 20 molecular species have been detected which together account for about 90 percent of the total lecithin present in the erythrocyte and plasma of man and rabbit. For each mammalian species it can be concluded that qualitatively the make-up of the lecithin of the membrane is similar to that of the environment of the red blood corpuscles. Quantitatively the following differences are apparent. In the human erythrocyte the content of 16:0/16:0-PC, 18:0/16:0-PC, and 18:1/18:1-PC is higher when compared with plasma. In the latter the relative amount of the species 16:0/18:2 and 16:0/20:4-PC dominates that of the erythrocytes.

For rabbit similar differences are to be noted with respect to the species 16:0/16:0-PC. The content of 16:0/18:1-PC is higher in erythrocytes, whereas the reverse is true for the species 18:0/18:2-PC. It will be of interest to elucidate which processes govern the differences in make-up of the molecular species of lecithin between red cell membrane and serum lipoproteins.

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