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Different effects of two structurally similar carotenoids, lutein and β-carotene, on the thermotropic behaviour of phosphatidylcholine liposomes. Calorimetric evidence of their hindered transport through biomembranes

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Abstract

The effect exerted by two structurally similar carotenoids, lutein and β-carotene, possessing antioxidant activity on the thermotropic behavior of model membranes constituted by dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles, was studied by differential scanning calorimetry (DSC). Attention was directed to evaluating eventual modifications in drug–lipid interaction induced by drug structure.

The two compounds examined, when dispersed in liposomes during their preparation, were found to modify the gel-to-liquid crystal phase transition of DMPC vesicles differently. Only the lutein caused a large effect on the transition temperature (T_m), shifting it toward lower values and decreasing the enthalpy (ΔH) associated with the $L_\beta - L_\alpha$ lipidic phase transition. These modifications were a function of the drug concentration, and modulated by the different polarity induced by the presence of hydroxyl groups in the terminal aromatic rings.

By carrying out a different liposomes loading procedure, namely putting solid carotenoids and MLV or LUV aqueous dispersions in contact and leaving these mixtures for long incubation times at temperatures higher than transitional lipid temperatures, no interaction was detected for both the carotenoids with lipid vesicles, implying a hindered transfer of the carotenoids through the aqueous medium. Also, the drugs transfer from carotenoid-loaded MLV to empty vesicles was studied and no effect was detected on the empty membranes. The results can be explained in terms of compound hydrophobicity which allows the membrane interaction with carotenoids and, at the same time, a low water solubility which avoids the transfer through the aqueous medium as well as the matching of the hydrocarbons chains of the carotenoid between lipid bilayer chains, suggesting a relation between drugs structure, bilayer thickness and membrane interaction.

Keywords: Phosphatidylcholine; Differential scanning calorimetry; Membranes; Lutein; Carotenoids

1. Introduction

A major class of compounds found in all fruits and vegetables are the carotenoids, which are powerful antioxidants. Between these classes of compounds are β-carotene, lutein and zeaxanthin. Lutein is the most prevalent carotenoid, found in several vegetables (2–4 times that of β-carotene) and has been shown to be an important antioxidant that is more effective, compared to other carotenoids, in inhibiting lipid peroxidation (one of the oxidation process that occurs in the human

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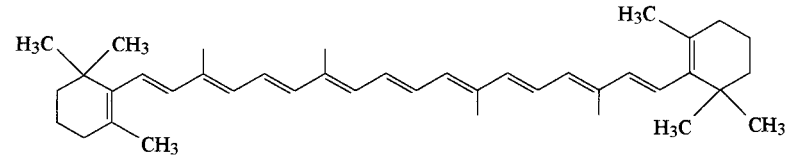
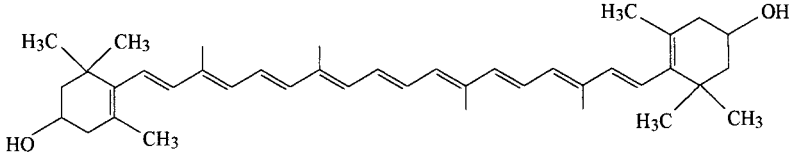


(A)



(B)

Fig. 1. Structural formula of β-carotene (A) and lutein (B).

serum and in the eye) [1]. Lutein has been found to have specific applications for the eye; lutein and its isomer, zeaxanthin, are the only two carotenoids found in the eye. The body absorbs lutein from foods and then specifically deposits it in two different parts of the eye: the region of the retina called macula and the lens of the eye [2,3]. Several studies related the dietary-rich composition in lutein with lower prevalence of the age-related diseases, namely macular degeneration and cataract formation [4–6]. The carotenoids play different roles in living cells, their function is explained in terms of membrane reinforcement, presenting affinity with cholesterol [7–9]. The interaction and the organization of carotenoids in membrane raised an interest, and studies were carried out investigating the effect of zeaxanthin on the thickness of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine vesicles used as membrane model [8,10,11]. Several techniques have been employed to carry out studies on the effect of increasing incorporation of carotene, zeaxanthin [7,8] and retinoids [9] on the phosphatidylcholine membrane. One of these techniques was the differential scanning calorimetry (DSC), a non-perturbative tool that permits study of the effect exerted by a substance on the well-known phase transition gel-to-liquid crystal exhibited by a phospholipid species. In fact, when an amphiphilic substance is dissolved in the lipidic sea, it acts as a solute in a solvent and a decrease in the phase-transition temperature can be introduced. This interaction is related to the amount of drug dissolved in the lipidic structure as well as to the matching of the length of the phospholipid with respect to dissolved molecules and its hydrophobicity. By the DSC method, it is also possible to monitor the eventual uptake process of a solid compound on a model membrane surface, processes which are modulated by drug lipophilicity, solubility in water, phospholipid membrane composition and phase separation presence [12,13].

Previously, we described the interaction of carotenoid derivatives, retinol, retinal and retinoic acids with dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles [14,15] putting in evidence, by DSC studies, that lipophilicity of these compounds combined with the polar groups can influence their effect on the thermotropic behaviour of the model membrane.

Thus, in the present work, we planned to investigate the possible effects of such structural differences between carotenoid molecules (compounds shown in Fig. 1) on their interaction with DMPC vesicles.

To study this interaction, we have used the L-α-dimyristoylphosphatidylcholine vesicles (multilamellar, MLV, and unilamellar, LUV, vesicles) as a synthetic simplified model membrane, which show a change of their thermotropic behaviour if other mole-

cules are dissolved in their ordered structure (see Refs. [16–19]).

On comparing the results obtained by 'classical' carotenoid–membrane interaction (interaction in organic solvent before multilamellar vesicles preparation) with kinetic experiments of the transfer of compounds to empty membranes, directly or mediated by β-cyclodextrin, the differences in the drug facility to interact and penetrate the lipid bilayer of bio-membranes, causing variations in their structure and fluidity, should be detectable. In this way, it is possible to examine the steric hindrance and electronic properties, which are known to modulate the interaction and/or penetration of foreign molecules into cell membranes as well as the lipophilicity, which should be more involved in such penetration phenomena.

The results should then give useful indications to an understanding of the role of substituents present in the drug molecule during interaction with the microenvironment of the lipid bilayer, causing also a variation in their transport across natural membranes.

2. Materials and methods

2.1. Chemicals

Synthetic L-α-dimyristoylphosphatidylcholine was obtained from Fluka (Buchs, Switzerland). Solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). Lipid concentrations were determined by phosphorus analysis by the method of Bartlett [20].

Buffer, consisting in Tris (50 mM) was adjusted to pH 7.4 with HCl. Lutein was obtained from Extrasynthese (France), Fluka supplied β-carotene and both were checked by TLC on silica gel.

2.2. Liposomes preparation

Multilamellar liposomes were prepared in the presence, and absence, of carotenoids following the usual procedure. Chloroform-methanol (1 : 1, v : v) stock solutions of lipid and drugs were mixed to obtain the chosen mole fraction of drugs. The solvents were removed under a nitrogen flow and the resulting film was freeze-dried to remove the residual solvents. Liposomes were prepared by adding 50 mM Tris buffer (pH 7.4) to the film, then heating at 37°C, a temperature above that of the gel–liquid crystalline phase transition, and vortexing thrice for 1 min.

The samples were shaken for 1 h in a water bath at 37°C to homogenize the liposomes. Afterwards, aliquots of 120 μl (4.5 mg of lipid) were transferred in a 150 μl DSC aluminium pan, sealed, and submitted to DSC analysis.

2.3. Differential scanning calorimetry

DSC was performed by using a Mettler TA 4000 system equipped with a DSC-30 cell and a TC-11 processor. The scan rate employed was 2°C/min in the 2–37°C range. The sensitivity was 1.72 mW, and the reference pan was filled with Tris buffer solution. After the factory-suggested calibration of the calorimetric system, a successive fine, in a narrow range, calibration of temperature was carried out by using indium, stearic acid and water. Indium was employed to calibrate the transitional enthalpies (ΔH). Temperature and enthalpies were checked also by using palmitic acid. Enthalpies were evaluated from the peak areas using the integration programme of the TC11 processor or by using the software Mettler TA72, permitting choice of different baselines and ranges of integration. The areas calculated with these different methods lie within the experimental error (±5%).

The samples were cooled and heated four times to achieve the reproducibility of results. All samples, after calorimetric scans, were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorus assay.

2.4. Permeation experiments

In order to study the capacity of the two carotenoids to permeate the model membrane, a kinetic experiment was carried out by putting in contact the DMPC aqueous suspension (MLV or LUV) with a fixed amount of finely powdered insoluble drug (0.12 molar fraction) placed in the bottom of the DSC crucible. The samples, after gently shaking for 10 s, were submitted to different calorimetric cycles in heating, isothermal and cooling mode by using the following step procedure.

1. a first scan between two and 37°C, to detect the degree of interaction between the compound,

eventually transferred, and the model membrane;
2. an isothermal period of 1 h at 37°C to permit to the drug to permeate (if able) the lipid layer/s staying in a disordered state at a temperature over the lipid transitional temperature; and
3. a cooling scan from 37 to 2°C before restarting the heating programme.

This procedure was run several times to detect variations in the temperature of the calorimetric peak (signal of drug partitioning between water and lipid membrane).

2.5. Transmembrane transport experiments

To examine the possibility of a transmembrane transport of the compounds the same procedure as reported before was applied to mixed aliquots (60 μl each, 2.25 mg of phospholipid) of 0.12 molar fraction loaded liposomes with empty liposomes. In this way, the transfer from loaded liposomes to empty vesicles will be followed by the relative T_m shifts of the calorimetric peaks. The experiments were also carried out in the presence of β-cyclodextrin, with an equimolar amount to the carotenoid present in the loaded vesicles, to investigate the eventual capacity of this complexing agent to extract carotenoid molecules inserted in the lipidic bilayer and modulating their transfer through the aqueous medium to empty liposomes.

3. Results and discussions

Multilayered DMPC vesicles were chosen as an experimental model to assess the capacity of the examined compounds (Fig. 1) to interact with a biomembrane model in relation to their lipophilicity, arising because of the presence of OH group substituents in the position 3,3′-. It is noteworthy that this 'three-dimensional' model can more widely resemble the micro-environment of a cell membrane, and it has already been shown to give better correlation with biological data than the classical two-solvent system.

In Fig. 2(A and B), we report the calorimetric heating curves of DMPC liposomes in the presence of different molar fractions of β-carotene and lutein, respectively. There are obvious differences between the two examined carotenoids in the figure. Only lutein seems to deeply interact with DMPC bilayers causing, by increasing the amount of lutein, an increasing shift of the transitional temperature (T_m)

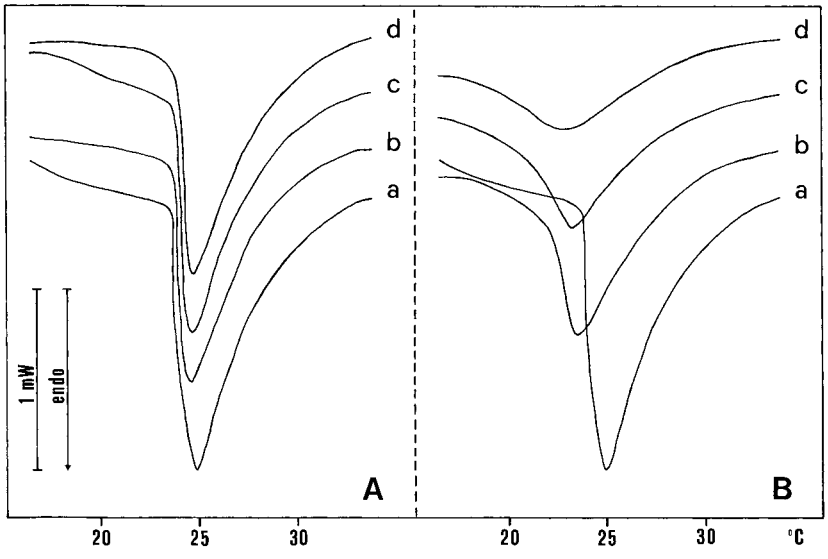


Fig. 2. Differential scanning calorimetry heating curves of hydrated DMPC containing: (A) β-carotene and (B) lutein, obtained from organic solvent solutions, at a drug molar fraction of: a, 0; b, 0.03; c, 0.06; and d, 0.12.

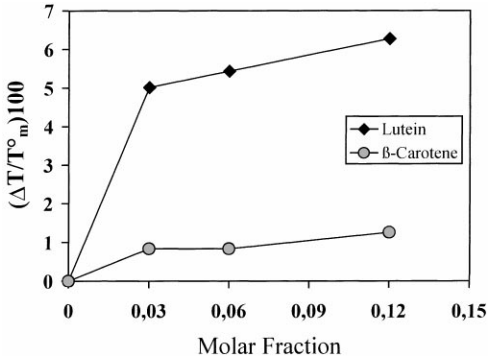


Fig. 3. Transition temperature ($\Delta T/T_m^0$) values (average of at least four runs), in heating mode, as a function of mole fraction of carotenoid.

of the calorimetric peak associated with the well-known gel-to-liquid crystal phase transition typical for DMPC multilayers (Fig. 2(B), curves a–c, Fig. 3 and Table 1). The enthalpy change (ΔH), related to the peak area, was also affected (Table 1). On the other hand, β-carotene has only a small effect on T_m and ΔH data, as is evident from the calorimetric curves (Fig. 2(A)).

This behaviour should find justification from a consideration of the formulae of the compounds. The presence of different polar groups at the end of a very long apolar all-*trans* chain should permit the lutein collocation through the lipidic layer in opposition to the β-carotene. These results are in agreement with the literature, whence it is evident that the lutein isomer, zeaxanthin, is incorporated in liposomial vesicles when prepared using carotenoid–phospholipid mixtures submitted to sonication during the preparation and the finding that carotenoids are more easily incorporated in DMPC for reasons of steric mismatching. Its length is closer to DMPC double layer than to DPPC [9,21], suggesting the positioning of zeaxanthin in a lipidic bilayer. Suggestions on the different collocation of β-carotene and zeaxanthin in a lipidic bilayer were also made [10,11] concluding as their position, with respect to the polyenic chains, oriented parallel or normal to the lipids lamellar plane, its concentration playing a great role in the lipidic packing by exerting a disturbing action on the order of lipidic chains only for low concentrations. For both the carotenoids, a phase separation in carotenoid-lipidic ideal solution and a crystalline carotenoid phase also seem to occur [10].

Table 1
Main transition peak temperature (T_m, °C) and enthalpy changes (ΔH, J/g) of DMPC dispersions at different molar fractions of carotenoids

Molar fractions	Lutein		β-Carotene	
	T_m	ΔH	T_m	ΔH
0.0	23.9	34.6	23.9	34.6
0.03	22.7	27.2	23.7	34.4
0.06	22.6	25.4	23.7	34.9
0.12	22.4	16.1	23.6	35.0

The interaction between drugs and DMPC liposomes was largely explained by us, as in Refs. [16,22–28], in terms of a 'fluidizing' effect due to the introduction of lipophilic drug molecules into the ordered structure of the lipidic bilayer. Drug molecules act as spacers in such a structure, causing a destabilization of the lipid mosaic with a decrease in T_m of the gel-to-liquid crystal phase transition. While a negligible variation in ΔH is explained as a superficial interaction between amphiphatic molecules and DMPC polar heads, occurring only at the surface of lipid layers without deeply involving the acyl chains, variations in ΔH are due to a variation in the cooperativity of the acyl chains. The presence of different substituents in the backbone structure should influence the incorporation. But the interaction of molecules, like the carotenoids, possessing a very long acylic chain is more complex than a simple interaction with a low amphiphatic drug. In fact, while the carotene, being a totally hydrophobic molecule, is compelled to place itself with an arrangement parallel to the bilayers lamellae, lutein – like zeaxanthin – is able to traverse the double lipidic layer anchoring the hydroxyl groups to the polar heads of phospholipids remaining immobilized inside the lipidic layer [8–11]. This interaction was found, for zeaxanthin, to be related to the matching with the lipidic bilayer length, which will change by passing from the DMPC to the DPPC as well as by passing through the transitional temperature of the bilayer. The length of a bilayer is deeply influenced by the phase (gel or liquid crystal) of the lipids modifying the depth of the bilayer; there was evidence of the different tilting angle assumed by carotenoids derivatives in the phospholipid packing

depending on the phase and the molar fraction of carotenoid present. The possibility of autoaggregation of the carotenoid molecules should to be taken into account. In fact, over an experimental carotenoids concentration value, it is possible that molecules are withdrawn from the lipidic dispersion to form aggregates, resulting in a nearly insensible variation of the transitional temperature (without any further effect on the depression of the phase transition on the model membrane) [10].

From the 'kinetic' measures to try to transfer the two compounds from the solid through the aqueous medium to LUV or MLV vesicles (data not reported), it was evident that both samples could not be extracted by the aqueous medium dissolving in the lipidic bilayer even after several heating and isothermal cycles. The detection of any effect on T_m and ΔH of the lipidic phase transition suggests the difficulty in transferring to the vesicles by a probable micellar formation in the aqueous medium.

Fig. 4 presents a comparison of the calorimetric curves of pure DMPC (a), DMPC charged with 0.12 molar fraction of lutein put in touch with empty DMPC vesicles (b) and in the presence of β-cyclodextrin (c), and the effect of 0.12 molar fraction of Lutein on DMPC vesicles (d). The corresponding curves obtained for the DMPC/β-carotene sample mixed with pure DMPC as well as in the presence of β-cyclodextrin are not shown because no effect was detectable. It appears evident that both the samples, lutein charged and empty DMPC vesicles put in touch with each other, maintain their identity showing a calorimetric peak with two components: the first at a lower temperature to be assigned to DMPC charged with lutein and the other to a higher temperature to the pure DMPC (Fig. 4 curve b). This behaviour also remains constant after several incubation periods at temperatures higher than the lipid transition temperature in order to facilitate an eventual lutein exchange. When the sample, like that showing the curve b, was prepared in the presence of an amount of β-cyclodextrin equal to the lutein content (Fig. 4 curve c) only a reduction of the peak of lutein-rich vesicles was observed with a concomitant increase of the pure phospholipid peak meaning thereby that cyclodextrin was able to extract the lutein but was not active in bringing about its transport to the surrounding empty vesicles.

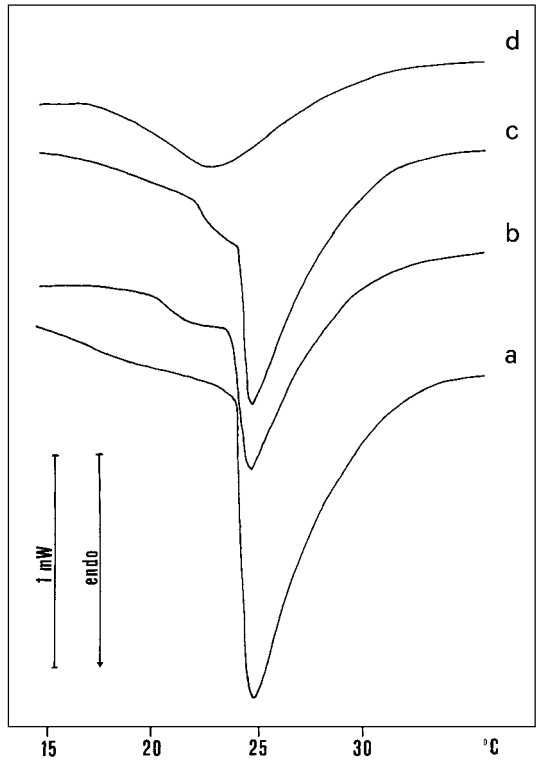


Fig. 4. Differential scanning calorimetry heating curves of (a) hydrated pure DMPC; (b) hydrated pure DMPC left in contact of DMPC lutein-charged liposomes (X=0.12) after 4 h of incubation at 37°C: (c) same sample as 'b', but in the presence of β-cyclodextrin in amounts equal to the lutein content; and (d) hydrated pure DMPC lutein-charged liposomes (X=0.12).

4. Conclusions

The aim of this research was twofold:

(A) to demonstrate that lutein, like zeaxanthin, is able to interact with a biological membrane, being able to find collocation inside the lipidic bilayer because of its length and terminal polar groups which matches the double layer DMPC length;
(B) to suggest that lutein transports through the human body, as is shown by increasing the amount of lutein in the intake of food, could occur not by a simple passive transport through biological membranes, but a transport mediated by carriers. In fact, the unsuccessful kinetics attempts to transfer the examined carotenoids from a solid form or from charged phospholipids (X=0.12 of lutein) to

empty membranes, it appears evident that lutein entrapped in the lipid bilayer prefers to stay there instead of being transferred to another empty membrane; also in view of the high lipophilicity, the carotenoid molecule is unable to diffuse in the aqueous medium from the solid form to reach the DMPC model membrane and to fluidize it. Besides, carriers like β-cyclodextrin, well known for their capacity to increase the solubility of poor soluble molecules, are unable to transport the lutein near the membrane and release it inside, even if it seems able to extract lutein from the DMPC charged membranes.

We can stress the importance of the calorimetric technique to detect indirectly transport processes trying to clarify the factors influencing the possibility to absorb a substance by the biological membranes.

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