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Interaction of lutein with phosphatidylcholine bilayers

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The interaction of lutein, an inhibitory uncoupler of mitochondrial oxidative phosphorylation, with phospholipid bilayers has been examined employing a variety of physical methods. Differential calorimetric scans revealed that incorporation of lutein into aqueous dispersions of dipalmitoylphosphatidylcholine broadened the main transition endotherm and decreased the transition enthalpy as well as the size of the cooperative unit without change in the temperature of transition. Permeability of the bilayer to ascorbate was enhanced significantly by the presence of lutein. The trapped volume of the vesicles was decreased. Incorporation of lutein broadened the NMR peaks of both the acyl side-chain methylene and terminal methyl protons without any change in the line-width of the choline head-group methyl proton signal. This indicated the ability of the compound to integrate deep into the hydrophobic regions of the phospholipid bilayer distal to the polar regions. These results support our earlier biochemical studies which revealed that the deleterious action of lutein on mitochondrial oxidative phosphorylation could be due to its ability to interact with membrane components and perturb the structure.

Introduction

There is increasing evidence that interaction between the two energy-transducing organelles of higher plants, namely the chloroplast and the mitochondrion, plays an important role in the regulation of energy metabolism [1–5]. In conformity with this, we reported recently that chloroplasts uncouple mitochondrial oxidative phosphorylation [6]. A factor with uncoupling and inhibitory actions on oxidative phosphorylation was purified from chloroplasts and identified as the xanthophyll lutein [7]. Lutein is a lipophile containing two hydroxylated asymmetric β-ionone rings bridged by a long chain of four isoprene units [8].

Purified preparations of the factor showed a tendency to bind firmly to mitochondria and to induce swelling. It stimulated the dormant ATPase of tightly coupled mitochondria for which the presence of Mg^{2+} in the reaction systems was obligatory [6,7]. These properties suggested that disruption of membrane organization may hold the key to the mode of action of the compound. This aspect merited deeper study. The mitochondrial membrane which is composed of a great variety of proteins and lipids is too complex to be amenable to exploratory studies. Therefore, as a first step, we have opted for the simpler model of dipalmitoylphosphatidylcholine (DPPC) vesicles for gleaning basic information on the interaction of the factor with bilayers of membranes. For the study, DPPC was chosen because it is the most used and best-characterized vesicular system [9]. Moreover, phosphatidylcholine is the predominant

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Abbreviations: DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; 5-SASL, *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; $t_{1/2}$, half-life; MLV, multilamellar vesicles; n, cooperative unit; T_m, temperature of phase transition.

lipid in the mitochondrial membrane.

It is well-recognized that the phase state of component lipids plays a major role in the control of membrane-mediated functions [10]. The influence of lutein on the themrotropic phase behaviour of aqueous dispersions of DPPC was investigated by differential scanning calorimetry and electron spin resonance. Proton-NMR was used to visualize the interaction of lutein with acyl side-chains. Alterations in the permeability profile of the vesicles were also monitored. The data presented in this paper reveal that the compound penetrates deep into the hydrophobic domains of the bilayer, disrupting cooperative interactions among the acyl side-chains of phospholipids and increasing permeability.

Materials and Methods

Materials

The phospholipid (DPPC) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and the spin labels (TEMPO and 5-SASL) from Syva Research Chemicals, U.S.A. The uncoupling factor (lutein) was isolated in pure form from alfalfa (*Medicago sativa*) chloroplasts as described earlier [7]. The purity was confirmed by high-performance liquid chromatography. In view of its unstable nature, fresh preparations of the compound were used for experiments as far as possible. All other chemicals used were of the purest grade available. Solutions were prepared in water double-distilled in an all-quartz apparatus.

Methods

Scanning calorimetry. Multilamellar vesicles of DPPC were prepared by dispersing 15 mg of the lipid as a thin film in 0.5 ml of double-distilled, deionized water. Dispersion was achieved by agitation on a vortex mixer for a few minutes after heating to 50–55°C [11]. Incorporation of lutein into the vesicles was achieved by adding a solution in chloroform at the time of formation of the lipid thin film.

Thermograms were obtained using a Perkin-Elmer DSC-20 scanning calorimeter at a scanning speed of 2.5 Cdeg/min and a sensitivity of 1 mcal/s. The sample of MLV (10 μl) was hermetically sealed in an aluminium sample pan. Indium and cyclohexane were used as standards for calibration. Change in enthalpy (ΔH) was calculated from the area of the transition curves. An approximate size of the cooperative unit (n) was calculated from the equation

$$n = \frac{6.9 T_m^2}{(\Delta T_{m1/2} \times \Delta H)}$$

where T_m is the transition temperature, $\Delta T_{m1/2}$ the transition width at half-height and ΔH the transition enthalpy [12–14]. Analysis of the samples by thin-layer chromatography confirmed that no decomposition of the phospholipid or the xanthophyll had taken place during the runs.

Nuclear magnetic resonance. From MLV dispersed in H_2O, unilamellar vesicles were prepared by sonic disruption with a Branson B-12 sonifier fitted with a microtip (25 min, power output 60%; temperature 45°C). The slightly turbid sonicate was centrifuged in a Beckman L5-50 ultracentrifuge at 100 000 × g for 30 min to remove any multilamellar structures present [15]. A vesicular diameter of 26 nm was obtained by the lanthanide shift reagent method [16,17]. That sonic disruption had not produced any chemical degradation was confirmed by thin-layer chromatography [18].

Proton NMR spectra were recorded on a Bruker WH-270 spectrometer operating at 50°C in the Fourier transform mode at 270 MHz. Field frequency lock was provided by the deuterium signal of solvent ^2H_2O.

Electron spin resonance. Vesicles were prepared in 10 mM phosphate buffer, pH 7.4. The spin label was incorporated at a molar ratio of 100 (lipid) : 1 (probe) and 50 : 1 for TEMPO and 5-SASL, respectively. Spectra were recorded in an X-band ESR spectrometer with a Varian 12 inch magnet and associated field dial and a VFR 2501 power supply coupled to a Varian V-45601, 100 kHz field modulation and detection unit. Temperatures were controlled to ±1 Cdeg by a Varian V-4540 regulator.

Phase transition was monitored by the temperature-dependent changes in TEMPO partition in the MLV bilayer as given by the parameter 'f' which was calculated as

$$f = \frac{H}{H + P}$$

where H and P were the heights of the high-field signal in lipid and aqueous phase, respectively [19,20]. The permeability of unilamellar vesicles to ascorbate was assessed by monitoring the kinetics of decay of the ESR signal of 5-SASL on addition of 20 mM ascorbate at 45°C.

Trapped volume. Vesicles were made in 0.1 M acetate buffer, pH 5.4, in the presence of 0.5 M $K_3Fe(CN)_6$. After incubation at 45°C for 3 h, the preparation was filtered through a Sephadex G-50 column (1 × 28 cm) using the buffer. Fractions containing the vesicles were pooled and the trapped $K_3Fe(CN)_6$ was released by the addition of *n*-propanol (40% v/v) and estimated by absorbance at 420 nm [21]. Absorbance was recorded with a Shimadzu UV-200S spectrophotometer. The concentration of lipid was estimated by the phosphorus method [22]. A high concentration of ferricyanide was used to eliminate any error due to binding to the phospholipid [23]. In all experiments, incorporation of lutein into the liposomes was monitored by gel filtration and chromatography.

Results

Scanning calorimetry

Calorimetric heating scans of DPPC vesicles containing increasing concentrations of lutein are depicted in Fig. 1. In the absence of lutein, the thermogram is characterized by a sharp peak at 41.5°C, signifying the gel to liquid-crystalline phase transition of the phospholipid, and a pre-transition peak at 34.5°C [24]. The enthalpy change (8.66 kcal/mol) and the half-height width (0.4 Cdeg) also agree well with the reported values [25]. Incorporation of lutein causes the main endotherm to broaden and decrease in height without any significant change in the midpoint of the transition (Table I). Pre-transition is abolished at the lowest concentration of lutein used.

The decrease in ΔH with increase in the molar ratio of lutein was linear and extrapolation of the regression line (correlation coefficient 0.998) indicated that the transition would be abolished ($\Delta H = 0$) at 2 mol% of lutein (data not shown). The broadening of the endotherm indicates that the factor decreases the cooperativity of transition [12,13,26] The effect is quite significant, even the lowest concentration shrinking the size of the co-operative unit to one-third (Table I).

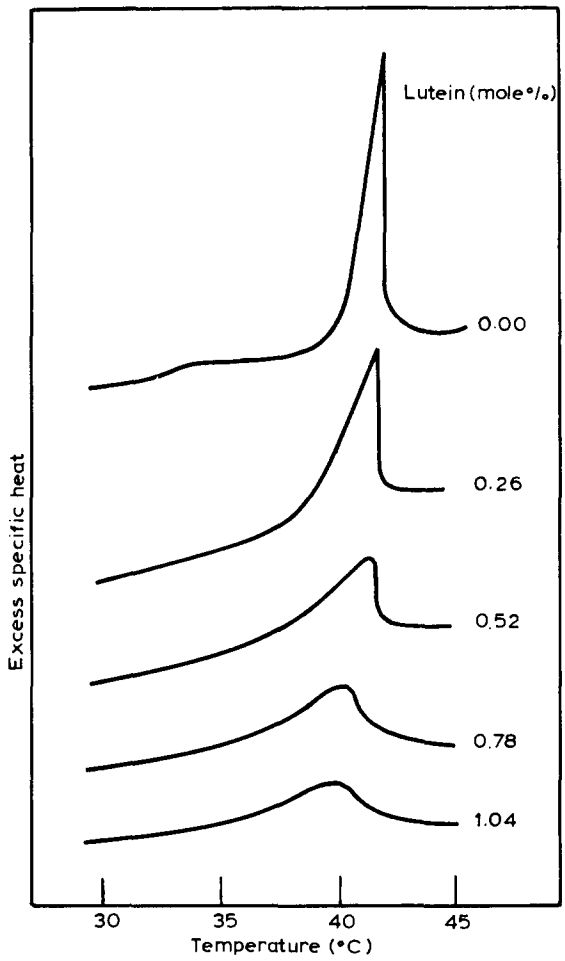

Fig. 1. Effect of incorporation of lutein on the thermotropic transition of multilamellar vesicles of DPPC. Differential calorimetric scans of DPPC multilayers in excess water with increasing concentrations of lutein are shown. For each scan 400 nmol DPPC were used. Other details are given in Materials and Methods.

Electron spin resonance

A plot of the TEMPO partition parameter 'f' as a function of temperature (Fig. 2) shows that the incorporation of lutein into MLV changes the amplitude of phase transition without change in T_m (41°C). This is in close agreement with the results obtained in calorimetry.

The effect of the factor on the permeability of unilamellar vesicles to ascorbate as monitored by

TABLE I

EFFECT OF INCORPORATION OF LUTEIN ON THE THERMOTROPIC TRANSITION OF MULTILAMELLAR VESICLES OF DPPC

The temperatures of pre-transition, main transition (T_m) and full width at half maximum of main transition ($T_{m1/2}$) are given. The size of the cooperative unit (n) and enthalpy change (ΔH) were calculated as described in the text.

Lutein (mol%)	Pretransition (°C)	Main transition T_m (°C)	$T_{m1/2}$ (°C)	Enthalpy change ΔH (kcal/mol)	Cooperative unit (n)
0.00	34.5	41.5	0.4	8.66	197
0.26	–	41.5	1.5	7.33	62
0.52	–	41.0	1.9	5.97	60
0.78	–	41.0	2.3	4.88	60
1.04	–	41.0	3.0	4.03	56

the kinetics of reduction of the bilayer-embedded 5-SASL is presented in Fig. 3. As expected, the reduction follows a biphasic pattern, the initial faster and the subsequent slower phases corresponding to the rate of diffusion of ascorbate into the outer and inner monolayers, respectively [27,28]. Incorporation of lutein increases the rate of reduction in both layers. This is clearly shown

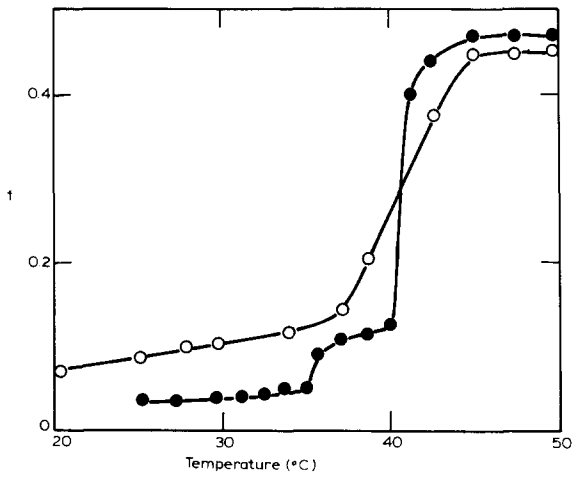

Fig. 2. Effect of incorporation of lutein on the partition of TEMPO into multilamellar vesicles of DPPC. The order parameters 'f' calculated from ESR signal heights of TEMPO incorporated into multilamellar vesicles of DPPC in the absence (●) and presence of 0.44 mol% of lutein (○) are given as a function of temperature. Other details are given in Materials and Methods.

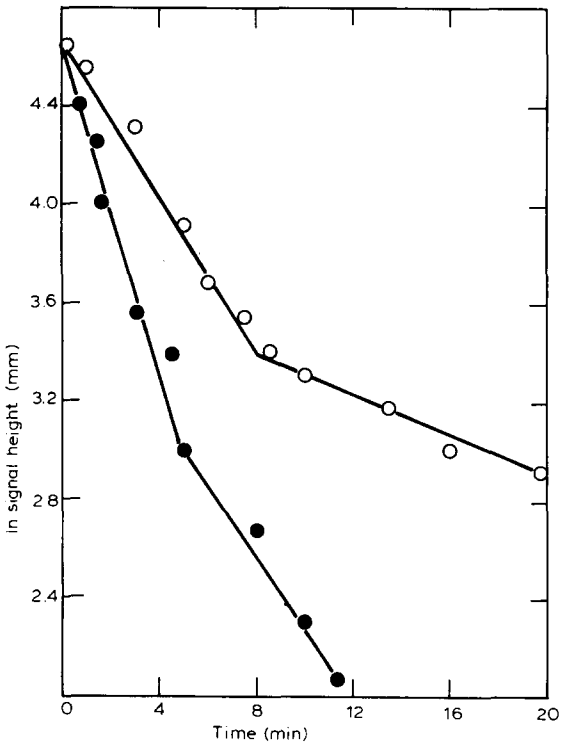

Fig. 3. Effect of incorporation of lutein on the permeability of DPPC bilayers to ascorbate. Permeability was monitored by the decrease in ESR signal of 5-SASL on addition of 20 mM ascorbate to unilamellar vesicles of DPPC. The logarithm of signal height is plotted as a function of time for DPPC alone (○) and for DPPC containing 0.44 mol% of lutein (●). The measurements were carried out at 45°C. The regression lines fitted by the least-squares method are drawn.

TABLE II

EFFECT OF INCORPORATION OF LUTEIN ON THE HALF-LIFE OF REDUCTION OF 5-SASL BY ASCORBATE

Half life ($t_{1/2}$) of reduction of 5-SASL was calculated from regression equations by the formula $t_{1/2} = 0.693/K$ where K is the slope of lines depicted in Fig. 3. The values of correlation coefficients (r) for regression are also given.

Lutein (mol%)		Outer bilayer	Inner bilayer
0	r	0.99	0.97
	$t_{1/2}$ (min)	4.4	16.6
	$t_{1/2}$ (min)	2.4	4.5
0.44	r	0.97	0.93

by the $t_{1/2}$ of reduction calculated from regression equations (Table II). In lutein-incorporated vesicles, the $t_{1/2}$ of reduction is decreased substantially in both layers of the bilayers, indicating a significant increase in the permeability to ascorbate.

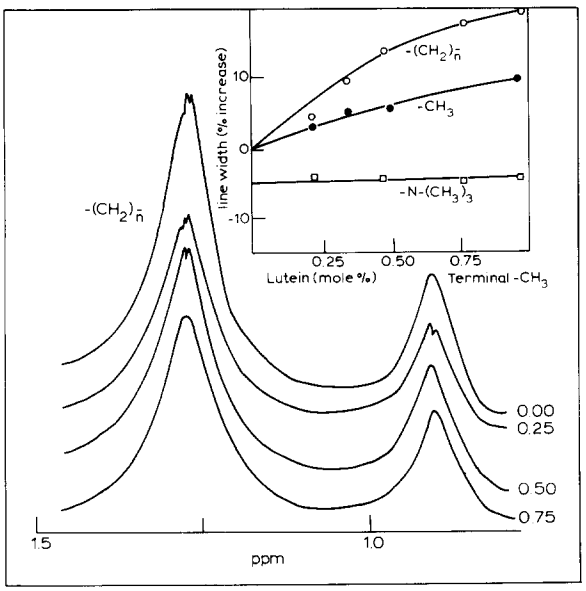

Fig. 4. Effect of incorporation of lutein on the proton NMR spectra of DPPC bilayers. The terminal $-CH_3$ and acyl-chain $-CH_2-$ spectral regions at varying concentrations of lutein are shown. Inset shows the percent increase in line-width on incorporation of lutein. The spectra were recorded at 50°C in ^2H_2O.

TABLE III

EFFECT OF INCORPORATION OF LUTEIN ON THE TRAPPED VOLUME OF UNILAMELLAR VESICLES OF DPPC

Measurements were made using $K_3Fe(CN)_6$. Details are given in the Materials and Methods section.

Lutein (mol%)	Trapped volume (litre/mol)
0.00	0.44
0.25	0.20
0.50	0.15
0.75	0.15

Nuclear magnetic resonance

The high-resolution proton NMR spectrum of sonicated vesicles of pure DPPC showed a strong and sharp signal at 3.24 ppm [$-N^+-(CH_3)_3$ proton] and peaks at 1.29 ppm [$-(CH_2)_n-$ proton], 0.89 ppm [terminal $-CH_3$ proton] and 4.56 ppm (H^2HO) as reported by Darke et al. [29]. Incorporation of lutein produces broadening of both fatty acyl methylene and terminal methyl protons (Fig. 4) without any change in the line width of the choline signal, indicating that the presence of lutein is sensed only by the hydrophobic side chains in the fluid state.

Trapped volume

Incorporation of lutein decreases the trapped volume of unilamellar vesicles (Table III). The internal volume of pure DPPC vesicles agrees well with the reported values [30]. It may be mentioned that the incorporation of lutein does not alter the elution profile of the vesicles on filtration through Sepharose 2B or sephadex G-25. Therefore the decrease in trapped volume does not indicate a change in size, but only the leakage of trapped ferricyanide from lutein-incorporated vesicles, during isolation.

Discussion

The thermotropic behaivour of aqueous dispersions of DPPC on incorporation of lutein reveals that the xanthophyll perturbs the bilayer. In lowering ΔH and the size of the cooperative unit,

the factor emulates cholesterol [31], cardiotoxin [26,32] and certain integral proteins of membranes [33,34]. It has been reported that steroids which depressed and broadened the thermotropic phase transition of DPPC were localized within the acyl region of the bilayer and that effects of the steroid did not extend to the head-group or interface regions [35]. Our experimental data indicate a similar action for lutein also. The decreases in enthalpy and size of the cooperative unit suggest disruption of cooperative interactions among the fatty acyl chains.

The interaction between the packed acyl chains of the bilayer and the xanthophyll seems stoichiometric in view of the excellent correlation between the decrease in ΔH and concentration of lutein. It would appear that one molecule of the pigment prevents 50 molecules of the phospholipid from undergoing phase transition ($\Delta H = 0$ at 2 mol% lutein). This concentration is considerably lower than that of cytochrome b_5 (7 mol%), cardiotoxin (10–20 mol%) or of cholesterol (33 mol%) required for the abolition of phase transition [13,25,32].

A shrinkage of the cluster size of the molecules in the region of phase transition may lead to general disruption of cooperative thermodynamic linkages among the membrane components and to alterations in the kinetics of passive transport. Local disruption of acyl chain packing without significant influence on bulk-phase chain motion may also be envisaged. The disappearance of pretransition at a relatively low concentration of the factor may reflect a transformation in the structure of the bilayer and the formation or elimination of 'ripples' [36]. It may be pointed out that the shape of the endotherm reveals that incorporation of lutein does not lead to phase separation. Cholesterol, for example, splits the endotherm into two at high concentrations [13].

The inference that lutein perturbs acyl chain packing is supported by the observed increase in the permeability of the bilayer to ascorbate and the decrease in the concentration of trapped ferricyanide. The broadening of the methylene and methyl proton signals indicates that the factor restricts their motional freedom [37]. In other words, lutein integrates deep into the hydrophobic domains of the bilayer distal from the choline head group of DPPC. This would facilitate the disruption of hydrophobic and Van der Waal's forces and loosening of fatty acid chain packing. The possibility that the increase in permeability results from the induction of 'aqueous pores' as has been proposed to explain the action of polyene antibodies which also decrease trapped volume [38] cannot, however, be excluded. We have observed that Lutein increases the permeability of phospholipid vesicles and mitochondrial membrane to Ca^{2+} (unpublished observations).

It may be pertinent to mention in this context that the effect of uncouplers of oxidative phosphorylation on the thermotropic properties of lipid bilayers is quite different from that observed with lutein. Thus the classical uncoupler 2,4-dinitrophenol lowered the temperature of the main transition and split the endotherm [39]. It may also be mentioned that other lipophilic compounds which contain an isoprenoid chain also do not produce the effects shown by lutein. Thus, incorporation of ubiquinone (25 mol%) produces very little change in the thermal properties of phospholipids [40,41]. The effects of lutein in general appear to resemble those of cholesterol [13] and mellitin [42], to some extent.

In conclusion it may be stated that the studies presented in this paper reveal that the complex effects of lutein on mitochondrial function [7] reflect the membrane-perturbing action of the lipophile. Shuttling of protons across the mitochondrial membrane as demanded by the chemiosmotic hypothesis [43] is a function which lutein is unlikely to perform because of its structural peculiarities.

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