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THE PREFERENTIAL INTERACTION OF CHOLESTEROL WITH DIFFERENT CLASSES OF PHOSPHOLIPIDS

R.A. DEMEL, J.W.C.M. JANSEN, P.W.M. VAN DIJCK and L.L.M. VAN DEENEN

Laboratory of Biochemistry, State University of Utrecht, University Centre "De Uithof", Padualaan 8, Transitorium 3, Utrecht (The Netherlands)

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Summary

1. By differential scanning calorimetry a preferential affinity of cholesterol for sphingomyelin was established in mixtures of sphingomyelin and phosphatidylcholine where sphingomyelin was either the higher or the lower melting phospholipid.

2. A preferential affinity of cholesterol for sphingomyelin was also found in mixtures of sphingomyelin and phosphatidylethanolamine where sphingomyelin was either the higher or the lower melting phospholipid. The sphingomyelin used was isolated from beef erythrocytes or synthetic palmitoyl sphingomyelin.

3. In mixtures of phosphatidylserine with phosphatidylethanolamine, or phosphatidylserine with phosphatidylcholine, cholesterol showed the highest affinity for the lower melting phospholipid.

4. In a previous paper (van Dijck et al. (1976) Biochim. Biophys. Acta 455, 576—588) it was established that cholesterol has a higher affinity for phosphatidylcholine than for phosphatidylethanolamine.

The affinity order of cholesterol for the neutral phospholipids which can be deduced from these experiments is sphingomyelin > phosphatidylcholine > phosphatidylethanolamine.

Introduction

In the last decade of membrane research physical studies on model membrane systems have contributed substantially to the understanding of the architecture of biomembranes. With respect to the presence of cholesterol it has been shown recently that this lipid is not necessarily homogeneously distributed in a membrane. In mixtures of different phosphatidylcholines [1—3] and phosphatidylethanolamines [4], respectively, showing phase separation, choles-

terol showed the highest affinity for the most liquid species. In mixtures of a phosphatidylcholine and a phosphatidylethanolamine, showing phase separation, where the phosphatidylcholine was either the lower or the higher melting phospholipid in the mixture, cholesterol showed the highest affinity for the phosphatidylcholine [4]. Most biological membranes have a complex lipid composition containing a variety of lipid classes. The predominant lipids of the erythrocyte membrane are next to cholesterol, phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine. It has been shown that the phospholipids are asymmetrically distributed in the erythrocyte membrane with most of the phosphatidylcholine and sphingomyelin in the outer layer and phosphatidylethanolamine and phosphatidylserine in the inner layer [5].

Differences in affinity of cholesterol for different phospholipid classes as already shown for mixtures of phosphatidylcholine and phosphatidylethanolamine could possibly affect the distribution of cholesterol in the plane of the membrane and over both sides of the membrane.

In this paper the preferential interaction of cholesterol is studied in mixtures of sphingomyelin and phosphatidylcholine; sphingomyelin and phosphatidylethanolamine; phosphatidylserine and phosphatidylcholine; and phosphatidylserine and phosphatidylethanolamine and its possible biological relevance is discussed.

Materials and Methods

The phospholipids used were synthesized by Mrs. A. Lancée-Hermkens in our laboratory. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, by the method of van Deenen and de Haas [6]; 1,2-didocosanoyl-*sn*-glycero-3-phosphocholine was obtained by hydrogenation of 1,2-didocosenoyl-*sn*-glycero-3-phosphocholine; 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine by the method of Dawson and Hemington [7] adapted by Cullis and de Kruyff [8]; 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine was obtained by hydrogenation of 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine. Synthetic palmitoyl sphingomyelin was a generous gift of Dr. D. Shapiro (Department of agricultural Biochemistry, Rehovot, Israel).

Phosphatidylserine and sphingomyelin were isolated from beef erythrocyte ghost prepared by the method of Dodge et al. [9]. The extraction and purification procedure was described by van Dijck et al. [10]. Part of the phosphatidylserine was hydrogenated. 1,2-Dimyristoyl-*sn*-glycero-3-phospho-1'-*sn*-glycerol was prepared by base exchange from the corresponding phosphatidylcholine as described before [11]. Cholesterol was obtained from Merck Darmstadt. Differential scanning calorimetry measurements were performed on a Perkin-Elmer DSC 2B apparatus, with a heating and cooling rate of 5°C/min. 5 μM of lipid was suspended in 40 μl Tris/acetate (40 mM), pH 7.0, containing 100 mM NaCl, 15 μl of the suspension was transferred to the sample pan. For the lipids with a phase transition below 0°C the buffer was diluted with ethylene glycol (1 : 1, v/v). The amount of phospholipid in the sample pan was determined by the methods of Fiske-SubbaRow [12] as described by Barlett [13].

TABLE I

Chain length and unsaturation	Spingomyelin	Phosphatidylserine	Hydrogenated phosphatidylserine
14 : 0	0.5		
16 : 0	22.5	9.1	10.8
16 : 1		1.8	
18 : 0	5.2	30.0	80.3
18 : 1	3.9	33.4	
18 : 2	6.5	15.8	
18 : 3	1.5	1.5	
20 : 0			5.8
20 : 3		1.3	
20 : 4		3.6	
22 : 0	8.8		
22 : 5		1.0	
23 : 0	2.6		
24 : 0	39.5		
24 : 1	6.5		

The fatty acid composition of the beef erythrocyte sphingomyelin, phosphatidylserine and hydrogenated phosphatidylserine is given in ref. 10 and in Table I.

Results

Fig. 1 shows the effect of cholesterol on the endothermic phase transition of sphingomyelin isolated from beef erythrocytes. Sphingomyelin shows a gradual reduction in the heat content of the phase of transition with increasing concentrations of cholesterol as has been shown for the other classes of phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol. The phase transition is no longer apparent at about 35 mol% of cholesterol (Fig. 1B).

To establish a possible preferential interaction of cholesterol for one class of phospholipids, mixtures were made which showed phase separation. First a mixture was formed where one phospholipid was the lower melting lipid and than one where it is the higher melting lipid. Fig. 2 shows a mixture of dioleoyl phosphatidylcholine and beef erythrocyte sphingomyelin. In this mixture sphingomyelin is the higher melting lipid. The pure phospholipids have phase transitions of −16 and +20° C, respectively. The equimolar mixture of both phospholipids shows two distinct phase transitions. The phase transition temperature is shifted about 5 degrees to lower temperatures. The incorporation of increasing amounts of cholesterol shows a fast disappearance of the sphingomyelin endothermic phase transition. Already at a concentration of 10 mol% of cholesterol the sphingomyelin phase transition is no longer apparent. (Fig. 2B) and only at higher cholesterol concentrations the dioleoyl phosphatidylcholine phase transition is eliminated.

A similar result is observed with a mixture of dioleoyl phosphatidylcholine and synthetic palmitoyl sphingomyelin (Fig. 3). The phase transition temperature of palmitoyl sphingomyelin is at 44° C and has a heat content of 8.3 kcal/

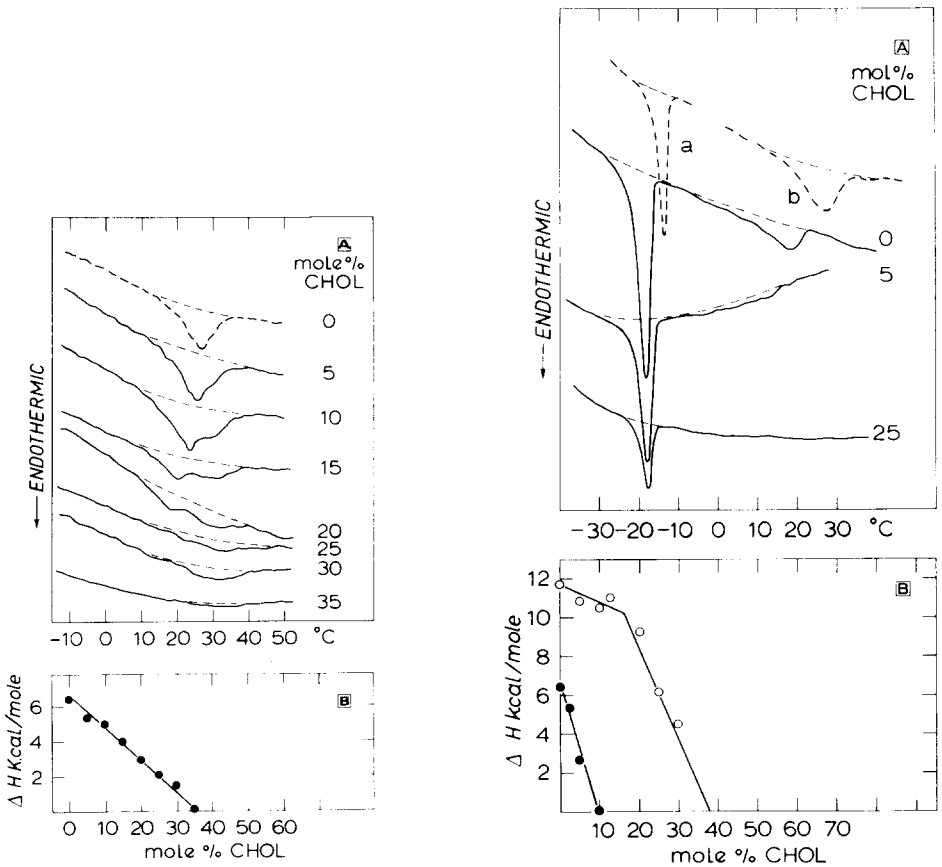


Fig. 1. (A) Calorimetric scans of sphingomyelin isolated from beef erythrocytes containing increasing percentages of cholesterol (CHOL). (B) Effect of cholesterol upon the energy contents of the phase transition of sphingomyelin isolated from beef erythrocytes.

Fig. 2. (A) Calorimetric scans of the equimolar mixture of dioleoyl phosphatidylcholine and sphingomyelin isolated from beef erythrocytes. The dotted curves represent the phase transitions of pure dioleoyl phosphatidylcholine (a) and pure sphingomyelin isolated from beef erythroyctes (b). (B) Effect of cholesterol upon the energy contents of the individual phase transitions occurring in a equimolar mixture of dioleoyl phosphatidylcholine (○) and sphingomyelin isolated from beef erythrocytes (●).

mol. There is a considerable broadening of the sphingomyelin phase transition after mixing with the lower melting dioleoyl phosphatidylcholine. Addition of cholesterol shows a disappearance of the sphingomyelin phase transition before the heat content of the dioleoyl phosphatidylcholine is reduced.

A mixture of beef erythrocyte sphingomyelin and a higher melting phosphatidylcholine as distearoyl phosphatidylcholine did not show a complete phase separation and for that reason a very long chain phosphatidylcholine has to be used. For phosphatidylcholines and phosphatidylethanolamines with a chain length of up to 18 carbon atoms the phase transition was no longer apparent at 33 mol% cholesterol, with didocosanoyl phosphatidylcholine the normal decrease in heat content is observed untill 15 mol% cholesterol.

Higher cholesterol concentrations do not produce more than a 50% reduc-

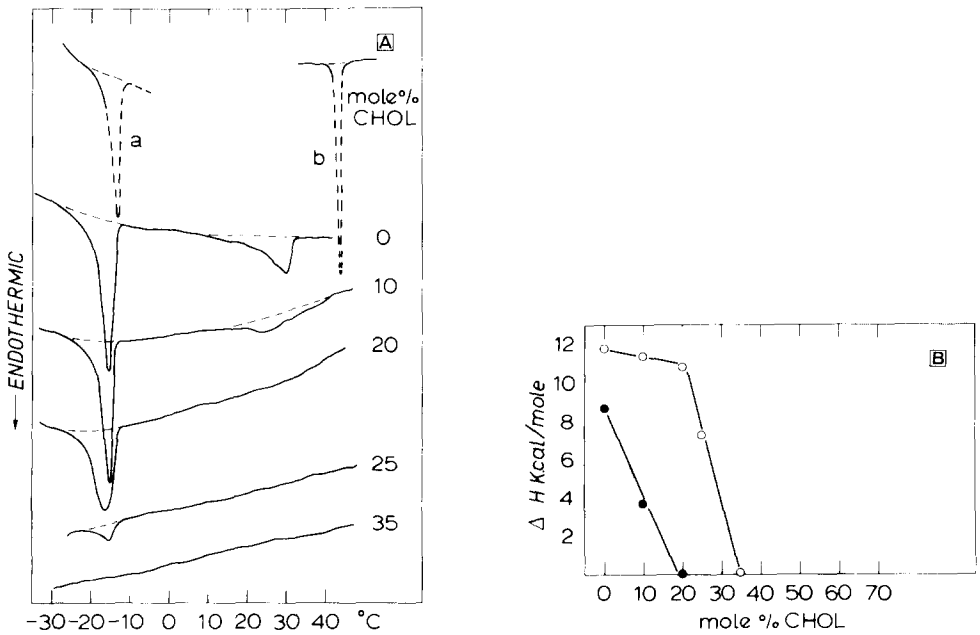


Fig. 3. (A) Calorimetric scans of the equimolar mixture of dioleoyl phosphatidylcholine and palmitoyl sphingomyelin. The dotted curves represent the phase transitions of pure dioleoyl phosphatidylcholine (a) and pure palmitoyl sphingomyelin (b). (B) Effect of cholesterol upon the energy contents of the individual phase transitions occurring in an equimolar mixture of dioleoyl phosphatidylcholine (○) and palmitoyl sphingomyelin (●).

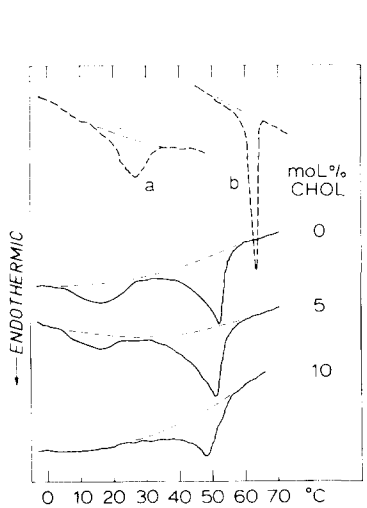
tion in heat content. Even at 50 mol% cholesterol the transition is still apparent. A complete phase separation was found for a mixture of beef erythrocyte sphingomyelin and didocosanoyl phosphatidylcholine. In this case the sphingomyelin is the lower melting phospholipid. Addition of cholesterol shows a disappearance of the sphingomyelin phase transition before there is any reduction in heat content of the docosanoyl phosphatidylcholine. A complete reduction of the lecithin phase transition cannot be obtained even at 50 mol% cholesterol.

A similar result was observed with a mixture of palmitoyl sphingomyelin and didocosanoyl phosphatidylcholine. In the 1 : 1 molar mixture of the two phospholipids a broadening of the higher melting didocosanoyl phosphatidylcholine phase transition is observed. The addition of cholesterol causes first the disappearance of the phase transition of the lower melting palmitoyl sphingomyelin and then reduces that of the didocosanoyl phosphatidylcholine. From the above results it can be concluded that in mixtures of sphingomyelin and phosphatidylcholine, when sphingomyelin is either the higher or the lower melting phospholipid, cholesterol shows a higher affinity for sphingomyelin than for phosphatidylcholine. In the same way as described above the preference of cholesterol between sphingomyelin and phosphatidylethanolamine was tested. In a mixture of dioleoyl phosphatidylethanolamine and beef erythrocyte sphingomyelin the phase transition of the higher melting sphingomyelin is eliminated first by addition of cholesterol. For a mixture of beef erythrocyte sphingomyelin and dipalmitoyl phosphatidylethanolamine, where the

sphingomyelin is the lower melting phospholipid, it could also be shown that the heat content of the sphingomyelin phase transitions is reduced first (Fig. 4). Also for these mixtures of sphingomyelin and phosphatidylethanolamine where sphingomyelin is either the higher or lower melting phospholipid, it can be concluded that cholesterol shows a higher affinity for sphingomyelin than for phosphatidylethanolamine.

To form mixtures of phosphatidylcholine and phosphatidylserine as well as of phosphatidylethanolamine and phosphatidylserine with a clear phase separation, phosphatidylserine was hydrogenated when it was the higher melting phospholipid. The heat content of beef erythrocyte phosphatidylserine is 4.9 kcal/mol and of the hydrogenated phosphatidylserine 7.8 kcal/mol. In a mixture of dioleoyl phosphatidylcholine the addition of cholesterol reduces the heat content of both phospholipid phase transitions, although the phase transitions of the phosphatidylcholine is reduced completely before that of the phosphatidylserine. This could mean that in this mixture cholesterol has a higher affinity for the lower melting lipid which is the phosphatidylcholine. In a mixture of beef erythrocyte phosphatidylserine and docosanoyl phosphatidylcholine (Fig. 5) the addition of cholesterol results in a fast disappearance of the phase transition of the phosphatidylserine. Also from this result it could be concluded that cholesterol shows the highest affinity for the lower melting phospholipid which is in this case phosphatidylserine. In mixtures of phosphatidylserine and phosphatidylethanolamine the phase transition of the lower melting phospholipid was affected first.

In a mixture of beef erythrocyte phosphatidylserine and dipalmitoyl phosphatidylethanolamine the phase transition of the lower melting phosphatidylserine disappears first. In a mixture of dioleoyl phosphatidylethanolamine and



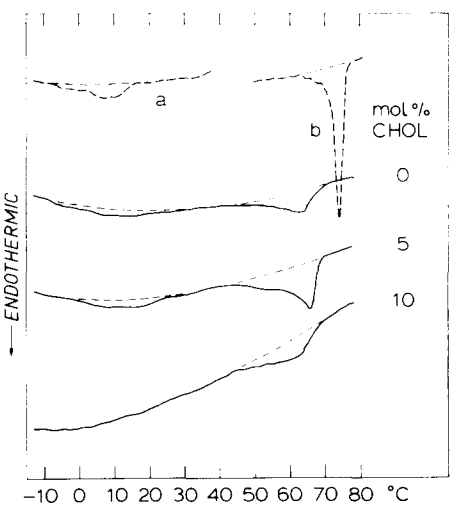


Fig. 4. Calorimetric scans of the equimolar mixture of sphingomyelin isolated from beef erythrocytes and dipalmitoyl phosphatidylethanolamine. The dotted curves represent the phase transition of pure sphingomyelin isolated from beef erythrocytes (a) and dipalmitoyl phosphatidylethanolamine (b).

Fig. 5. Calorimetric scans of the equimolar mixture of phosphatidylserine isolated from beef erythrocytes and didocosanoyl phosphatidylcholine. The dotted curves represent the phase transitions of the pure phosphatidylserine isolated from beef erythrocytes (a) and didocosanoyl phosphatidylcholine (b).

hydrogenated beef erythrocyte phosphatidylserine the phase transition of the lower melting phosphatidylethanolamine disappears first. The above results could be confirmed when phosphatidylglycerol was used in mixtures instead of phosphatidylserine. In a mixture of dioleoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol (the pure lipid showed a phase transition at —16 and +23° C, respectively), the endothermic phase transition of dioleoyl phosphatidylcholine is affected first. In a mixture of dioleoyl phosphatidylethanolamine and dimyristoyl phosphatidylglycerol the phase transition of the lower melting phosphatidylethanolamine is eliminated first. With the samples available for this study no mixtures of phosphatidylserine and sphingomyelin could be formed which give a clear phase separation.

Discussion

The differential scanning calorimetry measurements of mixtures of sphingomyelines and phosphatidylcholines where sphingomyelin is either the higher or the lower melting phospholipid showed that the phase transitions of the sphingomyelin is always reduced first. It is concluded that cholesterol has a higher affinity for sphingomyelin than for phosphatidylcholine. Also from mixtures of sphingomyelines and phosphatidylethanolamines the same conclusion is apparent, that cholesterol has a higher affinity for sphingomyelin than for phosphatidylethanolamine. It has been shown before that in mixtures of phosphatidylcholines and phosphatidylethanolamines where the phospholipids are saturated or unsaturated cholesterol had always a higher affinity for the phosphatidylcholine than for the phosphatidylethanolamine [4]. In mixtures of a negatively charged phospholipid such as phosphatidylserine and phosphatidylcholine or phosphatidylethanolamine; the phase transition of the lowest melting phospholipid is affected first. Also mixtures of phosphatidylglycerol and phosphatidylcholine or phosphatidylethanolamine seem to support this conclusion. From the above experiments the following order of cholesterol affinity for the neutral phospholipids studied can be concluded: sphingomyelin > phosphatidylcholine > phosphatidylethanolamine.

With all the phospholipids tested so far the liquid crystalline liquid phase transition was no longer apparent at 33 mol% cholesterol. With the long chain didocosanoyl phosphatidylcholine a normal decrease in heat content was observed till 15 mol% cholesterol. Higher cholesterol concentrations did not give a further reduction. It might be possible that the length of the cholesterol molecule is insufficient to liquefy this long chain saturated phospholipid completely, or that the solubility of cholesterol is reduced.

It has been suggested before that there is a possible correlation between the cholesterol concentration and the presence of certain phospholipid classes in membranes. The mitochondrial membrane is relatively high in phosphatidylethanolamine and low in cholesterol. This could possibly be related to the lower affinity of cholesterol for phosphatidylethanolamine as was demonstrated in mixtures of phosphatidylcholines and phosphatidylethanolamines. A correlative relationship of the occurrence of cholesterol and sphingomyelin in biological membranes has also been proposed [14]. High sphingomyelin (and other sphingolipids) and high cholesterol concentrations are for example found in the

central nervous system and peripheral nervous system and the erythrocyte membranes especially of the ruminants (beef and sheep). Low sphingomyelin and cholesterol concentrations are found in mitochondria microsomes, rough reticulum, nuclear membranes and the rat liver golgi apparatus (Fig. 6A). In most cases the high sphingomyelin concentrations are compensated by low phosphatidylcholine concentrations so that membranes high in cholesterol are relatively low in phosphatidylcholine (Fig. 6B). The erythrocytes are a somewhat special case. The cholesterol concentration of erythrocytes of all species is very high (39—46 mol%). The erythrocyte membranes of the ruminants contain 28—32 mol% sphingomyelin and practically no phosphatidylcholine. The erythrocytes of the non-ruminants contain 15 mol% sphingomyelin next to 12—15 mol% phosphatidylcholine.

Extremely interesting membranes with respect to the lipid composition are those of the lens cortex and nucleus (refs. 21 and 22 and Broekhuyse, R.M., personal communication). At an age of 28 years in the lens cortex membrane a cholesterol/phospholipid ratio of 1.3 : 1 is found. These cholesterol-rich membranes contain also a very high amount of sphingomyelin being 46 mol% of the phospholipid content. At an age of 70 years the cholesterol/phospholipid ratio is increased to 1.8 : 1 whereas also the sphingomyelin content is increased to 63 mol% of the phospholipid content. Of the nuclear membranes at an age of 70 years even 72 mol% of the phospholipid is sphingomyelin. It is also of interest to note that these membranes contain high amounts of lysophospholipids. From the experiments of Klopfenstein et al. [23] it can be concluded that lysophospholipids are well able to bind high ratio's of cholesterol.

In some classes of biological membranes where the cholesterol content is high the phosphatidylethanolamine concentration is lower than 20 mol% of the total lipid (e.g. erythrocytes, central and peripheral nervous system membranes) (Fig. 6C). When the phosphatidylethanolamine content is more than 20 mol% it is attended by a cholesterol content of less than 15 mol% (e.g. mitochondrial, microsomes nuclear, and rough reticulum membranes) (Fig. 6C). No particular correlation between the presence of negatively charged lipids as phosphatidylserine, phosphatidylinositol, phosphatidylglycerol or cardiolipin and the presence of cholesterol could be determined in biological membranes (Fig. 6D). The total amount of negatively charged lipids does normally not exceed 20 mol%.

Differential scanning calorimetry and monolayer measurements of the interaction of cholesterol with phospholipid analogs showed that the presence of oxygen atoms of the acylester linkages and of the oxygen atoms connecting phosphorus and carbon are not essential for the interaction [24]. $^{31}P\{^{1}H\}$ Nuclear Overhauser effect studies on cholesterol-lecithin vesicles [25] and ^{31}P NMR studies on cholesterol-lecithin liposomes [26] showed that cholesterol does not interact with the phosphate groups of lecithin. Measurements with monoglucosyl diglyceride and diglucosyl diglyceride even revealed that the phosphorus and choline moiety are not required [24]. These observations made a specific binding, of the sterol OH group with any polar part of the lipid molecule unlikely. On the other hand the sterol 3β-OH group was found to be essential for the lipid-sterol interaction [27—30]. Based on the present information hydrogen bonds with the bound water system were suggested as most likely

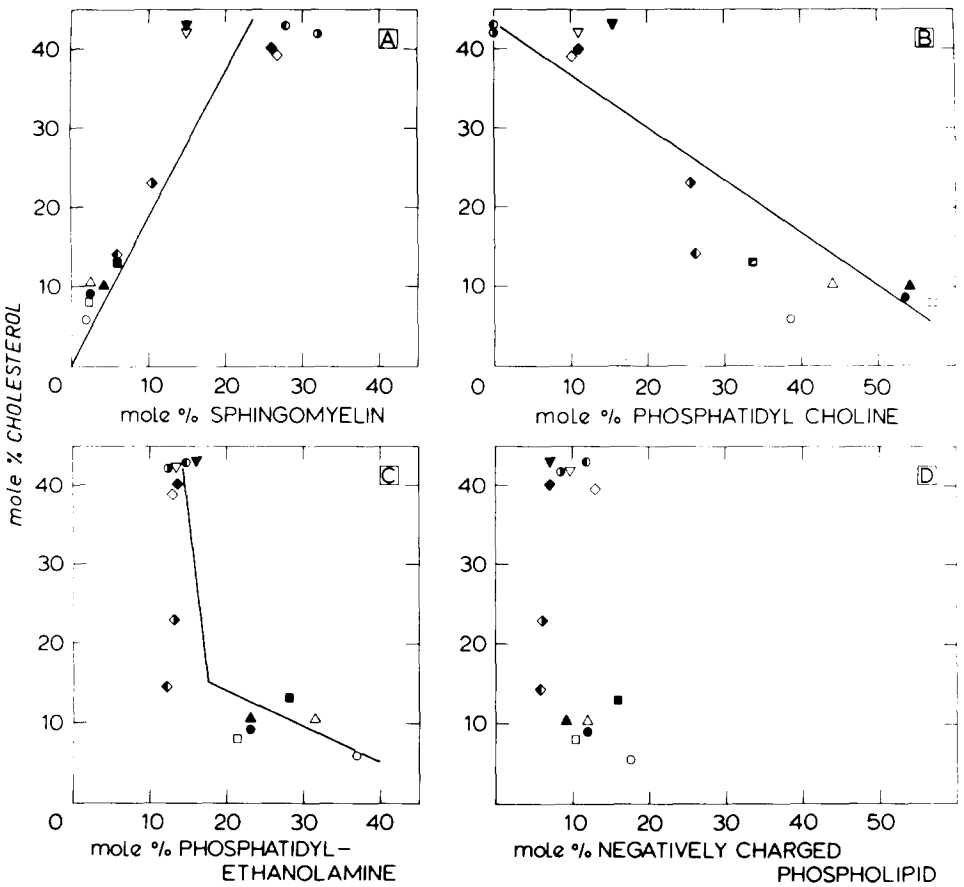


Fig. 6. The relationships (mol%) between cholesterol and sphingomyelin (A); phosphatidylcholine (B); phosphatidylethanolamine (C) and negatively charged lipids (phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidic acid, phosphatidylglycerol) (□) in: rat liver nuclear membrane [20] (●); rat liver mitochondrial inner membrane [16] (○); rat liver mitochondrial outer membrane [16] (△); microsomes [16] (▲); rough reticulum [16] (□); guinea pig brain mitochondria [18] (■); pig erythrocyte membranes [17] (▽); human erythrocyte membranes [18] (▼); sheep erythrocyte membranes [17] (◑); beef erythrocyte membranes [17] (◐); human central nervous system membranes [18] (◆); beef peripheral nervous system membranes [18] (◇); rat liver plasma membranes [19] (◈); rat liver golgi apparatus [19] (◈).

[30]. A closer packing of the headgroups and a lower water binding is observed for phosphatidylethanolamine compared to phosphatidylcholine [31] whereas for the first phospholipid a lower cholesterol affinity was found [4]. From the amounts of glucose trapped by phosphatidylcholine and sphingomyelin it is suggested that sphingomyelin binds more water than phosphatidylcholine [32]. The sphingosine OH moiety and the amide bond of sphingomyelin could possibly play an important role in the water binding and the cholesterol interaction. The properties of sphingomyelin and negatively charged lipids are now under further investigation.

The higher levels of cholesterol and sphingomyelin in many natural membranes can be correlated with the preferential interactions of cholesterol with sphingomyelin as demonstrated in this paper. In the erythrocyte membrane the

phospholipids with the highest cholesterol affinity, sphingomyelin and phosphatidylcholine are nearly exclusively located on the outer side of the membrane whereas most of the phosphatidylethanolamine and all of the phosphatidylserine is located on the inner side of the membrane. The different cholesterol affinities for species and classes of phospholipids might produce a non-random distribution of cholesterol in the plane of the bilayer as well as over the inner and the outer side of the membrane.

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