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THE RAPID TRANSBILAYER MOVEMENT OF THIOCHOLESTEROL IN SMALL UNILAMELLAR PHOSPHOLIPID VESICLES

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Cholesterol is a major component of biological membranes, yet there is very little information concerning its distribution across the membrane. Recent experiments in our laboratory, using cholesterol oxidase, have demonstrated that cholesterol can undergo a rapid transbilayer movement in lecithin-cholesterol vesicles in a half-time of 1 min or less at 37°C. In order to support this conclusion, we have sought other approaches to the measurement of this process. We now report our finding that the transbilayer movement of thiocholesterol in phospholipid vesicles occurs in a half-time of 1 min or less at 20°C.

The distribution of phospholipids in small unilamellar vesicles has been determined either impermeable labelling reagents [1] or from the splitting of a characteristic peak in the NMR spectrum induced by a chemical shift reagent added to the external vesicle surface [2]. Due to a lack of reactivity and absence of a shiftable NMR peak, neither of these approaches can be applied to cholesterol. However, Huang et al. [3] were able to measure the distribution of a cholesterol analog, thiocholesterol, by direct means. These authors reported that titrations of egg lecithin-thiocholesterol vesicles with Ellman's reagent [4,5] 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) exhibited biphasic kinetics. These two kinetically defined pools appeared to correlate with the amounts of the sterol present on the inner and outer vesicle surfaces. It was later reported [5] that a rate for the transbilayer exchange of thiocholesterol calculated from this data had a half-time of 42 min at room temperature.

We have used a new approach to measure the transbilayer movement of thiocholesterol. Small unilamellar vesicles composed of egg lecithin (from Makor Chemicals, Jerusalem, Israel) were prepared with the sulfhydryl reagent DTNB trapped solely in their internal aqueous compartment by a method analogous to that described for trapping 2,4,6-trinitrobenzene sulfonic acid in similar vesicles [6]. In order to demonstrate the absence of any externally located DTNB in a given vesicle preparation, 25 μl of 100 mM glutathione was added to a 2-ml aliquot of the vesicles. Unless there was no detectable change in the absorbance at 412 nm, followed by a large rise after the subsequent addition of deoxycholate (to a final concentration of 1%) to lyse the vesicles, the preparation was discarded.

Thiocholesterol, cholest-5-ene-3β-thiol (Eastman Organic Chemicals, Rochester, NY), was recrystallized three times from absolute alcohol in the presence of Norit. The resulting compound gave a single spot after thin-layer chromatography on Silica gel G with $CHCl_3$/MeOH (100 : 2, v/v) as the eluting solvent. A 250 μM solution of the purified sterol was prepared in absolute alcohol. Titrations of this stock solution into a final volume of 2 ml containing 10 mM DTNB, 100 mM Tris-HCl, and 3% Triton X-100 at pH 7.4 obeyed Beer's Law with

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

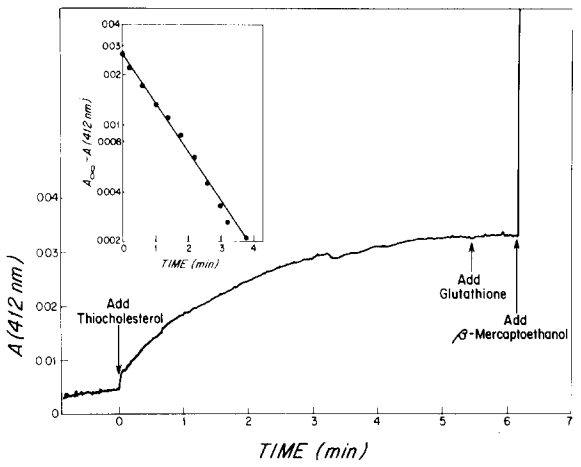

Fig. 1. Transbilayer movement of thiocholesterol. 25 μl of thiocholesterol in alcohol (250 μM) was added as indicated to 2 ml of egg lecithin vesicles (2.7 mM lipid) containing DTNB at 20°C, with continuous stirring. The vesicles were prepared by dispersing lyophilized egg lecithin in 100 mM Tris-HCl containing 10 mM DTNB pH 7.4. After sonication and centrifugation, vesicles were eluted from Sepharose 4B in 100 mM Tris-HCl, pH 7.4. The transbilayer movement of thiocholesterol was followed at 412 nm. 25 μl of glutathione (100 mM in 100 mM Tris-HCl, pH 7.4) and 25 μl of β-mercaptoethanol was added as controls where indicated. From titrations of the stock solution of thiocholesterol in alcohol, one would predict an absorbance change of 0.043 at 412 nm for the quantity used in the above experiment (see text). The observed absorbance change of 0.03 indicates that only 70% of the added sterol had undergone transbilayer movement. Although the reasons for this result are unclear, they have no effect on the conclusion of this study that the transbilayer movement of thiocholesterol is a very rapid process.

0.144 μmol of thiocholesterol resulting in 1 absorbance unit at 412 nm. This is in excellent agreement with the predicted value of 0.147 μmol/absorbance unit based on the molar extinction coefficient of $1.36 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for the anion of thionitrobenzoic acid [4].

Twenty five microliters of the ethanolic solution of thiocholesterol were added to 2 ml of DTNB containing vesicles in a cuvette at 20°C, with continuous stirring [7], monitored at 412 nm. A typical increase in absorbance at 412 nm is shown in Fig. 1. After the reaction had reached an end-point, glutathione was added where indicated to demonstrate that DTNB had not leaked out of the vesicles during the experiment. Subsequent addition of β-mercaptoethanol was used to indicate that the initial end-point was not a result of insufficient DTNB in the vesicles. Since Ellman's reagent is very sensitive in defecting sulfhydryl containing compounds, the amount of thiocholesterol used in this experiment represents on the average 3 mol of thiocholesterol per vesicle (based on 2 700 phospholipid molecules/vesicle [9]). This small amount is not fusogenic and has no effect on the size of the vesicle [3]. In order to demonstrate that the observed absorbance changes at 412 nm were due to the reaction of the thiol group with DTNB, control experiments were performed as above with the exception that pure ethanol replaced the ethanolic solution of thiocholesterol. No changes in absorbance at 412 nm were detected, which demonstrates that the absorbance change shown in Fig. 1 is due to the reaction between thiocholesterol and DTNB.

The control experiments in Fig. 1, clearly indicate that DTNB remains exclusively on the inside of the vesicles throughout the course of the measurement. Thiocholesterol is initially added to the outer membrane surface; consequently the increase in absorbance observed at 412 nm is the result of the transbilayer movement of thiocholesterol. This increase can be described by a single exponential (see inset Fig. 1) which reveals that the processes of diffusion to the membrane followed by transbilayer movement and subsequent reaction with DTNB cannot be distinguished kinetically. Therefore, we conclude that the rate of transbilayer movement of thiocholesterol is a very rapid process occurring in a half-time of 1 min or less at 20°C. A similar rapid rate for the transmembrane movement of cholesterol has recently been determined in our laboratory [10].

The results presented in this study indicate that thiocholesterol, a compound closely related to cholesterol, is capable of very rapid movement across a lipid bilayer. Combining this conclusion with results from our exchange studies [8] and our studies involving cholesterol oxidase [10] strongly supports our contention that the transbilayer movement of cholesterol in lipid vesicles is a very rapid process.

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