BBA 76006

PREFERENTIAL BINDING OF SPHINGOMYELIN BY MEMBRANE PROTEINS OF THE SHEEP RED CELL

R. KRAMER, CH. SCHLATTER AND P. ZAHLER

Theodor Kocher Institute and Institute of Forensic Medicine, University of Berne, Central Laboratories of the Swiss Blood Transfusion Service SRK, Berne (Switzerland)

(Received February 22nd, 1972)

SUMMARY

Sheep red cell membranes are characterized by their low phosphatidylcholine and high sphingomyelin contents as compared to the same membranes of other species.

A study of the selective lipid-binding capacity of human and sheep red cell membrane proteins was carried out by comparative recombination experiments. Isolated lipid-free human and sheep erythrocyte membrane protein fractions were separately recombined with a total lipid fraction of human stroma. The recombined lipoproteins were isolated by density-gradient centrifugation and the lipids analyzed for phosphatidylcholine and sphingomyelin. The results of chemical and radiochemical analyses clearly demonstrate preferential binding of sphingomyelin by sheep membrane proteins. This favours the hypothesis that membrane proteins at least partly influence the lipid composition of a given membrane.

INTRODUCTION

The phospholipid composition of mammalian red cell membranes shows a considerable, species dependent variation[1]. For the series rat, human, pig, ox and sheep the phosphatidylcholine content decreases from 56% in the rat to 1% in the sheep, with a concurrent increase in sphingomyelin content from about 25 to 63%. Extreme dietary manipulations do not influence the proportion of the main phospholipid classes[2], although their fatty acid composition adapts partially to the ingested lipids. It is accordingly unclear what mechanisms regulate the proportions of phospholipids in the erythrocyte membranes of various species.

Studies on the extractability of lipids from the erythrocyte membrane indicate a large heterogeneity of their binding forces[3,4]. Most of the phosphatides, the "strongly bound fraction", are considered to be bound to membrane protein by electrostatic and hydrophobic interactions. The remaining phospholipids and sterols, representing the "loosely bound fraction", are considered to be associated predominantly with other lipids through apolar interactions[4]. In all the species studied the "loosely bound fraction" revealed a similar composition[3], whereas the characteristic differences in phospholipid composition known to exist between the erythrocytes of diverse mammals are limited to the "strongly bound fraction" (Table I). The fact that the fatty

TABLE I

COMPOSITION OF PHOSPHOLIPIDS FROM HUMAN AND SHEEP RED CELL GHOSTS

Species	*Total phospholipids* *			*"Loosely bound lipids"* **			*"Strongly bound lipids"* **		
	%PC	*%S*	*PC/S*	*%PC*	*%S*	*PC/S*	*%PC*	*%S*	*PC/S*
Human	39	37	1.05	24	22	1.1	40.5	28	1.4
Sheep	1.0	63	0.02	5	23.5	0.2	4.5	57	0.08

Abbreviations: PC, phosphatidylcholine; S, sphingomyelin.
* Data from de Gier and van Deenen[5].
** Data from Roelofsen *et al.*[3].

acid distribution in the phosphatidylcholine portion of the "strongly bound" lipid fraction was identical to that of the "loosely bound lipids" may indicate that similar lipid molecules are bound differently in the same membranes[4]. In *Bacillus subtilis*[6] the membrane composition appears to be controlled by the regulation and synchronization of the biosynthesis of the membrane proteins and lipids and this may also be true for the biosynthesis of cytochrome oxidase in mitochondria[7]. However, studies on the biosynthesis of membrane protein and lipid in *Mycoplasma laidlawii* have led to contrary findings[8]. When membrane protein synthesis is arrested by treating the cells with chloramphenicol, membrane lipid synthesis is not affected and a concurrent increase in the lipid content of the membranes occurs.

Most results favour the general concept that membrane proteins contain the necessary information to direct the binding of specific lipids and hence partly regulate the overall lipid composition of a given membrane. Anionic lipids seem to be bound to the structural protein of myelin more specifically than other lipids. This is shown by the fact that they form insoluble, aggregated lipoprotein complexes, whereas lipids which are not negatively charged result in "soluble" products[9]. Additional evidence for specific lipid–protein interactions is the observation that the above mentioned aggregation phenomen is unaffected by the presence of non-acidic lipids. Succinylation of the lysine amino groups of myelin structural protein almost completely abolishes the capacity of this protein to associate with lipids. This effect most likely is a consequence of drastic alterations in the three-dimensional structure of the protein which are induced by the negatively charged succinyl groups[9].

Furthermore mitochondrial structural proteins bind lipids almost stoichiometrically[10], and the binding of certain lipids with apo-proteins from chloroplast lamellae not only is quantitative but highly selective as well[11]. The relative proportion of the main lipid classes in reconstituted erythrocyte membranes is closely similar to that of native stroma[12]. A similar finding has been reported for chloroplast membrane recombinates. In this report we present a comparative study of the lipid-binding capacity of human and sheep erythrocyte membrane proteins. The results obtained favour the hypothesis that membrane proteins influence at least in part the lipid composition of a given membrane.

MATERIALS AND METHODS

Our general experimental approach is summarized in Fig. 1. Lipid-free human and sheep erythrocyte membrane protein were isolated by solubilization and gel

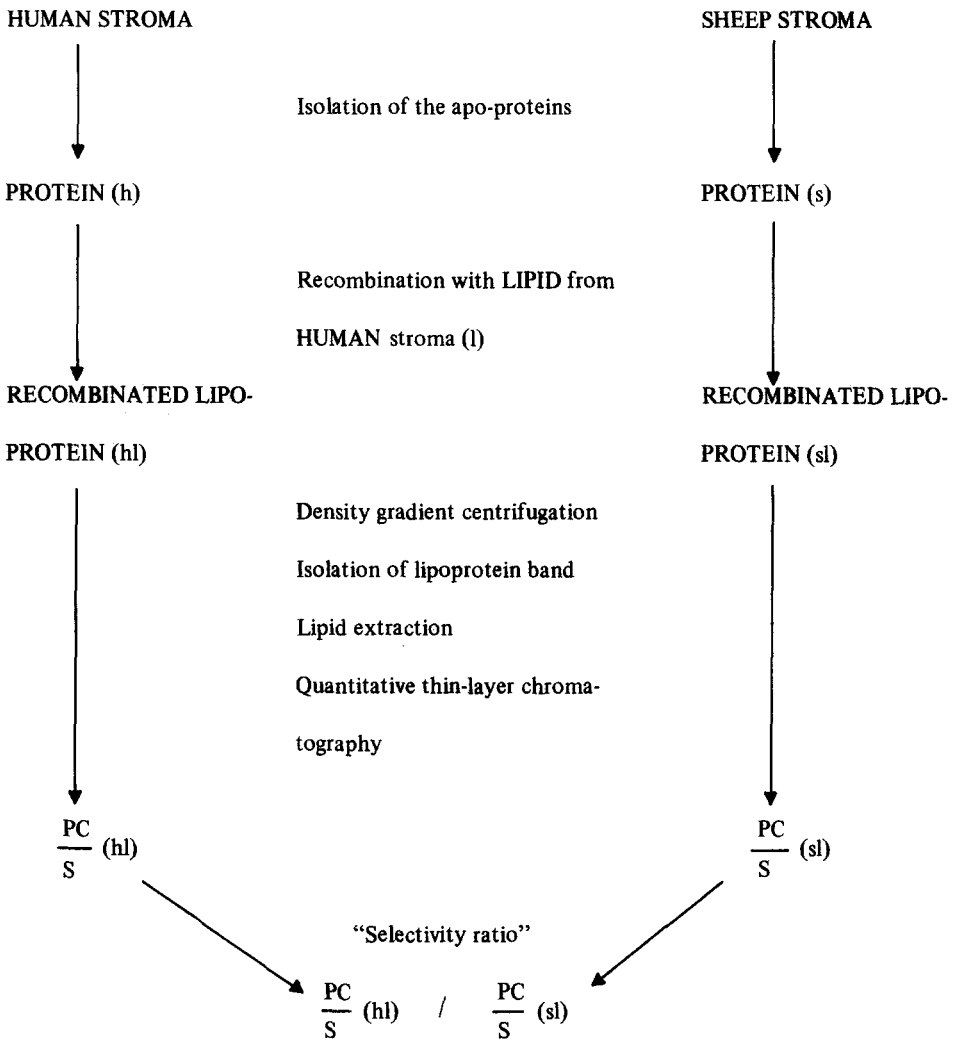


Fig. 1. Schematic outline of the solubilization, fractionation, recombination, density-gradient centrifugation and analysis of human and sheep red cell membranes.

filtration in 2-chloroethanol as previously described[12,13]. Each of the protein fractions thus obtained was then recombined with a total lipid fraction from human stroma. The organic solvent was removed by dialysis against an aqueous buffer and the lipoproteins, containing either human or sheep proteins, were isolated by density-gradient centrifugation. After isolation of the lipoprotein bands the material of a first series of experiments was analysed for lipid composition by quantitative thin-layer chromatography. In the second series the lipoprotein was directly measured for radioactivity.

Red cell membranes were isolated according to the method of Dodge *et al.*[14], modified as in refs 12 and 15.

Isolation and labeling of membrane lipids

Membrane lipids were extracted with 20 vol. of chloroform–methanol (2:1, v/v), the extracts processed as described in ref. 16, and the final chloroform phase dried under N_2. Phosphatidyl[^{14}C]choline (uniformly labeled, prepared from *Chlorella pyrenoidosa*), was obtained from New England Nuclear.

For the preparation of [^{3}H]sphingomyelin the concentrated lipid extract was chromatographed on silica gel HR thin-layer plates with chloroform–methanol–water (14:6:1, v/v/v). After verification of its localization with iodine vapour at a marginal position of the thin-layer plates, the sphingomyelin was eluted with chloroform–methanol (1:1, v/v) and assayed gravimetrically. It was rechromatographed in order to make sure that no visible lipid contamination was detectable. Tritium exchange was achieved by heterogeneous catalysis[17] as follows: Purified sphingomyelin (1–2 mg) was dissolved in 10–15 ml of glacial acetic acid and enclosed in a sealed, evacuated tube containing tritiated methanol (10–20 mCi) and platinum black. After shaking for 24 h at 70 °C and after removal of the solvent at reduced pressure, labile tritium was removed by repeated equilibration with methanol. The labeled sphingomyelin was purified once by thin-layer chromatography.

Isolation of membrane proteins

Red cell membranes were dissolved in 2-chloroethanol[12] and the membrane proteins separated from the lipids by molecular sieving on Sephadex LH 20 in 2-chloroethanol–water (9:1, v/v)[12]. The lipid-free protein was pooled and the subsequent recombination experiments performed immediately.

Recombination

Two series of recombination experiments were carried out with the isolated lipid-free membrane proteins from human (h) and sheep (s) erythrocytes and the lipid fraction isolated from human red cell stroma. In the first series of experiments, solutions of protein and lipid in 2-chloroethanol were combined to give mixtures containing 1 mg of protein and 0.8 mg of lipid.

In the second series, phosphatidyl[^{14}C]choline ($5.6 \cdot 10^4$ dpm) and [^{3}H]sphingomyelin ($8.7 \cdot 10^5$ dpm) were added to the human erythrocyte membrane lipids in 2-chloroethanol. This lipid solution was then combined with the protein fractions from human and sheep red cell membranes to give the mixtures (h+l) and (s+l), containing 1 mg of protein and 0.8 mg of lipid per ml. In both series, recombination was carried out by dialysis against 10 mM Tris–HCl, 10 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH 7.6[12]. The resulting suspension was centrifugated (27 000 rev./min, 30 min, 4 °C), the pellet washed with 10 mM Tris–HCl (pH 7.6), the final sediment taken up in 5 ml of 10 mM Tris–HCl (pH 7.6), 0.25 M sucrose and sonicated for 30 s with a Branson sonifier (setting 8) at 20 °C. Sonication was carried out to separate possible uncombined micellar lipid from the recombinate.

Density-gradient centrifugation

The sonicated suspensions (1–2 mg/ml:4.5 ml) were layered on top of linear sucrose gradients (density 1.1–1.2) and centrifugated to isopycnic equilibrium as described earlier[12]. Centrifugation separates two major fractions: The free lipid material at the top of the gradients and the reconstituted membrane layer. These were washed on the centrifuge with 10 mM Tris–HCl (pH 7.6) and analysed for

protein[18]. The ratio of protein to total lipid in the reconstituted membranes could be estimated from the density of the band.

Chemical analysis

In the first series of experiments, the lipids were extracted by the method of Renkonen *et al.*[19] and separated by thin-layer chromatography with chloroform–methanol–water (14:6:1, v/v/v)[20]. The pertinent lipid spots were detected with iodine vapour, scraped from the chromatogram and eluted with chloroform–methanol (1:1, v/v); the solvent was evaporated and the lipid samples were assayed for phosphorus[21].

The identification of each separated lipid spot was done by comparison with a lipid standard mixture containing phosphatidylcholine, sphingomyelin and lysophosphatidylcholine. The phosphatidylethanolamine spot was not included in the analysis, neither were taken into consideration the minor phospholipid fractions like phosphatidic acid, phosphatidylserine, plasmalogens *etc.* These fractions partially appearing within the spots of phosphatidylcholine and sphingomyelin may therefore have falsified slightly the values obtained for phosphatidylcholine + sphingomyelin by our technique.

Radiochemical analysis

In the second series, one half of the isolated hl and sl bands were extracted with diethyl ether to remove the "loosely bound lipids" as described by Roelofsen *et al.*[3]. To determine the radioactivities of the non-extracted and ether-extracted recombinates, 0.5-ml aliquots were transferred to scintillation vials and warmed with 0.5 ml 2 M NaOH for 30 min at 80 °C. The residues were dissolved in 12.5 ml of a special neutralizing scintillation fluid [toluene containing 7 g of 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (Ciba-Geigy, Basel)–Beckman-BBS-2–Beckman-BBS-3, (10:2:0.5, v/v/v)], and the ^{3}H and ^{14}C activities were determined in a Beckman scintillation counter LS 150. Isotope spectrum discrimination and efficiences were obtained by the use of [^{14}C]- and [^{3}H]hexadecane standards.

RESULTS

Density-gradient centrifugation

Density-gradient centrifugation of the recombined membranes hl and sl resulted in the formation of two major bands in each case (Figs 2b, 2c). The upper or low-density band consisted of free lipids, indicating that not all lipids had recombined with the protein. In both hl and sl recombinates a lipoprotein layer, located in the density range of 1.14–1.23, was found (Fig. 2). As reported before[12], the recombined lipoprotein had a lower proportion of lipid than the starting membranes.

Chemical analysis

An average of 90% of the protein and 70% of the lipid was recovered in the lipoprotein layer. The uncombined lipid (30%) remained at the top of the gradient (Figs 2b, 2c). Human and sheep membrane proteins show similar binding capacities for human membrane lipid, as indicated by the comparable recoveries of lipid and protein in the sl and hl fractions. Qualitative thin-layer chromato-

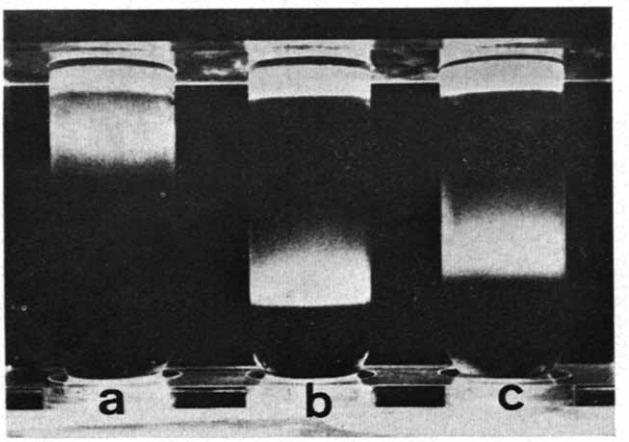


Fig. 2. Photograph of equilibrium centrifugation in sucrose gradient of (a) human red cell membrane, (b) recombinate of human red cell membrane protein with lipid from human red cell membrane, (c) recombinate of sheep red cell membrane with lipid from human red cell membrane.

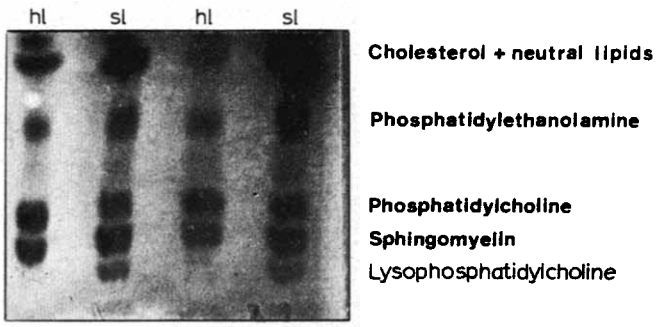


Fig. 3. Thin-layer chromatogram of the lipid extracts after density-gradient centrifugation. hl, recombinate of human red cell membrane protein with lipid from human red cell membrane; sl, recombinate of sheep red cell membrane with lipid from human red cell membrane.

graphy of the extracted lipids from the hl and sl recombinates revealed that all the major lipid components were present in both, although the proportions of phosphatidylcholine, lysophosphatidylcholine and sphingomyelin in the two fractions differed (Fig. 3). In all experiments the thin-layer plates revealed a higher content of lysophosphatidylcholine in the sl recombinates as compared with the hl material. A comparison of the ratios phosphatidylcholine/sphingomyelin calculated from the quantitative assay of the two phosphatides is shown in Table II. Strikingly, membrane protein from sheep erythrocytes binds sphingomyelin more avidly than the membrane protein from human erythrocyte. This preferential binding of sphingomyelin by sheep erythrocyte protein is reflected in all experiments by lower phosphatidylcholine/sphingomyelin ratio of the sl recombinate (1.2) as compared with the hl recombinate (2.4). This preferential binding can be demonstrated in a different manner by calculating the "specifity ratio" phosphatidylcholine/sphingomyelin (human): phosphatidylcholine/sphingomyelin (sheep), which as an average, gives a value of 2.15 (1.9–2.5).

TABLE II

PHOSPHATIDYLCHOLINE AND SPHINGOMYELIN CONTENT OF RECOMBINED MEMBRANES hl AND sl

The data were obtained by chemical analyses (phosphatide phosphorus) as described in Methods.

Experiment	*Recombined lipoprotein hl*			*Recombined lipoprotein sl*			*"Selectivity ratio"*
	PC (*ng*)	*S* (*ng*)	*PC/S*	*PC* (*ng*)	*S* (*ng*)	*PC/S*	$\frac{PC_{hl}}{S_{hl}} / \frac{PC_{sl}}{S_{sl}}$
III, Plate I	37.5	10.4	3.6	62.5	39.0	1.6	2.5
	40.0	10.4	3.8	50.0	39.0	1.3	
II	42.5	15.6	2.7	27.5	23.4	1.2	2.0
	40.0	18.2	2.2	27.5	20.8	1.3	
III	50.0	18.2	2.8	42.5	33.0	1.3	1.9
				30.0	18.2	1.6	
				35.0	23.4	1.5	
IV, Plate I	31.2	14.3	2.2	20.2	28.6	0.7	2.2
	37.5	19.5	1.9	25.0	20.8	1.2	
II	35.0	20.8	1.7	30.8	44.2	0.7	2.2
	32.5	20.8	1.6	32.2	41.6	0.8	
III	38.8	20.8	1.9	25.0	31.2	0.8	2.1
				30.0	28.6	1.0	

Abbreviations: see Table I.

TABLE III

RELATIVE PHOSPHATIDYLCHOLINE AND SPHINGOMYELIN CONTENT OF RECOMBINED MEMBRANES hl AND sl

The data were obtained by radiochemical analyses as described in Methods.

Experiment	*Recombined lipoprotein*	*Radioactivity (dpm/0.5 ml of the recombinate)*			*"Selectivity ratio"*
		PC (^{14}C)	*S* (^{3}H)	*PC/S*	$\frac{PC_{hl}}{S_{hl}} / \frac{PC_{sl}}{S_{sl}}$
I	Recombinate hl	1342	435	3.1	1.4
	sl	1135	503	2.2	
II	Recombinate hl	1234	312	4.0	1.3
	sl	1503	500	3.0	
III	Recombinate hl	1440	730	2.0	1.5
	sl	1500	1180	1.3	
IV	Recombinate hl	2018	483	4.2	1.2
	sl	1689	481	3.5	

Abbreviations: see Table I.

Radiochemical analysis

The amounts of phosphatidylcholine and sphingomyelin present in the recombinates as determined by radiochemical analysis are compared in Table III. This data give no information about the absolute quantities of the two phosphatides, because the specific activities of phosphatidylcholine and sphingomyelin were not the same; however, the phosphatidylcholine/sphingomyelin ratio of the sl recombinate is consistantly lower than the one of the hl recombinate. The preferential sphingomyelin binding by sheep membrane protein is indicated in the average "selectivity ratio" of about 1.4 (Table III) and can be even more clearly demonstrated when the "loosely bounds lipids" are extracted with ether from the recombined lipoprotein. The respective "selectivity ratio" in this case gives an average of about 2.7 (2.4–3.6) (Table IV).

TABLE IV

RELATIVE PHOSPHATIDYLCHOLINE AND SPHINGOMYELIN CONTENT OF THE ETHER-EXTRACTED RECOMBINED MEMBRANES hl AND sl AS DETERMINED BY RADIOCHEMICAL ANALYSES

Experiment	*Recombined lipoprotein*	*Radioactivity (dpm/0.5 ml of the ether-extracted recombinate)*			*"Selectivity ratio"* $\frac{PC_{hl}}{S_{hl}} / \frac{PC_{sl}}{S_{sl}}$
		PC (^{14}C)	*S* (^{3}H)	*PC/S*	
II	Recombinate hl	1153	262	4.4	2.4
	sl	635	352	1.8	
III	Recombinate hl	1395	495	2.8	2.5
	sl	730	655	1.1	
IV	Recombinate hl	1247	250	5.0	3.6
	sl	438	317	1.4	

Abbreviations: see Table I.

DISCUSSION

The results of the chemical and radiochemical analysis of the lipids in the recombined human (hl) and sheep (sl) lipoproteins clearly demonstrate preferential binding of sphingomyelin by sheep membrane proteins. Quantitative thin-layer chromatography of the extracted lipids from hl and sl recombinates shows a considerable scattering for phosphatidylcholine and sphingomyelin values from one experiment to another (Table II), mainly due to variations of the applied volumes to the thin-layer plates. However, the relative proportion of phosphatidylcholine and sphingomyelin within one series of experiments performed on the same plate is in good agreement. Differences between two experiments are probably due to variations in the lipid binding during recombination dialysis, a step which with our procedure is not fully reproducible. The phosphatidylcholine/sphingomyelin ratios calculated from the radiochemical analysis may not be compared as such to the respective chemical data, because the activities of the phosphatidyl[^{14}C]choline and [^{3}H]sphingomyelin added to the human lipid solution were not proportional to the absolute amounts of phosphatidylcholine and sphingomyelin present in this lipid mixture. However, the

"selectivity ratios" calculated from the chemical and the radiochemical data are directly comparable.

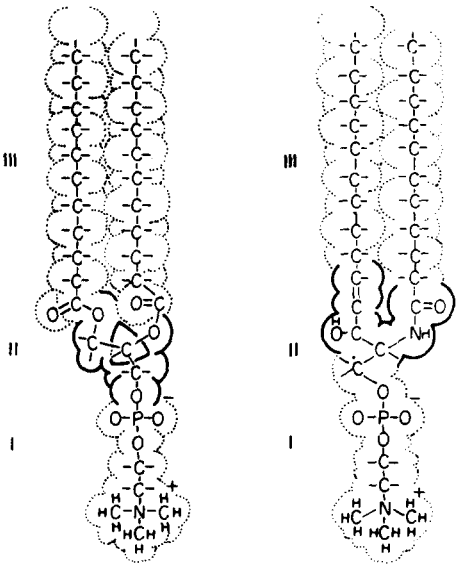
In contrast to hl, the sl recombinates contain a significant amount of lysophosphatidylcholine. Subsequent assays of the lysophosphatidylcholine content showed, however, that the "selectivity ratio" decreases only slightly and leads to values of 1.3 to 1.5 which are in good agreement with the radiochemical results. In the radiochemical measurements, both the ^{14}C-labeled phosphatidylcholine and lysophosphatidylcholine were included, so that possible lysophosphatidylcholine formed during the recombination would not falsify the results. Therefore the "selectivity ratio" calculated from these data reflects the total quantity of phosphatidylcholine and lysophosphatidylcholine. The interesting possibility that the membranes of sheep red cells contain a phospholipase A activity which can be reactivated during the recombination step is now being investigated.

The ratios of phosphatidylcholine/sphingomyelin in the recombined membranes sl are significantly higher than in the original membranes. Electron microscopy studies have revealed the occurrence of varying amounts of free lipid within the recombined lipoprotein vesicles. These free lipids have no relation whatsoever to membrane proteins, but reflect the composition of the added lipid mixture. From this one would predict a less pronounced selectivity in recombined membrane compared to native membrane. This interpretation is supported by the finding that the difference between the ratios of phosphatidylcholine/sphingomyelin in hl and sl is increased when recombined materials are extracted with ether. The remaining phospholipids representing the "strongly bound fraction" show a more pronounced difference in the distribution of phosphatidylcholine and sphingomyelin between hl and sl. These findings are in agreement with studies of Roelofsen *et al.*[3] indicating that it is in the first place the "strongly bound fraction" of the extracted lipids which reflects the compositional differences in the phospholipids of the red cells from various species. Taking into account these considerations, it must however be stressed that the relatively poor preference towards sphingomyelin found in the sl recombinates is far from explaining the extreme phosphatidylcholine/sphingomyelin ratio in sheep red cell membrane.

Optical measurements of rotatory dispersion and circular dichroism have revealed strong evidence suggesting that the α-helical content of the membrane proteins is retained or even increased by solubilization in 2-chloroethanol[22,23]. Since most functional activities of the membrane tested so far are lost by treatment with 2-chloroethanol, it must be concluded that the tertiary structure of the membrane proteins is destroyed by this solvent. The fact that a preferential binding of certain lipids is retained by this recombination technique, is concordent with our hypothesis[23] that secondary structure or more precisely the α-helical segments of the membrane proteins are the topographical areas on the protein molecule where the membrane lipids interact and bind.

A comparison of the structure of phosphatidylcholine and sphingomyelin offers an interesting insight into the problem how the discrimination and the specific binding of the lipid by the protein may be operating. It is evident (Fig. 4) that the charged region (I) is identical in both lipids whereas the polar region (II) and the hydrocarbon region (III) are different. As a consequence the protein has to interact with Region II or III in order to distinguish between phosphatidylcholine and sphingomyelin, whereas the ionic binding with the phosphatidylcholine group would be the

same for both. Many observations on the binding of lipids by membrane proteins support indeed the concept that at least two sites of a lipid molecule interact with the protein, *i.e.* the ionic region and the apolar hydrocarbon moiety. Zwaal and van Deenen[24] observed initial ionic binding between negatively charged lipid vesicles and



Phosphatidylcholine Sphingomyelin

Fig. 4. Structural formulas of phosphatidylcholine and sphingomyelin. I, charged region; II, polar region; III, apolar region.

positively charged membrane protein from erythrocytes isolated by the butanol procedure. These authors subsequently demonstrated that hydrophobic interactions take place and that these interactions stabilize the lipoprotein complex. Similar findings were reported by Ward and Pollak[25] on the binding of lipids to proteins in liver microsomal fractions. The results of Camejo *et al.*[27] suggest some discrimination by proteins of lipid molecules with respect to their non-polar region. It is therefore conceivable that the code for specific bindings is given by a topographically defined arrangement of polar and hydrophobic groups on the protein molecule dictated by the α-helical region[23].

The hypothesis that the lipid composition of a given membrane is regulated by synchronization of protein and lipid biosynthesis[5,6] should be extended to include the findings that the selective lipid binding capacity of membrane protein may regulate the selection of those lipids interacting directly with the proteins, whereas the composition of the "loosely bound" lipids in the bilayer region of the membrane may be regulated predominantly by a biosynthetic feed-back mechanism.

ACKNOWLEDGEMENT

The expert technical assistance of Susanne Moser and Rudolf Flückiger is gratefully acknowledged.

This investigation was supported by grant 3.602.71 of the Swiss National Science Foundation.

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