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INTERACTION OF α-TOCOPHEROL WITH PHOSPHOLIPID LIPOSOMES: ABSENCE OF TRANSBILAYER MOBILITY

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Active attempts have been made in recent years to use vitamin E as a therapeutic substance in clinical practice [2-4]. Fundamentally new opportunities are deemed to have been provided by the specific delivery of vitamin E to target organs in phospholipid liposomes [7]. Liposomes circulating in the blood stream clearly interact with the internal medium of the body through their own outer surface, i.e., through their outer monolayer, whereas the inner monolayer of liposomes is in contact only with the internal contents of the liposomes and is inaccessible for macromolecular and cellular components of blood. Hence it follows that compounds built into the two monolayers of liposomes are accessible for target organs only if they possess sufficiently high transbilayer mobility (the so-called "flip-flop"). In the case of vitamin E, the main component of which is α-tocopherol (TP), this problem is of fundamental importance because the limited degree of incorporability of TP into phospholipid vesicles has been demonstrated [4], and this naturally limits the saturation of biological structures (biomembranes, lipoproteins) with vitamin E.

In the investigation described below a method of fluorescence analysis was used to study interaction between TP and liposomes, obtained from saturated and unsaturated phospholipids, and the possibility of its "flip-flop" in them.

EXPERIMENTAL METHOD

Liposomes were obtained from phosphatidylcholines of different nature (from egg, dimyristoyl-, dipalmitoyl-, and dioleyl-phosphatidylcholine). Liposomes were obtained by evaporation of the phosphlipids under argon, followed by shaking in Tris-HCl buffer (50 mM), NaCl (100 mM), pH 7.4, and treatment on an MSE ultrasonic disintegrator (22 kHz) by repeated sonication (12 × 15 sec with intervals of 30 sec). Ultrasonic treatment was carried out at a temperature above the phase transition temperature until the liposomes were completely translucent. The liposomes thus obtained were centrifuged (90,000g, 20 min), and supernatant containing monolayer liposomes [5] was used for the measurements. To incorporate TP into the outer and inner monolayers of the liposomes, solutions of phospholipids and TP in chloroform were mixed in definite proportions. The mixture was evaporated in a current of argon, and liposomes prepared from it. Fluorescence spectra of TP were recorded on "Perkin-Elmer MPF-44B"

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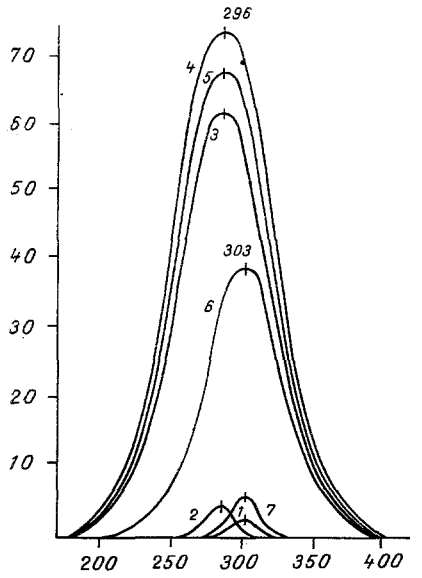

Fig. 1. Fluorescence excitation spectra of TP in various media at 37°C. Abscissa, wavelength of excitation (in nm); ordinate, intensity of fluorescence (in relative units). Concentration of TP $2 \cdot 10^{-4}$ M. 1) Tris-HCl buffer 50 mM, NaCl 100 mM, pH 7.4; 2-7) methyl, ethyl, propyl, butyl, amyl, and capryl alcohols, respectively. Width of slits 2 nm. Maximum of fluorescence 325 nm in 2-5, 322 nm in 1, 6, and 7.

(USA) and Hitachi SPF-850 (Japan) spectrofluorometers in a cuvette (1 cm) with slits 5 nm wide. The TP was oxidized with the aid of potassium ferricyanide. Disturbances of the structure of the liposomes and induction of "flip-flop" of TP were induced by addition of 0.075% sodium dodecylsulfate to the suspension of liposomes. Lipid phosphorus was determined by the method in [8].

The TP, phospholipids, and sodium dodecylsulfate were obtained from Serva, West Germany. The alcohols, hexane, and chloroform, obtained from Merck, West Germany, were purified by distillation. The remaining reagents were of the chemically pure grade.

EXPERIMENTAL RESULTS

It is convenient to study interaction with TP, incorporated into liposomes by recording its characteristic fluorescence at 321 nm, excited by light with a wavelength of 302 nm, whose intensity depends on the polarity of the medium [6]. The intensity of fluorescence of TP, dispersed in Tris-HCl buffer, is considerably below the intensity of fluorescence of the same quantity of TP, dissolved in aliphatic alcohols (Fig. 1). The intensity of fluorescence of TP depends at the extrema on the hydrophobicity of the solvent among the spectra studied and is maximal in propyl alcohol.

On addition of phosphatidylcholine liposomes to a dispersion of TP in Tris-HCl buffer, a rapid rise of fluorescence of TP, taking place in 1-2 sec, was recorded (Fig. 2a). With phospholipid and TP in the ratio of 20:1 and 10:1 moles % the intensity of fluorescence increased stepwise by 3.5-4 times, and then increased a further 1.5 times during the subsequent 5-7 min of incubation. Further incubation of liposomes with TP for 2-4 h led to only a very small increase in fluorescence, not exceeding 20% of its value recorded during the first 5-7 min of incubation. Incubation for a longer time (20-40 h) did not lead to any further increase in the intensity of fluorescence. During long-term incubation for several hours, in the case of unsaturated phospholipids, a very slow decrease was observed in the intensity of fluorescence of TP (5-10%) probably due to its oxidation.

It can be tentatively suggested that the sudden increase in the intensity of fluorescence of TP during the first minutes of incubation is due to its incorporation into phospholipid vesicles. Under these circumstances the TP ought to be localized either entirely in the outer monolayer of the liposomes, if no "flip-flop" has taken place, or in both monolayers of the liposomes in the case of sufficiently rapid "flip-flop."

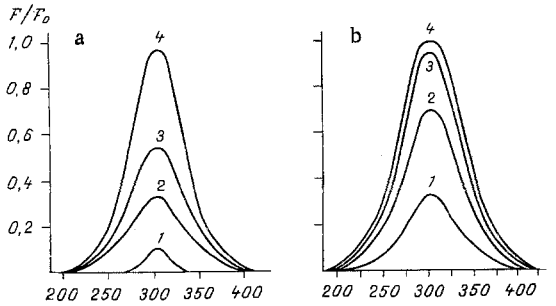

Fig. 2. Fluorescence excitation spectra of TP in phospholipid liposomes from egg phosphatidylcholine at 37°C. Ordinate, reduced intensity of fluorescence. Abscissa, excitation wavelength (in nm); concentration of lipids 0.5 mg/ml, ratio of phospholipid to TP 20:1 mmole %. a) Different conditions of incorporation of TP into liposomes: 1) micelles of TP in Tris-HCl buffer; 2) alcoholic solution of TP added to suspension of liposomes (alcohol concentration 0.001%), 3) the same, 5 min after addition, 4) homogeneous mixture of TP and phospholipids in film was suspended in Tris-HCl buffer and subjected to ultrasonic treatment; b) effect of sodium dodecylsulfate on incorporation of TP into liposomes: 1) the same as in (a), 2; 2) the same as in 1 + 0.075% sodium dodecylsulfate; 3) the same as (a), 4 + 0.075% sodium dodecylsulfate; 4) the same as in (a), 4. Width of slits 5 nm, $\lambda_{m.ex.}$ = 303 nm; $\lambda_{m.em.}$ = 322 nm.

The choice of one particular possibility may be made experimentally with the aid of procedures facilitating incorporation of TP into both monolayers of liposomes. It was shown that the intensity of fluorescence of TP in liposomes obtained by ultrasonic treatment of phospholipids and TP, present simultaneously in the buffer and mixed beforehand, is significantly higher (by 2-2.5 times) than in the case when TP was added to a suspension of already sonicated liposomes (Fig. 2a). This probably means that the TP added to a suspension of ready-made liposomes is incorporated only into the outer monolayer of phospholipids, whereas ultrasonic treatment of the mixture of phospholipids and TP enables the latter to be incorporated into both outer and inner monolayers of the liposomes. This hypothesis is confirmed by the results of experiments to study oxidation of TP by a nonpenetrating agent — potassium ferricyanide (Fig. 3). Addition of potassium ferricyanide to liposomes into which TP has been added without the sonication procedure completely "quenches" its fluorescence. Meanwhile introduction of potassium ferricyanide into a suspension of liposomes into which TP has been incorporated by ultrasonic treatment, reduced the intensity of fluorescence only approximately by half. The residual signal remained unchanged during incubation for many hours.

As a whole it can be concluded from the results that TP, incorporated into the outer and inner monolayers, is preserved in each of them for quite a long time (tens of hours), and it does not take part in transbilayer migration from the outer into the inner monolayer and vice versa. This preservation of TP in each monolayer was found in liposomes prepared from both saturated and unsaturated phospholipids (from egg, dimyristoyl-, dipalmitoyl-, and dioleylphosphatidylcholine) and, consequently, it is not determined by the fatty acid composition of the phospholipids.

The rate of "flip-flop" of phospholipids in liposomes from saturated or unoxidized, unsaturated phospholipids is known to be very slow, and to rise sharply on the appearance of lipid peroxidation products [1]. In this connection it is important to note that transbilayer migration of TP was not found in liposomes from phospholipids containing their peroxidation products. It may be that this is due to the formation of complexes of TP with phospholipids, in which the carboxyl group of TP forms a hydrogen bond with the carbonyl atom of the oxygen of the fatty-acid chain of the phospholipid [6].

It can be postulated that introduction of substances forming defects and inducing translation mobility of lipids into the liposomal membrane [1] ought to lead also to intensification of intermonolayer transfer of TP. In the present experiments introduction of 0.075%

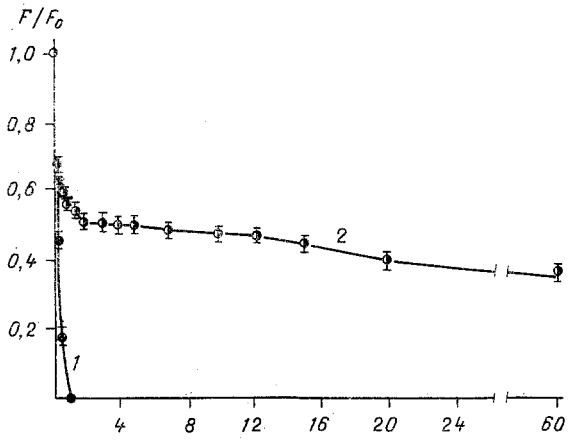

Fig. 3. Change in intensity of fluorescence of TP in liposomes from egg phosphatidylcholine on addition of potassium ferricyanide ($2 \cdot 10^{-5}$ M). Abscissa, incubation time (in min); ordinate, reduced intensity of fluorescence. Concentration of lipids 0.5 mg/ml, ratio of phospholipid to TP 20:1 moles %. Conditions of recording spectra as in Fig. 2. 1) Alcoholic solution of TP added to suspension of liposomes + 5 min for incorporation of TP into liposomes; 2) homogeneous mixture of TP and phospholipids in a film was suspended in Tris-HCl buffer and subjected to sonication.

sodium dodecylsulfate into a suspension of liposomes caused a marked increase in fluorescence of TP, when it was added to the suspension of previously sonicated liposomes, but had virtually no effect on the intensity of fluorescence of the TP signal in liposomes into which it was incorporated by the sonication procedure (Fig. 2b).

Interaction of exogenous TP with liposomes thus leads to its incorporation only into the outer monolayer, in which it remains for some tens of hours, without penetrating into the inner monolayer. If TP is incorporated into both monolayers, it likewise does not migrate between the outer and inner monolayers. These facts are essential when the choice of optimal conditions of saturation of the membrane structures in vivo with TP delivered with the aid of liposomes, are to be chosen.

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