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Quantitation of cholesterol incorporation into extruded lipid bilayers

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ABSTRACT

Cholesterol incorporation into lipid bilayers, in the form of multilamellar vesicles or extruded large unilamellar vesicles, has been quantitated. To this aim, the cholesterol contents of bilayers prepared from phospholipid:cholesterol mixtures 33–75 mol% cholesterol have been measured and compared with the original mixture before lipid hydration. There is a great diversity of cases, but under most conditions the actual cholesterol proportion present in the extruded bilayers is much lower than predicted. A quantitative analysis of the vesicles is thus required before any experimental study is undertaken.

1. Introduction

Cholesterol (Chol) is an essential lipid component in mammalian membranes. Either Chol or other related sterols are found in all eukaryotic membranes. There are examples in biology in which high Chol concentrations, i.e. mole fractions of 0.4 and above, are found. This is the case of the ocular lens membrane [1], or of the so-called nuclear envelope remnants [2]. Beginning with the seminal studies by Chapman and co-workers [3,4] innumerable publications have dealt with the physical properties of Chol in lipid bilayers. To mention a few contributions, the reader is directed to Refs. [5–9]. In spite of all efforts, the effects of cholesterol on the biophysical properties of membranes are far from being adequately understood.

Many of the studies on cholesterol in membranes are carried out on model membranes, particularly multilamellar or large unilamellar vesicles (MLV, LUV, respectively). It has usually been taken for granted that all the lipids would be equally incorporated into the bilayers, i.e. that the liposome lipid composition would be the same as that of the original mixture from which the vesicles were formed. It occurred to us, however, that, particularly in mixtures with high cholesterol contents, this might not be the case. Consequently a number of experiments were undertaken, in which the chemical composition of LUV was determined, and compared with the original lipid mixture. Several studies have been carried out on the solubility of Chol in phospholipid bilayers [10–12] but we have focused our attention on the fate of cholesterol during vesicle preparation, particularly when extrusion methods are involved. The results summarized below indicate important deviations from the predicted behaviour, i.e. liposome cholesterol content is often much lower than intended. Conversely, none of the experimental values for Chol incorporation in this study is beyond the maximum solubilities measured by Huang et al. [11].

2. Materials and methods

2.1. Materials

Chol, DPPC, POPC, DOPC, DLPC, DAPC and SM were purchased from Avanti Polar Lipids (Alabaster, AL). Egg PE, egg PC and egg DAG were purchased from Lipid Products (South Nutfield, UK). Ferric chloride hexahydrate, sulfuric acid and phosphoric acid were purchased from Sigma. A kit for measuring cholesterol concentration was supplied by BioSystems (Barcelona, Spain). This kit is based on three coupled reactions using Chol esterase, Chol oxidase and peroxidase. Tests carried out in our laboratory with the Chol enzyme kit on appropriate standards provided a linear response ($r^2 = 0.99$) for Chol concentrations in the 1–4 mM range, in which our samples were included.

2.2. Multilamellar vesicle preparation

For multilamellar (MLV) liposome preparation the lipids were dissolved in chloroform/methanol (2:1) and mixed at the required proportions, and the solvent was evaporated to dryness under a stream

Abbreviations: Chol, cholesterol; DAG, diacylglycerol; DAPC, diarachidonoyl phosphatidylcholine; DLPC, dilinoleoyl phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; SM, sphingomyelin

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of nitrogen. Traces of the solvent were removed by evacuating the samples under high vacuum for at least 2 h. The samples were hydrated in 25 mM HEPES, 150 mM NaCl, pH 7.4 helping dispersion by stirring with a glass rod. When vesicles had to be prepared at a high temperature the hydration buffer was pre-heated. In order to ensure that all the lipid was incorporated the sample was subjected to a brief period of bath sonication. To ensure homogeneous dispersion the hydrated samples were passed between two syringes through a narrow tubing (0.5 mm internal diameter, 10 cm long) 100 times at 45 °C.

2.3. LUV preparation

LUV of diameters 100–150 nm were prepared by the extrusion method [13] using Nuclepore filters 0.1 μm pore diameter at room temperature, in 25 mM HEPES, 150 mM NaCl, pH 7.4.

2.4. Phosphorus and cholesterol assays

Phospholipid concentration was measured as lipid phosphorus [14]. Chol was usually assayed with an enzymatic procedure involving Chol oxidase and peroxidase (see section 2.1).

When the vesicles contained SM a non-enzymatic Chol quantification method was used. The reason for this was that according to Slotte [15,16] the activity of Chol oxidase is decreased when SM is present in the membrane. The protocol is based on Bowman and Wolf [17]. Briefly, 300 μl of sample were diluted to 3 ml with ethanol. To each tube 3 ml of working iron reagent were added. The working iron reagent is composed of 8 ml stock iron reagent + 92 ml concentrated sulfuric acid. The stock iron reagent contains 2.5 g ferric chloride hexahydrate + 100 ml phosphoric acid. The tubes were carefully vortexed and absorbance read at 550 nm. Both enzymatic and chemical methods of Chol determination gave coherent results in the absence of SM.

2.5. Sonication procedure

For Chol assays using the enzymatic procedure, MLV were sonicated prior to the assay in order to make Chol fully accessible to the enzyme. The lipid suspension was sonicated with on/off intervals of 10 seconds. The total sonication time ("on" intervals) was 10 min. The vials were kept on ice during the process to avoid sample overheating.

2.6. Statistics

Unless otherwise indicated, data are average values of 3–5 independent measurements ± one standard deviation. Student's *t*-test was used in order to assess the significance of observed differences.

3. Results and discussion

An extensive series of lipid mixtures containing Chol was prepared under different conditions. After hydration and, eventually, extrusion through polycarbonate filters (100 nm pore diameter) the actual proportion of cholesterol was quantitated and compared with the theoretically predicted (calculated) value, should all the cholesterol and all the phospholipid have been incorporated into the lipid suspension. Before cholesterol assays, non-extruded preparations were subjected to sonication, in order to make Chol fully accessible to the enzyme because it is known that multilamellarity affects oxidation rate. Results, as "predicted" and "experimental" mol% Chol in the extruded mixtures, are summarized in Tables 1–4. The absolute figures of phospholipid and cholesterol are given in the Supplementary Material, Tables S1-S4.

Cholesterol solubility is not uniform for all phospholipids. Huang et al. [11] reported $X_{Chol} = 0.66$ as the solubility limit for DOPC and DPPC. Using different methods, Epand et al. [18] found phase separation in POPC bilayers at $X_{Chol} > 0.5$. Brzustowicz et al. [19] found evidence of Chol crystallization in DAPC already at $X_{Chol} = 0.17$. In gel phase DPPC, Chol phase separation was observed at $X_{Chol} = 0.5$ [4,20]. Predicted Chol concentrations in our lipid mixtures ranged from 25 to 75 mol%. Even if the highest Chol concentrations predicted were above the solubility limits of Chol in phospholipids, we assayed them in order to confirm the previous data, and to detect possible patterns of Chol incorporation as a function of Chol/phospholipid ratio. Perusal of the data indicates that, in most cases, the actual proportion of Chol was lower, even much lower, than predicted. Also, in agreement with Pan et al. [10], the incorporation patterns differed considerably with the phospholipid acyl chain.

Table 1
Cholesterol incorporation into lípid bilayers. A comparison of predicted and experimental values. "Predicted" refers to the calculated data assuming 100% phospholipid and Chol incorporation into the bilayers. Data are given as cholesterol molar fraction × 100 (or mol% cholesterol). Average values ± S.D. ($n = 3–5$).

#	Lipid mixture	$X_{Chol} \times 100$ (mol% Chol)				
1	DOPC/Chol	Predicted	33	50	67	75
		Experimental (LUV)	30 ± 4.3	43 ± 8.3	57 ± 6.4	59 ± 6.1
2	POPC/Chol	Predicted	33	50	67	75
		Experimental (non extruded)	34 ± 0			80 ± 4
		Experimental (LUV)	15 ± 4.1	54 ± 6.8	59 ± 9.0	59 ± 4.0
		Filter	19 ± 1.9			17 ± 5
3	DLPC/Chol	Predicted	33	50	67	
		Experimental (non extruded)			63 ± 5	
		Experimental (LUV)	35 ± 8.3	49 ± 2.9	50 ± 14.8	
		Filter			20 ± 9	
4	DAPC/Chol	Predicted	33	50	67	75
		Experimental (non extruded)	32 ± 1			80 ± 2.5
		Experimental (LUV)	22 ± 1.5	9 ± 0	18 ± 1.1	10 ± 1.8
		Filter	11 ± 1.3			63 ± 7.5

3.1. PC containing monounsaturated acyl chains

Liposomes based on DOPC or POPC are often used to mimic the physical properties of cell membranes. In mixtures with DOPC (Table 1, #1), when the predicted Chol concentration was 33 mol%, the experimental value for LUV was about the same, but with predicted Chol concentrations ≥ 50 mol%, Chol appeared to be incorporated only partially, with a maximum at ≈60 mol% irrespective of the original proportion of lipids before hydration.

Table 2
Cholesterol incorporation into lipid bilayers in the gel or fluid state. A comparison of predicted and experimental values. "Predicted" refers to the calculated data assuming 100 % phospholipid and Chol incorporation into the bilayers. Data are given as cholesterol molar fraction × 100 (or mol% cholesterol). Average values ± S.D. ($n = 3–5$).

#	Lipid mixture	T (°C)	$X_{Chol} \times 100$ (mol% Chol)				
1	DPPC/Chol	45	Predicted	33	50	67	75
			Experimental (LUV)	26 ± 0	30 ± 2.8	42 ± 2.7	47 ± 6.4
2	DPPC/Chol	22	Predicted	33	50	67	75
			Experimental (non extruded)	33 ± 1.3			80 ± 0.7
			Experimental (LUV)	25 ± 1.5	22 ± 0$_+$	20 ± 2.4$_{+++}$	31 ± 0$_+$
			Filter	15 ± 6			50 ± 9
3	SM/Chol	45	Predicted	33	50	67	75
			Experimental (LUV)	29 ± 0.95	43 ± 2.1	48 ± 4	37 ± 4.5
			Filter	8 ± 3	15 ± 0.8	28 ± 8	35 ± 5
4	SM/Chol	22	Predicted	33	50	67	75
			Experimental (LUV)	34 ± 2.8$_+$	44 ± 3	40 ± 5	41 ± 3
			Filter	5 ± 2	12 ± 3	29 ± 5	25 ± 3

Statistical significance (Student's *t*-test). +, ++, +++ represent, respectively, $p < 0.05$, $p < 0.01$, $p < 0.001$ between 22 °C and 45 °C, all other conditions being the same.

Table 3
Cholesterol incorporation into lamellar or inverted hexagonal lipidic structures. A comparison of predicted and experimental values. "Predicted" refers to the calculated data assuming 100 % phospholipid and Chol incorporation into the bilayers. Data are given as cholesterol molar fraction × 100 (or mol% cholesterol). Average values ± S.D. ($n = 3$–5).

#	Lipid mixture	T (°C)	pH	Phase	$X_{Chol} \times 100$ (mol% Chol)		
1	PE/Chol	45	9.5	lamellar	Predicted	50	67
					Experimental (non extruded)	54 ± 4	63 ± 3.8
					Experimental (LUV)	44 ± 1.3	44 ± 0.5
2	PE/Chol	45	5.0	inverted hexagonal	Predicted	50	67
					Experimental (non extruded)	41 ± 5	46 ± 3
					Experimental (hexagonal)	35 ± 9.6	43 ± 6.7
3	PE/Chol	25	5.0	lamellar	Predicted	50	67
					Experimental (non extruded)	46 ± 4	67 ± 3
					Experimental (LUV)	39 ± 4.7	61 ± 7.6

Statistical significance (Student's *t*-test). No significant differences were found between the experimental results displayed in this table.

Mixtures with POPC behaved differently (Table 1, #2) in that the proportion of incorporated cholesterol in LUV was always lower than predicted. Even at the lowest proportion tested (predicted 33 mol%), incorporation was poor. The highest experimental values of Chol incorporation remained at ca. 60 mol% Chol. This may correspond to the maximum solubility of Chol in bilayer in binary mixtures with phospholipids, in agreement with Feigenson and co-workers [11,21]. In order to find out the fate of the "missing" Chol, in selected assays the polycarbonate filters used in the extrusion procedure were extracted with organic solvents, and Chol assayed in those extracts. The results are shown in Table 1 ("Filter"). The sum of the amount of Chol in LUV + filter corresponded, within experimental error, to the predicted value, and to the experimental value obtained in the non-extruded samples. This suggests that Chol was incorporated in a metastable way in some bilayer samples, and extrusion drove the sterol out of the phospholipid-based lipid phase. In contrast to Chol, all the phospholipid was retrieved in the extruded samples (Tables S1–S4).

3.2. Polyunsaturated PC

Binary mixtures of DLPC and Chol incorporated the predicted amounts of Chol up to 50 mol%, but not beyond. Again the extrusion filters retained some of the Chol that had not been properly incorporated into the bilayers (Table 1, #3). Mixtures with DAPC, in turn, displayed a paradoxal behaviour (Table 1, #4), the higher the original Chol proportion, the lower the experimental value after vesicle formation. Missing Chol was recovered in the polycarbonate extrusion filter. DAPC/Chol mixtures were a prime example of differences between predicted and experimental Chol concentration, e.g. 50 mol% and 9 mol% respectively for LUV. Note as well the very low solubility measured for Chol in DAPC bilayers by other authors [19].

In general, fatty acyl polyunsaturation appeared to decrease Chol miscibility with phospholipids. This is in agreement with the ^{2}H-NMR and X-ray diffraction studies of Stilwell and co-workers [12,22,23] who found a markedly decreased solubility of Chol in di-C20:4 and di-C22:6 bilayers as compared to di-C18:2. Note however that, in the nuclear envelope remnants [2], up to 42 mol% Chol was found, solubilised by mainly polyunsaturated polyphosphoinositides. In this, and perhaps other cases, phospholipid polar head groups may be important in Chol stabilization in bilayers.

It may be mentioned in this context that, in our hands, diphytanoyl PC, containing branched fatty acyl chains, did not give rise to stable bilayers with Chol, even at low proportions, and experimental measurements provided erratic results. The fact that diphytanoyl PC in mixtures with Chol can give rise to four coexisting phases [24] may explain this complex behaviour.

3.3. Disaturated PC and sphingomyelin

In all DPPC/Chol mixtures tested, Chol incorporation was clearly below the predicted values, both above and below the gel-fluid transition temperature of the pure phospholipid (41 °C) (Table 2). In the gel state, incorporation was somewhat lower. Extrusion to form LUV at 22 °C, i.e. in the gel state, caused in most cases a decrease in Chol contents of the vesicles (Table 2, #2). Chol was recovered in the filters (Table 2, #2). It is remarkable that, according to our measurements, a commonly studied mixture such as DPPC/Chol at a 2:1 mol ratio might in fact contain, for LUV in the fluid state, only 26 mol% Chol, i.e. ≈3:1 DPPC/Chol mol ratio (Table 2, #1), and the corresponding values for a theoretical 1:1 mixture would actually be 30 mol%, i.e. ≈3.3:1 DPPC/Chol mol ratio.

Sphingomyelin exhibited, when fully hydrated, a gel-fluid phase transition at ca. 40 °C. Chol incorporation approached the predicted value only for the 33 mol% mixture, at 22 °C (34 ± 2.8 mol% see Table 2, #4). The incorporation of Chol was higher below the gel-fluid transition with a maximum incorporation of 40 mol%. The missing cholesterol was recovered in the filter that was used in the extrusion process. It is interesting in this context that ocular lens membranes, that are rich in SM and in dihydroSM, may contain 50 mol% Chol. In this case, dihydroSM appears to be able to solubilize larger amounts of Chol than SM [1].

3.4. Inverted hexagonal phases

Egg PE can exist in either lamellar or inverted hexagonal phases, depending on pH and temperature [25,26]. Three conditions were selected, namely pH 9.5, 45 °C, when PE was lamellar, pH 5.0, 45 °C, when PE was in the inverted hexagonal phase, and pH 5.0, 25 °C, when PE was again in the lamellar state (Table 3). In general, cholesterol increased the propensity of lipids to adopt the inverted hexagonal phase

Table 4
Cholesterol incorporation into complex lipidic mixtures. A comparison of predicted and experimental values. "Predicted" refers to the calculated data assuming 100 % phospholipid and Chol incorporation into the bilayers. Data are given as cholesterol molar fraction × 100 (or mol% cholesterol). Average values ± S.D. ($n = 3$–5).

#	Lipid mixture (mol ratio)	Phase	$X_{Chol} \times 100$ (mol% Chol)				
1	PC:PE:Chol (2:1:3)	lamellar	Predicted			50	
			Experimental (LUV)			46 ± 6.2	
2	PC:PE:Chol: DAG (2:1:X) + 5 mol% DAG	lamellar	Predicted	25	40	50	58
			Experimental (LUV)	26 ± 1.0	40 ± 3.0	45 ± 12.7	47 ± 7.6
			Filter			9 ± 3	10 ± 4
3	PC:PE:Chol: DAG (2:1:X) + 30 mol% DAG	inverted hexagonal	Predicted	25	40	50	58
			Experimental (LUV)	25 ± 0.75	35 ± 5	45 ± 5	50 ± 5

[27,28]. There was no significant difference in Chol incorporation to PE under conditions favouring the lamellar or the non-lamellar state (Table 3). In fact, the actual proportions of Chol incorporated to PE bilayers under any conditions were very similar to those found in mixtures with DOPC or POPC (Table 1, #1, 2). PE may be somewhat unstable at pH 9.5, but, according to our quantitative TLC data no significant degradation occurred within the experimental time range (<2% PE loss 2 h after extrusion, i.e. approximately 1 h after our experiment was completed).

3.5. Ternary and complex mixtures

PC:PE:Chol mixtures, sometimes with added diacylglycerol, have been often in use in ours and other laboratories in experiments involving vesicle fusion induced by phospholipases C [29,30]. Chol incorporation into this sort of mixtures was tested, and the results are summarized in Table 4. Chol was readily incorporated into the PC:PE:Chol mixture, even at a 2:1:3 mol ratio (Table 4, #1). In the presence of 5 mol% DAG (additional %) incorporation was also quantitative up to 50 mol% Chol (Table 4, #2). When DAG was added at 30 mol% (additional %), under conditions that favour inverted hexagonal phase formation, Chol incorporation occurred more or less to the same extent as in the lamellar phase (Table 4, #3). In general, Chol incorporation appeared to be easier in the PC:PE mixture, with and without DAG, than in the binary mixtures, in agreement with the notion that more degrees of freedom generate bilayers with higher stability [31].

4. Conclusion

The above results show that, under most conditions, and particularly in binary mixtures with phospholipids extruded through polycarbonate filters, Chol does not become quantitatively incorporated into lipid bilayers. It is therefore imperative to carry out quantitative tests of vesicle composition once the lipid mixtures have been hydrated and equilibrated, and before they are used for experimental purposes. Perhaps some previous results will require revision in the light of the present data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.06.004.

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