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Phospholipid vesicles as a drug delivery system. Part I. Interaction of cytidine-5′-diphosphate choline with charged and zwitterionic phospholipids

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Abstract

Charged (dipalmitoylphosphatidylserine (DPPS) and dipalmitoylphosphatidic acid (DPPA) and zwitterionic (dipalmitoylphosphatidylethanolamine (DPPE) and dipalmitoylphosphatidylcholine (DPPC) phospholipid vesicles were used as a drug delivery device containing cytidine-5′-diphosphate choline (CDP-choline). To evaluate the interaction between the membrane surface and CDP-choline, the thermodynamic behaviour, linked with gel–liquid crystal phase transition was analyzed by differential scanning calorimetry (DSC) and scanning dilatometry (SD). Analysis of thermodynamic parameters shows that the interaction between CDP-choline and phospholipid heads is very weak for DPPC, but is strong for DPPE and DPPA systems; DPPS interacts very strongly with CDP-choline so is not able to form liposomes.

INTRODUCTION

Cytidine-5′-diphosphate choline (CDP-choline), a key intermediate in the biosynthesis of choline phospholipids, is synthesized from phosphorylcholine and cytidine triphosphate (CTP), through the action of cholinephosphate cytidylyltransferase (EC 2.7.7.15). Moreover, CDP-choline can represent one of the precursors of choline, essential for the formation of acetylcholine [1].

Choline demand in the central nervous system is very relevant, so that it must be introduced from the outside, by the diet, as a lipotropic factor [2].

The highly polar nature of CDP-choline (Fig. 1) accounts for its limited ability to pass the blood–brain barrier and for the low amounts which may

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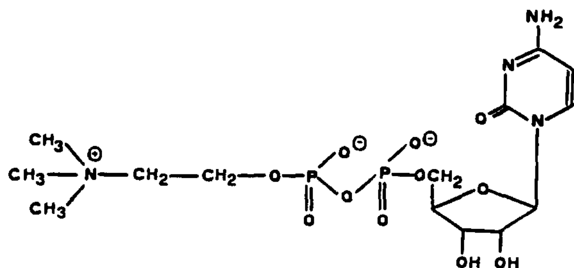

Fig. 1. Chemical structure of CDP-choline.

reach the cerebral phospholipids (about 0.25% of the total administered dose) [3].

Previous work has shown that the limiting factors in CDP-choline therapy were transport through cell membranes [4] and bioavailability [5]. A possible strategy to increase the drug transport across cell membranes could be the preparation of a pharmaceutical formulation able to decrease the hydrophilicity of the drug. Therefore, in this study it is important to clarify both bioavailability and interaction between the drug and cellular membrane. By considering biological membranes as complex structures composed of proteins, acid and neutral phospholipids and a variety of glucosides, it was thought a simple membrane model could be used as phospholipid vesicles.

Studies on liposomes require a good knowledge of the interactions occurring between host and guest, in order to design a perfectly efficient device [6–9]. Differential scanning calorimetry (DSC) is a powerful and non-perturbing thermodynamic technique which can be used to characterize the thermotropic behaviour of the lipid bilayers in model and biological membranes [10]. The presence of drugs in lipid chains, depending on their amphipatic or lipophilic nature, causes variations in the transition temperature of the pure lipid and/or changes in the enthalpy and volume of chain melting [11,12].

In this research we have undertaken to study neutral and charged phospholipid vesicles containing CDP-choline. Phospholipid molecules were selected from among the most abundant components of biological membranes. In particular, keeping the same hydrophobic tail of the lipids (palmitoyl residue), the polar heads were chosen in order to give neutral (phosphatidylcholine and phosphatidylethanolamine) and charged (phosphatidylserine and phosphatidic acid) phospholipids. Our study is divided into two parts: in this paper we study the interaction between CDP-choline and the membrane surface by scanning dilatometric and calorimetric techniques. We are now carrying out experiments to investigate the kinetic fusion phenomenon between CDP-choline and phospholipid vesicles of various compositions, the results of which will be the subject of a future paper.

EXPERIMENTAL

Materials

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE) and dipalmitoylphosphatidic acid (DPPA) were commercial products (Fluka Chemicals Co., Buchs, Switzerland); phosphatidylserine is a commercial product (Sigma Chemicals Co., St. Louis, U.S.A.); the purity was greater than 99% as assayed by two-dimensional thin-layer chromatography on a silica gel plate, loaded with solutions of the lipids in $CHCl_3$ and eluted first with $CHCl_3/CH_3OH/(7\ N)NH_4OH$ (60:3:5, v/v) and successively with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (12:60:8:2.5, v/v). Inorganic salts (NaCl), were recrystallized from twice distilled water and tested for the absence of calcium by atomic absorption. CDP-choline was a gift from Cyanamid-Italia. The purity was greater than 99% by high pressure liquid chromatography.

Preparation of liposomes

Phospholipid vesicles of pure DPPC, DPPE, DPPA and DPPS containing CDP-choline were prepared by the following procedure. Lipids were dissolved in $CH_3OH/CHCl_3$ 1:1 (v:v); then, the solvent was removed at 30 °C on a rotary evaporator by a nitrogen stream, followed by overnight high vacuum storage. Vesicles containing CDP-choline were prepared by adding an aqueous solution of the drug in 0.1 M NaCl to the phospholipid film; this procedure was carried out at a temperature greater than that of their gel–liquid crystal phase transition to allow the full hydration of the samples; thus the DPPC samples were heated at 55 °C, whereas all the other aqueous phospholipid dispersions were heated at 70 °C, then centrifuged twice for 1 min.

For DSC experiments, 120 μl of each sample, containing about 5 mg of lipid, were sealed in an aluminium pan and submitted to analysis. For SD experiments, 600 μl of each sample, containing about 20 mg of lipid, were sealed in a quartz cylinder and analyzed. In these experiments, the scanning procedure was started 1 h after sample preparation in order to obtain a better stabilization of volumes.

Samples for SD tests were always degassed for 10 min at 40 °C with a water-pump before measurements. After each DSC and SD experiment, the total inorganic phosphorus was determined spectrophotometrically [13].

Apparatus

Scanning dilatometry

A Mettler TC 10A processor equipped with a TMA 40 thermomechanical analyzer, previously calibrated for temperature and length with indium

and lauric, caprilic and myristic acids as standards, was used to obtain measurements of length as a function of temperature at constant pressure. A quartz cylinder with a tight but freely movable piston was used as sample holder. Movements of the piston were measured as a length change of the sample.

Further technical details are described in ref. 14.

In these systems, owing to the large mole fraction of water, we corrected for the previously measured thermal expansion of pure electrolyte solution by a computer program. The scanning rate adopted to follow the phase transition was 0.5 °C min^{-1}. The volume change versus temperature was detected both in heating and cooling modes in the 30–80 °C range with a precision of ±0.2 °C.

Differential scanning calorimetry

A Mettler TC 10A processor equipped with a DSC 20 measuring cell was used for calorimetric analysis, after calibration of the temperature and energy, using indium, lauric, capric and myristic acids as standards. The plotting range, as full scale deflection, was set to 1 mW; the noise (r.m.s.) was 0.006 mW. Enthalpy changes were evaluated using the integration program (Bezout method) of an IBM XT computer. Thermograms were detected both in heating and cooling modes in the 30–80 °C range with a precision of ±0.2 °C.

Density measurements

The calculation of sample density was carried out to obtain precise values of the volume change in SD experiments.

Densities were measured with an Anton Paar "vibrating tube" digital density-meter (DMA 602/60). The thermal stability of the liquid, flowing through the jacket around the density measuring cell, was maintained within ±0.001 °C by a Techne Tempette TE-8D temperature controller. The density-meter was calibrated every day with twice distilled water and air. The reproducibility of the density measurements was better than 15 ppm.

RESULTS AND DISCUSSION

Estimation of the interactions between drugs and surface membranes is of primary importance in studies on drug absorption. A very good approach in such a field is the use of artificial membranes, such as MLV vesicles, which also supply efficacious "host–guest" systems to deliver drugs showing some problems in their absorption.

Firstly the very good agreement between the temperature values of the dilatometry experiments and those obtained by calorimetric analysis, especially in heating mode, must be noted.

The SD technique revealed a greater sensitivity towards resolving thermal bands, such as those shown by the DPPE/CDP-choline system, which are sharply split. This event is not easily detectable by DSC measurements, while it is clearly evidenced by the thermodilatometric curves. It is evident that the different phases are characterized by different specific volumes.

In Table 1 we show the temperature, ΔH and ΔV changes linked with the gel–liquid crystal phase transition at various concentrations of CDP-choline for DPPC, DPPE and DPPA systems in heating modes. DPPS values are not shown because no gel–liquid crystal transition was observed for DPPS with CDP-choline at mole fractions of less than 0.1.

From gel to liquid crystal phase transition temperatures it is possible to gain information about the phospholipid–drug interactions and those among the phospholipids.

As a first approximation, the phase transition temperature can be deemed as being influenced by three energetic contributions: (a) hydrogen bond energy among polar heads, (b) reticular energy of hydrophobic chains, both of which contribute to increase the transition temperature, and (c) the electrostatic repulsion energy, which leads to lower temperatures [15]. Thus, the presence in the phospholipid system of an extraneous hydrophilic molecule, which is able to interact with the lipid polar heads creating hydrogen bonds, causes a transition temperature increase; instead, a hydrophobic compound is responsible for the temperature and ΔH decrease, because it can fit into the hydrocarbon matrix.

Gel–liquid crystal transition temperature for the DPPC system does not change greatly (1 °C) either in the absence or in the presence of the highest concentration of the drug (CDP-choline molar fraction, 0.5). This indicates a scarce interaction between the drug and DPPC polar heads. In fact, the choline head cannot form hydrogen bonds with CDP-choline because of the presence of methyl groups linked to the quaternary nitrogen atom. Furthermore, ΔH associated with the gel to liquid-crystal phase transition remains quite constant, demonstrating that CDP-choline, because of its polar nature, is not able to penetrate into the hydrocarbon core of the bilayers. The same trend is shown by the ΔV values associated with the phase transition. This latter evidence confirms our conclusion of a negligible interaction between DPPC and CDP-choline.

On the contrary, calorimetric and volumetric data relative to the DPPE–drug system clearly indicate that a considerable interaction occurs. The gel–liquid-crystal phase transition temperature appears (Table 1) about 4 °C higher in the presence of the drug, as a consequence of hydrogen bonds with DPPE. In this system, a phase segregation has been pointed out: domains of DPPE–CPD-choline aggregates and of unlinked DPPE are formed. The ΔH and ΔV values, related to the DPPE gel–liquid-crystal transition, indicate that CDP-choline is not able to penetrate

TABLE 1

Transition temperatures (T) enthalpy variation (ΔH) and relative volume variations accompanying the transition ($\Delta V / V$) of some MLVs, vesicles at various CDP-choline molar fractions obtained by DSC and SD techniques; these values were obtained in heating modes; Ionic strength was 0.1 M (NaCl)

X_{DRUG}	DPPC [a]			DPPE			DPPA		
	T (°C)	ΔH (kcal mol^{-1})	$\Delta V / V$ (%)	T (°C)	ΔH (kcal mol^{-1})	$\Delta V / V$ (%)	T (°C)	ΔH (kcal mol^{-1})	$\Delta V / V$ (%)
0	41.2	8.6	3.7	61.5 65.2	8.8	9.4	63.1	5.2	4.1
0.1	42.8	9.2	4.0	67.0 65.4	10.3	9.6	66.5	8.7	5.2
0.2	42.9	8.9	4.1	67.2 65.5	10.5	9.9	66.6	9.1	5.3
0.3	43.2	9.4	4.0	67.5 65.6	10.9	10.2	66.8	9.5	5.5
0.4	43.3	9.0	4.2	67.7 65.8	11.3	10.5	66.9	10.0	5.7
0.5	43.3	9.0	4.1	68.0	11.7	10.8	67.1	10.4	5.9

[a] Only in this system has a weak transition associated with the gel $L_{\beta'}$ to rippled $P_{\beta'}$ phase been detected. The corresponding parameters obtained by SD and DSC measurements of such a phase transition are respectively: $T = 35\,°C$; $\Delta V / V = 0.35\%$ and $T = 35\,°C$; $\Delta H = 1.85$ kcal mol^{-1}. This value also remains steady at a higher concentration of drug.

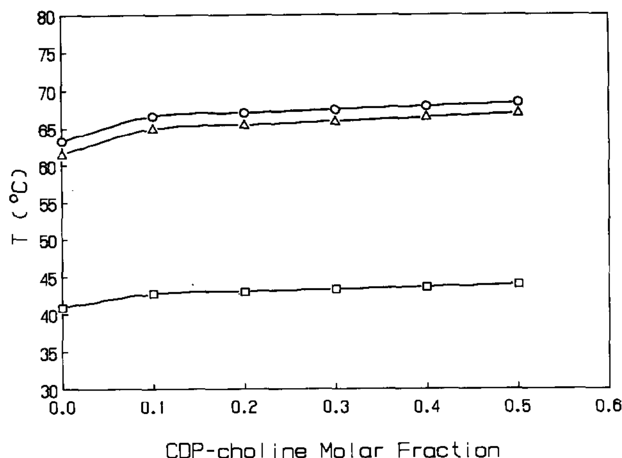

Fig. 2. Temperature of gel to liquid-crystal transition of some phospholipid vesicles in NaCl (0.1 M) versus CDP-choline molar fraction: □, DPPC; ○, DPPA; △ DPPE.

the hydrocarbon core of the bilayers and this limits its capacity to form hydrogen bonds with the phospholipid polar heads.

The DPPA–CDP-choline system shows the same rise of gel–liquid-crystal phase transition temperature as DPPE, but no phase segregation. Enthalpy linked to the gel–liquid-crystal transition increases markedly, whereas the ΔV values undergo just a modest enhancement. DPPA, like DPPE, can form hydrogen bonds with CDP-choline. The scant increase in ΔV may be due to a shielding effect of CDP-choline on the negative charge of the phosphorous group and to the small size of the DPPA polar head.

For all three phospholipids now analyzed, temperature, enthalpy and volume changes associated with the gel to liquid crystal transition increase in line with increasing CDP-choline concentration (Figs. 2 and 3).

Such an increase is more marked for DPPE, is less for DPPA and almost negligible for DPPC. It could be ascribed to the capacity to form hydrogen bonds of different strength. Also the volume changes, owing to the lateral phospholipid expansion, can be influenced by several factors: a positive volumetric variation is due to the enhancement of "gauche" conformers in the hydrocarbon chains, and to the electrostatic and steric repulsion forces; a negative factor is instead due to the higher solvation rate (as an effect of the electrostriction). As shown in Table 1, the volume change of DPPA, which has a small and negatively charged polar head, is comparable to that of DPPC, which presents a bigger and zwitterionic (choline) polar head.

The observed trend can be justified by considering the importance of the different factors: in fact, the electrostatic repulsion contributes more in the case of DPPA than DPPC; it is now known from the literature that the –OH group gives a relevant electrostriction effect (partial molar volume (p.m.v.) = -4.04 cm^3 mol^{-1}) [16] with respect to choline (p.m.v. = $+79.0$

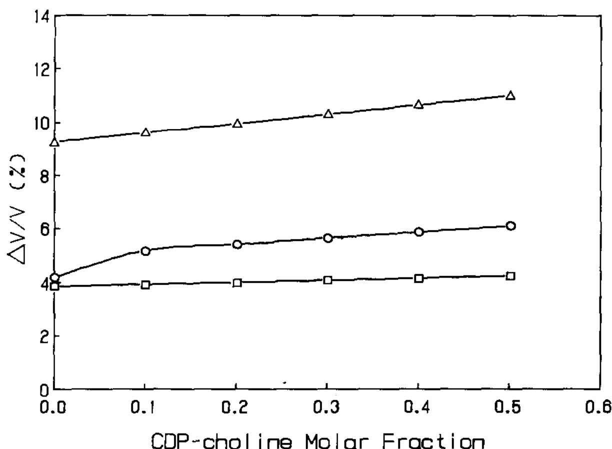

Fig. 3. Relative volume variation (%) consequent upon gel–liquid crystal transition versus CDP-choline molar fraction. Ionic medium was 0.1 M NaCl. □, DPPC; ○, DPPA; △, DPPE.

cm^3 mol^{-1}) [17]. Thus, the change of reticular distance after gel to liquid-crystal phase transition favours greater solvation of polar heads, with a volume reduction in DPPA.

From the balance of these contributions we can explain the likeness between the two volume changes of the transition. The high solvation degree of DPPA also influences the specific volume in the gel state, which is 4.5% higher than that of DPPC; moreover, DPPC gel to liquid-crystal transition is more cooperative, as is shown by the half-height SD peak widths (2.00 °C for DPPA and 0.75 °C for DPPC). All these factors can explain the small volume and enthalpy variations during the phase transition of a polar system which is able to form hydrogen bonds.

The DPPE volume change is 50% greater than that of DPPC. Such a difference is essentially due to the presence of hydrogen linkages which give a positive contribution to the transition volume variation.

On the contrary, DPPS, even if negatively charged, does not have a similar high degree of solvation (serine p.m.v. = 60.62 cm^3 mol^{-1} [18]; thus, the more determinant term in volume variation is the electrostatic repulsion.

The last examined phospholipid, DPPS, shows at CDP-choline mole fraction of 0.08 a broad and low DSC peak (half-height width of 4 °C) centred at 67.9 °C, with ΔH of 1.47 kcal mol^{-1}. Furthermore, the volume variation of the phase transition is 2.1%, while the transition temperature (68.0 °C) appears to be in very good agreement with DSC data.

Comparison of these results with the values for pure DPPS ($T = 60.8$ °C; $\Delta H = 9.10$ kcal mol^{-1}; $\Delta V = 20.0\%$) [19], shows that strong interactions exist between the lipid and CDP-choline; in fact, the small ΔH and ΔV

TABLE 2

Isobaric expansion coefficient α (°C) of some MLV vesicles at various molar fractions obtained by SD measurements; these values were obtained in heating modes; ionic strength was 0.1 M (NaCl)

X_{DRUG}	DPPC			DPPE		DPPA	
	αL_β	$\alpha P_{\beta'}$	αL_α	αL_β	αL_α	αL_β	αL_α
0	85.1	137.0	104.5	490.0	611.1	27.7	50.6
0.1	80.0	122.3	98.0	340.5	537.0	20.5	46.2
0.2	82.4	120.9	99.2	335.2	531.0	18.4	45.0
0.3	81.0	121.2	99.0	330.0	529.2	17.5	43.8
0.4	80.2	122.0	99.3	326.7	521.7	16.7	42.2
0.5	80.5	122.3	99.5	321.1	514.1	16.0	40.8

values are indicative of a scarcely cooperative system. Thus, any possibility for CDP-choline solubilizing itself in the hydrophobic bilayer of DPPS can be excluded and we can hypothesize the formation of very small vesicles with low radius of curvature.

The thermal dilation of a lipidic bilayer can be associated with the appearance of "holes" within the pseudo-crystalline reticle of the phospholipid molecules; since the formation of every "hole" is coupled with an increase of volume and then, proportionally to the lipid size, the volume variations are correlated with the enhancement of the number of "holes". An additional factor is the population relative to the various conformational lipidic tail structures, which can exist at the different temperatures.

All these considerations justified the small value of the linear expansion coefficient (α) (Table 2) for DPPA and the higher values for DPPC and DPPE. In fact, DPPA cannot give many conformational variations with its polar head, whereas DPPC, with a big polar head (about 70 Å) [20], can increase its conformational degrees of freedom. DPPE is able to form lipid–lipid hydrogen bonds with consequent decrease of conformational mobility and permit penetration of water into the bilayer surface, with a consequent enlargement of the polar head volume.

The DPPC–CDP-choline α value does not change appreciably during the three mesophases (L_β, P_β, and L_α), confirming the almost total absence of interactions. On the contrary, DPPE- and DPPA–CDP-choline systems, during the phase change, and also in relation to the CDP-choline concentration, show marked α value variations. This proves that the drug is adsorbed upon the membrane surface, even at high concentrations.

The thermal expansion coefficient of DPPS–CDP-choline system is about 5.2×10^{-5} °C^{-1}, whereas DPPS MLV vesicles supply a value of 4.2×10^{-3} °C^{-1}. The volumetric behaviour of DPPS is very strange both with and without drug. Previous measurements have shown that the DPPS α value is temperature dependent in exponential mode [19]; unfortunately,

it is impossible to explain this behaviour from thermodynamic data only. When CDP-choline is added the exponential temperature dependence of α values decreases. These values are not reported here because DPPS mixed with CDP-choline does not form liposome structures.

From the experimental data we deduce that the most suitable phospholipid for the CDP-choline delivery system should be DPPA.

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