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# Effect of cholesterol on the tight insertion of cytochrome $b_5$ into large unilamellar vesicles [1]

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**Abstract**

When cytochrome $b_5$ is added to large unilamellar vesicles (LUVs) of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), it binds predominantly in a 'loose,' or transferable form. Prolonged incubation at 30°C leads to insertion in the physiological 'tight,' nontransferable form, with a halftime for the loose → tight conversion of approx. 9 days. In this study, the effect of cholesterol on the rate of tight insertion was determined. Tight binding was assayed by depleting the LUVs of loose cytochrome $b_5$ with an excess of SUV acceptors and then separating the liposome populations by gel-filtration or velocity sedimentation. Incorporation of cholesterol into the LUVs was found to markedly increase the rate of tight insertion, even though cholesterol decreases the equilibrium binding constant and saturation level of protein binding. The effect is not a continuously increasing function of cholesterol content, but attains a maximum at 20–25 mol%, where the rate enhancement is approx. 10-fold over baseline. At higher cholesterol levels, the rate decreases, returning to baseline at 40 mol% cholesterol. These observations are highly unusual in that cholesterol generally decreases the membrane binding affinity and the permeability of solutes, and does so as a monotonic function of cholesterol concentration (above the liquid-crystalline phase transition of the phospholipids). It is suggested that tight insertion is enhanced by lipid-protein packing mismatches and by bilayer fluidity; the former increases monotonically with increasing cholesterol whereas the latter decreases monotonically. At 20–25 mol% cholesterol the optimum balance of these physical properties is obtained for tight insertion.

*Keywords:* Cytochrome $b_5$; Liposome; Cholesterol

## 1. Introduction

Many water soluble proteins spontaneously bind to and insert into biological membranes [1–3]. But even in those cases where insertion is clearly dependent upon a translocation apparatus and energy input, portions of polytropic integral membrane proteins may also insert spontaneously (reviewed by Gennis [4]). The thermodynamics, kinetics, and other mechanistic features of spontaneous protein insertion have been, and will continue to be, studied with liposomes as a model membrane system. The present report extends our studies of the spontaneous incorporation of cytochrome $b_5$ into LUVs.

Cytochrome $b_5$, a component of the microsomal electron transport system, is an integral membrane protein with an N-terminal water-soluble catalytic domain and a C-terminal nonpolar membrane-binding anchor [5]. Synthesized without a signal sequence on water-soluble ribosomes, this protein spontaneously inserts into intracellular membranes without using signal recognition particle, receptors, ATP hydrolysis, or a membrane potential [6–9].

The purified detergent-free protein is water-soluble as an equilibrium mixture of octomers and monomers [10], yet spontaneously binds to preformed lipid vesicles of phosphatidylcholine [11–17]. When added to preformed liposomes, cytochrome $b_5$ usually inserts in a 'loose' binding form, characterized by the ability of the protein to spontaneously transfer from one vesicle population to an-

Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine; [$^{14}$C]POPC, L-α-1-palmitoyl-2-[1-$^{14}$C]oleoylphosphatidylcholine; [$^{3}$H]triolein, [9,10(n)-$^{3}$H]triolein; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle.

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other [18,19]. In the loose configuration, the hydrophobic anchor forms a hairpin structure that partially penetrates into the bilayer, leaving three to eight C-terminal residues in the external aqueous phase alongside of the catalytic domain [5,20]. In contrast, the endogenous cytochrome $b_5$ in microsomes is bound in a nontransferable or 'tight' binding form [19]. The tight binding form can be generated in liposomes by using reconstitution procedures that are commonly employed to incorporate integral membrane proteins into lipid vesicles [19,21–23], or by incubating the proteoliposomes above 50°C [24]. The configuration of the hydrophobic tail in the tight binding form has not yet been resolved; photolabelling studies indicate that it spans the bilayer [25] whereas other types of studies indicate that it does not [5,26–28].

We have previously shown that 10–15% of the protein becomes tightly incorporated into LUVs within the first 2 h of incubation; prolonged incubation (> 24 h) leads to a much slower, but steady increase in the percentage of tightly bound form, with a halftime of approx. 10 days [29]. In the transitional period of 2–24 h, $t_{1/2} \approx 5$ days. The mechanism of tight insertion was found to be complex: Tightly bound cytochrome $b_5$ is concentrated in a small fraction of 'insertion-active' LUVs, which are generated during the incubation period.

In the present study we show that cholesterol can increase the rate of tight insertion by approximately a factor of 10. Also, the mechanism is apparently different, and simpler, with cholesterol-containing vesicles in that the increased amount of tightly inserted protein does not appear to require the formation of a new vesicle population.

To our knowledge, this is the first time that cholesterol has been found to enhance the rate of protein insertion above the phospholipid phase transition. Ordinarily, cholesterol decreases the membrane/water partition coefficient, and the permeability of small molecules [30–33].

A preliminary report of these results has been presented [34].

## 2. Materials and methods

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (POPC) was purchased from Avanti Polar Lipids and cholesterol was from Calbiochem Corporation. Both had > 99% purity and were used without further purification. L-α-1-Palmitoyl-2-[1-$^{14}$C]oleoylphosphatidylcholine ([$^{14}$C]POPC, specific activity = 58.0 μCi/μmol) and [9,10(n)-$^{3}$H]triolein ([$^{3}$H]triolein, specific activity = $2.68 \cdot 10^4$ μCi/μmol) were obtained from New England Nuclear.

Cytochrome $b_5$ was purified to homogeneity from bovine liver as previously described [35].

Liposomes were prepared from common chloroform stock solutions containing POPC and cholesterol at specified mole ratios. Either [$^{14}$C]POPC or [$^{3}$H]triolein was incorporated into the lipid vesicles by adding 1–2 μCi of the labelled compounds to the organic lipid solutions prior to forming the lipid dispersions.

Large liposomes of 800–1500 Å diameter were obtained by reverse-phase evaporation [36], using 20 mM Tris-acetate, 100 mM NaCl, 0.1 mM EDTA, pH 8.1 as the aqueous phase, followed by sequential extrusion through Nuclepore polycarbonate membranes with pore sizes of 0.4, 0.2, 0.1, and 0.08 μm. A model HPVE-10 high pressure extrusion apparatus from Sciema Technical Ltd., Vancouver, Canada was used to facilitate extrusion for liposomes that contained ≥ 30 mol% cholesterol. The preparation was fractionated by gel-filtration through a Sepharose 2B-CL column (1.6 × 55 cm); only liposomes that eluted in the peak void-volume fractions were collected. Lipid phosphorous was determined according to the Bartlett procedure [37].

Homogeneous populations of limit-size small unilamellar vesicles (SUVs) were obtained by ultrasonication of lipid dispersions followed by differential sedimentation (Ti50 fixed-angle rotor, 45 000 rpm, 1 h) of contaminating larger vesicles [35].

Cytochrome $b_5$ was reconstituted into POPC/cholesterol bilayers by incubating the detergent-free protein with preformed LUVs for 2 h at 30°C under argon. Under these conditions, a 2 h incubation is sufficient for the completion of the initial binding process [29].

The extent of tight insertion was then determined from the amount of nontransferable cytochrome $b_5$ remaining with the LUVs following incubation with acceptor SUVs, as described previously [29]. Briefly, LUVs were depleted of loosely bound cytochrome $b_5$ by incubating them with an excess of SUVs for 2 h at 30°C (under argon). The SUV/LUV phospholipid ratio was either 3:1 or 4:1, which is high enough to completely deplete the LUVs of loose cytochrome $b_5$ because SUVs have a significantly greater affinity [38,39] and capacity for the protein [39].

To ensure that a 2-h incubation period adequately depletes cholesterol-containing LUVs of loose cytochrome $b_5$, some transfers were also performed for 8 h. The results were indistinguishable from the 2-h transfer protocol.

The donor and acceptor liposome populations were then separated by either gel-filtration or velocity sedimentation, and the individual fractions were analyzed for cytochrome $b_5$ and phospholipid. The concentration of cytochrome $b_5$ was determined from the Soret band at 413 nm as described elsewhere [38]; liposomes were assayed from the $^{14}$C and $^{3}$H markers (60 s counting time) using Beckman HP liquid scintillation cocktail.

Although subsaturating levels of cytochrome $b_5$ were generally used in the initial incubation mixtures (1 cyt $b_5$/1300 POPC), in some experiments protein was added in excess (1 cyt $b_5$/50 POPC, at the same POPC concentration) to determine if the results are dependent upon the protein/lipid ratio. In the range of 20–35 mol% choles-

terol, adding the protein in excess increases the amount of cytochrome $b_5$ binding by approx. 2-fold. Unbound protein was removed by gel-filtration on a Sepharose 2B-CL column prior to incubation with SUV acceptors. In the absence of cholesterol, the rate of tight insertion is independent of protein/lipid ratio [29].

## 3. Results

### 3.1. Appearance of nontransferable cytochrome $b_5$ in LUVs containing cholesterol

Cytochrome $b_5$ was incubated for 2 h or 24 h at 30°C with POPC LUVs containing various levels of cholesterol.

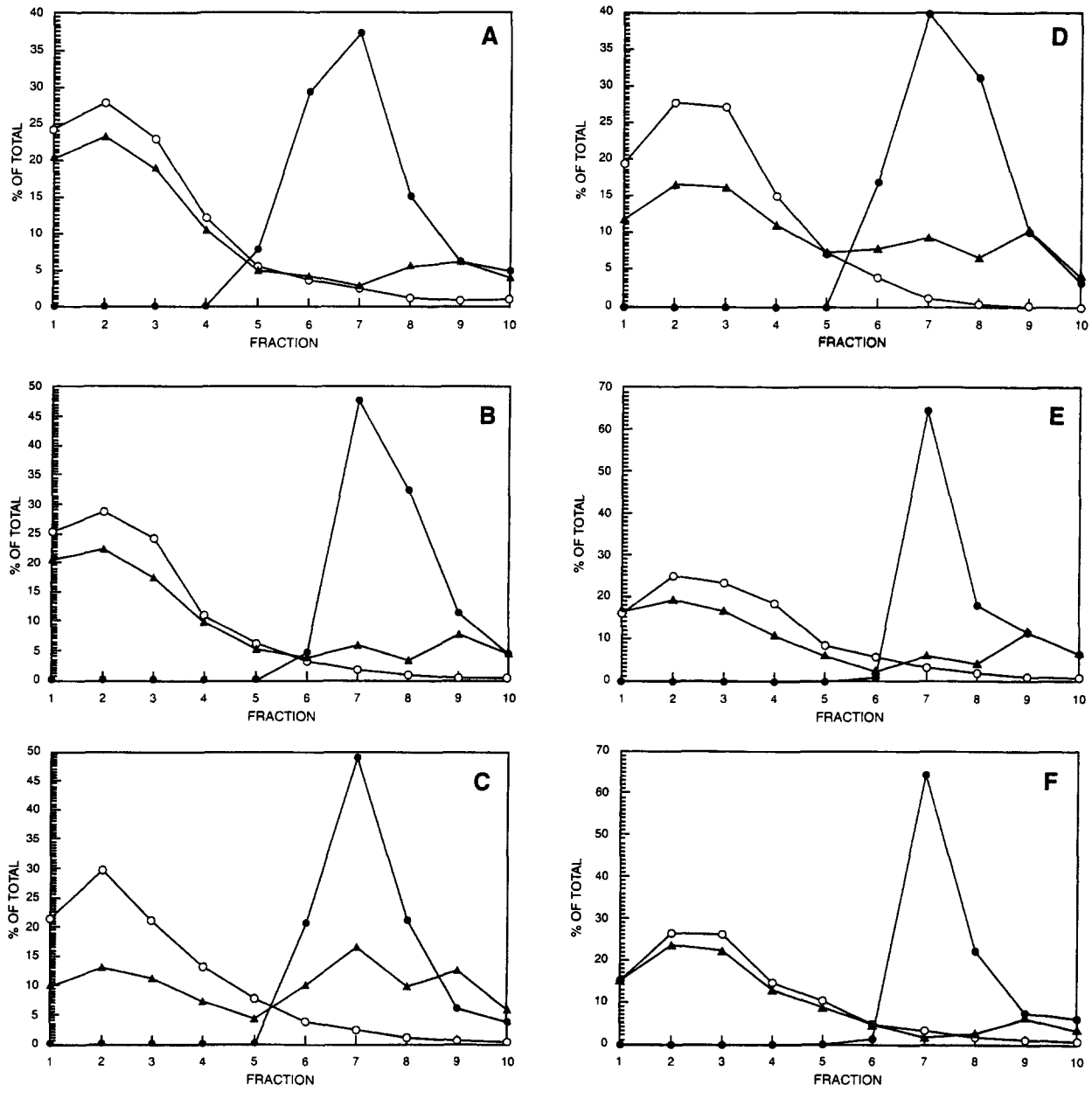


Fig. 1. Cytochrome $b_5$ transfer from POPC LUVs to acceptor POPC SUVs. (A) 0% cholesterol, (B) 10% cholesterol, (C) 25% cholesterol, (D) 30% cholesterol, (E) 35% cholesterol, and (F) 40% cholesterol. $^3$H-labelled POPC LUVs and $^{14}$C-labelled POPC SUVs were prepared from common chloroform stock solutions of POPC and cholesterol (see Section 2). Cytochrome $b_5$ (3.84 nmol) was incubated with POPC LUVs (5 $\mu$mol POPC, doped with $9.7 \cdot 10^4$–$1.6 \cdot 10^5$ dpm [$^3$H]triolein/$\mu$mol POPC) in Tris-acetate buffer for 2 h, 30°C, under argon. POPC SUV acceptors (15 $\mu$moles POPC, doped with $2.8 \cdot 10^4$–$4.1 \cdot 10^4$ dpm [$^{14}$C]POPC/$\mu$mol POPC) were then added and the mixture was incubated for an additional 2 h as before to deplete LUVs of loose protein. 0.6-ml aliquots of the transfer mixtures were then applied to glycerol step gradients of 1.3 ml 1% glycerol, Tris-acetate buffer and 0.1 ml 60% glycerol pad. The gradients were centrifuged in a 50-Ti fixed-angle rotor at 45,000 rpm, 25°C, for 1 h. Fractions of 0.2 ml were collected and assayed for LUVS (●), SUVs (○), and cytochrome $b_5$ (▲) by $^3$H, $^{14}$C dpm, and $A_{413}$, respectively. Nontransferable cytochrome $b_5$ remains with the LUVs following incubation with SUV acceptors.

The extent of tight binding was determined by adding SUV acceptors, followed by separation of donor and acceptor populations by gel-filtration or sedimentation through glycerol gradients. Fig. 1 shows the glycerol gradient profiles obtained after a 2-h initial incubation of LUVs with cytochrome $b_5$.

With 0% cholesterol (panel A), 10–20% of the cytochrome $b_5$ remains with the LUVs, indicating that this percentage of the protein is tightly inserted after 2 h. The glycerol gradients also reveal that tight insertion may be a complex process: the tightly bound cytochrome $b_5$ is not uniformly distributed among the liposomes but concentrated in a relatively small fraction of the vesicles toward the bottom of the gradient. We have previously determined that this tight insertion is due to 'insertion-active' vesicles, which form during the incubation [29].

As the LUV cholesterol content increases from 0 to 25 mol%, the total amount of cytochrome $b_5$ that becomes nontransferable significantly increases. However, the glycerol gradient profiles indicate separate fractions of nontransferable cytochrome $b_5$. As shown for liposomes containing 10 and 25 mol% cholesterol, two migratory bands of cytochrome $b_5$ become evident (panels B and C, respectively): one that is essentially coincident with the sedimenting liposomes and the second that is near the bottom of the gradient. Although both bands increase concomitantly with cholesterol content, the co-migrating cytochrome $b_5$ band becomes predominant at approx. 25% cholesterol. At cholesterol compositions greater than 25 mol%, this band decreases (panels D and E) and disappears completely at 40% cholesterol (panel F). Similarly, the fast-sedimenting, noncoincident fraction also decreases beyond 25% cholesterol, and eventually becomes the only band of tightly inserted cytochrome $b_5$.

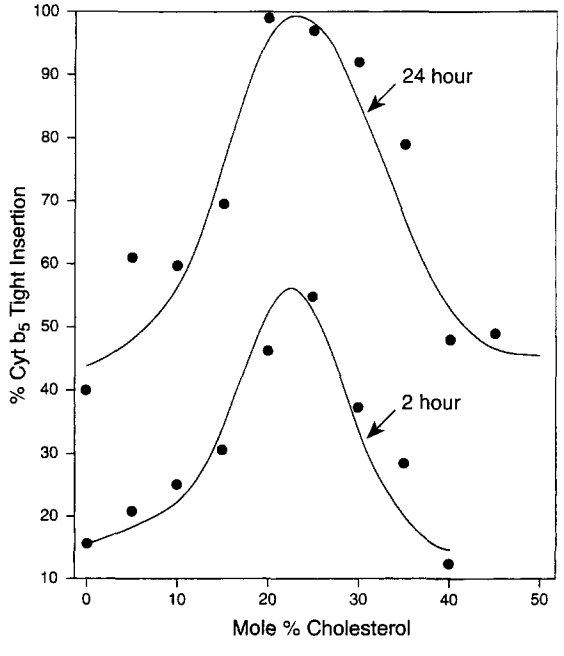


Fig. 2. Cytochrome $b_5$ tight insertion into POPC LUVs as a function of cholesterol mole percent. The extent of tight insertion is determined from the amount of nontransferable cytochrome $b_5$ remaining with POPC donor liposomes following incubation with acceptor POPC SUVs at 2 h (bottom curve) and 24 h (top curve) of binding.

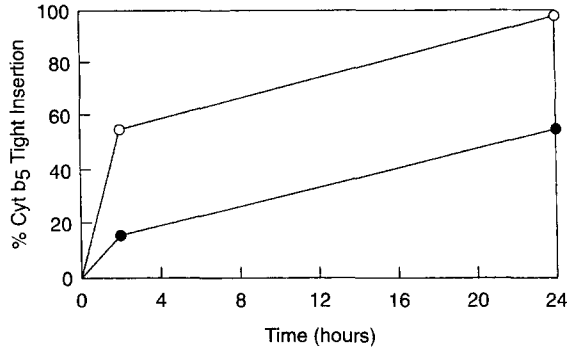


Fig. 3. Kinetics of cytochrome $b_5$ tight insertion. Interaction with LUVs of POPC (●) and with LUVs containing a 3:1 molar ratio of POPC to cholesterol (○).

The extent of cytochrome $b_5$ tight insertion into POPC LUVs at 2 and 24 h is shown in Fig. 2. As is apparent from Fig. 1, the amount of bound cytochrome $b_5$ that spontaneously inserts into the tight configuration increases from baseline values at 0% cholesterol to a maximum at approx. 20–25 mol% cholesterol, and then decreases to the original levels at about 40% cholesterol.

Fig. 3 shows the kinetics of tight insertion for 0% and 25% cholesterol. The tight insertion process involves an initial rapid phase followed by a significantly slower rate. Because the extent of tight binding cannot be determined prior to two hours, only the lower limit of the fast phase rate is estimated: for POPC LUVs without cholesterol this halftime is $\leq 8$ h, whereas for LUVs that contain 25 mol% cholesterol, the halftime is $\leq 2$ h. The halftime of tight insertion during the 2–24 h incubation period is approx. 2 days for POPC LUVs without cholesterol and 5 h for liposomes with a 3:1 molar ratio of POPC to cholesterol.

The cholesterol-mediated tight insertion appears to be unaffected by the initial cytochrome $b_5$ per phospholipid ratio, since essentially the same results were obtained when the ratio of bound protein to phospholipid was approximately doubled. This suggests that the cholesterol-related tight insertion does not involve protein–protein interactions.

## 4. Discussion

Above the liquid-crystalline phase transition temperatures of phospholipids, cholesterol has been observed to decrease bilayer fluidity, permeability, and equilibrium binding of small molecules [40]. Our previous observations that increasing cholesterol content progressively decreases the saturation level of cytochrome $b_5$ binding to phosphatidylcholine liposomes [34,39] is consistent with these

observations. Therefore, it is highly surprising that cholesterol should stimulate the rate of cytochrome $b_5$ tight insertion, which may be viewed as a permeability phenomenon. Moreover, the compositional dependence of this phenomenon, i.e., increasing to a maximum at 25 mol% cholesterol and then descending to baseline at 40%, has not to our knowledge been observed with any other property of liquid-crystalline phosphatidylcholine/cholesterol mixtures.

The mechanism of cholesterol induced tight insertion appears to be different, and is much more straightforward, than the mechanism which predominates in the absence of cholesterol. In the absence of cholesterol, tight insertion is accompanied by the formation of a subpopulation of 'insertion-active' vesicles, which is highly enriched in protein [29]. In contrast, cholesterol appears to facilitate insertion of the protein uniformly into the original liposome population.

At present, the exact kinetic mechanism of cholesterol-induced tight insertion cannot be determined because the topology of the tight binding configuration has not yet been conclusively established (see Introduction). In the absence of detailed structural models of the loose and tight binding forms, attempts to explain these kinetic phenomena are necessarily highly speculative. Nevertheless, the rate enhancement could be rationalized on the basis of the packing constraints that are likely to occur between any membrane protein and the lipids in a phosphatidylcholine/cholesterol mixture.

The 'ordered bimolecular mesomorphic lattice' model of liquid- crystalline POPC/cholesterol bilayers [41] posits very infrequent contacts among cholesterol molecules. A consequence of this central assumption is that at 20 mol% cholesterol, the bilayer resembles an array of clusters, each consisting of a central cholesterol surrounded by 4 phospholipids. At cholesterol compositions greater than 20 mol%, the number of cholesterol contacts per phospholipid increases (to a maximum of 4 contacts per phospholipid at 50% cholesterol) so that bilayer fluidity is predicted to decrease.

We suggest that these phospholipid/cholesterol clusters cannot effectively pack against the asymmetrically shaped membrane-anchoring domain of loosely bound cytochrome $b_5$, because their sides are too large and rigid. If so, this would result in local packing defects, which could conceivably facilitate the transition from loose to tight binding. The maximum in the insertion rate occurs at 20–25 mol% cholesterol because tight insertion is assumed to also require the additional factor of bilayer fluidity. At cholesterol compositions greater than 25 mol%, the decrease in bilayer fluidity predominates, thereby reducing the rate of tight insertion. At lesser concentrations of cholesterol, where fluidity is greater, the increase in packing mismatches predominates. Accordingly, the optimum balance between bilayer fluidity and bilayer defects occurs at approx. 20 mol% cholesterol.

Additional studies and theoretical analysis will be required to test the plausibility of this explanation.

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