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DELIVERY OF LIPOSOME MEMBRANE-ASSOCIATED STEROLS THROUGH SILASTIC MEMBRANES

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The transport of sterols incorporated into the lecithin bilayer of small unilamellar liposomes through a model membrane was studied. A two-chamber diffusion cell containing liposomes with incorporated [4-^{14}C]cholesterol or β-[4-^{14}C]sitosterol in the donor chamber and liposomes with unlabeled cholesterol in the receiver chamber was used. The permeability coefficients of the sterols through silastic rubber membranes which served as a model membrane were measured. The permeability for cholesterol incorporated into liposomes in a phosphatidyl choline/cholesterol molar ratio of 1 : 1, produced by sonication for 1 h, and subsequent centrifugation at 100 000 × *g* for 1 h, was $1.6 \cdot 10^{-8}$ cm sec^{-1}. Dilution of the liposome suspension did not change the permeability coefficient significantly. The permeability coefficient of sitosterol incorporated into liposomes was about 4-times smaller than that of cholesterol. These results suggest that the sterols were delivered to the silastic membrane by the intact liposomes and that free solute was not involved in the transport to the membrane to a significant degree. The large differences in the permeability coefficients between cholesterol and sitosterol indicate that an aqueous interfacial barrier was crossed by the sterol during the delivery to the membrane.

The mechanism of the drug delivery to the target cell has thus far not been totally explained. Four mechanisms, not necessarily exclusive, have been discussed [1]: (1) Cellular uptake of the liposomes via endocytosis [2–4]; (2) fusion of the liposomes with the cell membrane [2,3,5–8]; (3) facilitated transfer of drug by adsorption of the liposomes at the cell membrane surface [9–11]; and (4) exchange diffusion of drug from liposomes into cells [1,12]. The prevailing mechanism seems to depend on the chemical and physicochemical properties of the liposomes and the type of cells involved [13]. Fusion, for example, has been observed mainly with charged liposomes.

This study utilizes a simple method developed by Karth and Higuchi [14] for investigating various factors which influence the interfacial environment on the transport of molecules to model membranes. The method uses a diffusion cell in which a silicone rubber membrane separates two aqueous compartments. A silicone rubber membrane has been shown [14] to act almost like a perfect sink towards cholesterol, which was used as a model for compounds associated with the liposome bilayer. The bulk properties of the silicone membrane do not influence cholesterol transport for permeability coefficient values of much smaller than 10^{-5} cm/sec to membrane thicknesses of 100 to 500 μm.

The chemicals used in this study were all of analytical grade. L-α-Phosphatidyl choline from egg yolk, Type IX-E (Sigma Chem., St. Louis, MO USA) was purified as described by Singleton et al. [15] and after purification (as confirmed by TLC) stored in

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chloroform under nitrogen at −20°. The liposomes were produced according to the method of Papahadjopoulos and Watkins [16], using 1.1 μmol L-α-phosphatidyl choline/ml saline and 1.1 μmol/ml cholesterol for the production of liposomes having a 1 : 1 phosphatidyl choline : cholesterol molar ratio. Liposomes containing phosphatidyl choline and cholesterol in a 7 : 2 molar ratio were produced using 1 . 1 μmol/ml L-α-phosphatidyl choline and 0.31 μmol/ml cholesterol. Radioactively labeled solute liposomes were produced by addition of $1.39 \cdot 10^{-2}$ μmol/ml [4-^{14}C]cholesterol (50 mCi/mmol) (New England Nuclear, Boston, MA, USA) or $1.39 \cdot 10^{-2}$ μmol/ml β-[4-^{14}C]sitosterol (50 mCi/mmol) (Amersham, Arlington Heights, IL, USA) to the chloroform solution of the other compounds. In the case of addition of the labeled compounds, correspondingly smaller amounts of unlabeled cholesterol were added. The organic solvents were then evaporated under nitrogen. The saline (0.145 M NaCl) was then added and the solution was stored overnight under nitrogen at 4°C. This solution was then vortexed for 1 min and sonicated at 25 kHz in a bath-type sonicator (Branson, Shelton, CT, USA) for 1 h. The solutions were then used as such in the diffusion cell as described by Karth and Higuchi [14] or centrifuged (L3-40, Beckman, Palo Alto, CA, USA) for 1 h at 100 000 × g preceding addition of the supernatant to the diffusion cell. The silicone rubber membrane (Dow, Midland, MI, USA) in the diffusion cells had a thickness of 127 μm. In some experiments the supernatant was diluted 1 : 5 with saline prior to pipeting into the diffusion cell. Samples of 0.5 ml were drawn after an initial equilibration time of 0.5 h (time = zero) from the donor as well as from the receiver compartment, and at various time intervals (see Fig. 1) from the receiver compartment. The samples were replaced by the same volume of unlabeled liposome preparations. At the end of the experiments, another sample was drawn from both compartments. These samples were mixed with 0.5 ml of a 10% polysorbate 80 solution in order to lyse the liposomes. Then 10 ml scintillation cocktail (ACS, Amersham, Arlington Heights, IL, USA) was added and the samples counted in a scintillation counter (LS-150, Beckman, Palo Alto, CA, USA).

Aliquots of the donor and of the receiver solutions of some batches were tested for degradation products and radioactive impurities by TLC as described by Singleton et al. [15]. In addition, a n-hexane : diethyl ether : acetic acid, 95 : 7 : 1 (v/v) solvent system was used. No degradation products of the liposome components could be found. Radioactivity levels slightly above the background could be detected only in the cholesterol spot. These results indicate that radioactive impurities did not contribute significantly to the measured cholesterol transport. The amount of transported labeled sitosterol was too low to be detectable after TLC of the receiver solution.

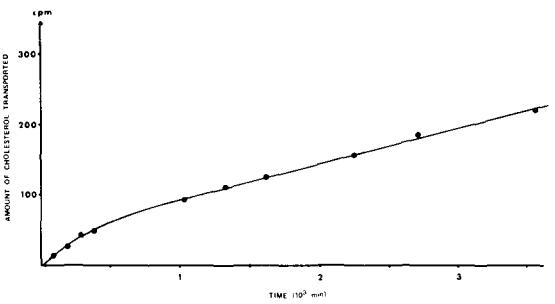

Fig. 1. Typical plot of the amount of sterol transported through the silastic rubber membrane versus time, measured by appearance of radioactivity in the receiver chamber.

The chemical composition of the liposomes was confirmed by passage over a Sephadex G-50 (Pharmacia AB, Uppsala, Sweden) column as described by Papahadjopoulos and Watkins [16]. 1 ml fractions were collected and assayed for radioactivity as described above and for phosphate using the method of Fiske and Subbarow [17]. The phosphate/cholesterol or the phosphate/sitosterol ratio, respectively, was the same in all fractions thus indicating a homogeneous chemical composition of the liposomes.

The transport of the sterols was measured by appearance of radioactivity in the receiver compartment. A typical curve is shown in Fig. 1. The amount of radioactivity transported per time unit is constant between 8–21 h, as indicated by the straight line. The biphasic slope is probably caused by an equilibrating process between liposome and environment at earlier times. The permeability coefficients at the steady-state phase were calculated using Eqn. 1,

$$P = \frac{V \cdot (dC/dt)}{A \cdot \Delta C} \qquad \text{eqn. 1}$$

TABLE I

PERMEABILITY COEFFICIENTS FOR THE TRANSPORT OF LIPOSOME BILAYER-ASSOCIATED STEROLS THROUGH SILICONE RUBBER MEMBRANES IN $cm \cdot s^{-1}$

Labeled sterol	Lecithin/cholesterol ratio	Treatment of liposomes	Permeability coefficients ± S.D.
[4-^{14}C]Cholesterol	7 : 2	non-centrifuged undiluted	$7.2 \cdot 10^{-9} \pm 0.5$
[4-^{14}C]Cholesterol	1 : 1	non-centrifuged undiluted	$8.7 \cdot 10^{-9} \pm 2.2$
[4-^{14}C]Cholesterol	1 : 1	centrifuged 100 000 × *g* undiluted	$1.6 \cdot 10^{-8} \pm 0.3$
[4-^{14}C]Cholesterol	1 : 1	centrifuged 100 000 × *g* diluted 1 : 5	$1.4 \cdot 10^{-8} \pm 1.2$
β-[4-^{14}C]Cholesterol	1 : 1	centrifuged 100 000 × *g* undiluted	$3.7 \cdot 10^{-9} \pm 0.7$

where P is the permeability coefficient, V is the half-cell volume, A is the diffusional area, dC/dt the steady-state slope in counts transported/s and ΔC the concentration differential across the membrane which was taken to be equal to the donor phase concentration at time zero.

The permeabilities are shown in Table I. A somewhat lower permeability was observed for the liposomes with a phosphatidyl choline : cholesterol molar ratio of 7 : 2 in comparison to the 1 : 1 molar ratio. This difference, however, was not significant according to Student's *t*-test.

After centrifugation at 100 000 *g* for 1 h, only small homogeneous unilamellar liposomes of a particle size of 250–300 Å were observed by electron microscopy (Temscan-100C, JEOC, Tokyo, Japan) after staining with a 2.5% ammonium molybdate solution with a pH adjusted to 7.4. No apparent difference between liposomes containing radioactively labeled sitosterol, radioactively labeled cholesterol, and unlabeled liposomes could be observed. The centrifugation pellet, however, contained larger multilamellar structures showing that 1 h sonication alone was not sufficient to obtain homogeneous small liposomes. The permeability coefficient of the noncentrifuged liposomes containing multilamellar larger vesicles was only half of that obtained after centrifugation at 100 000 × *g* ($2P < 0.02$).

Dilution of the liposome suspension did not increase the permeability coefficient, indicating that the liposome delivers the cholesterol to the membrane and that free solute is not involved in the transport to the membrane. If free solute were involved in the transition state, i.e., the rate determining step, one would expect P to be a function of the ratio of the free solute C_F to the total solute C_T so that:

$$P = P_F \frac{C_F}{C_T} \qquad \text{eqn. 2}$$

where P_F is the intrinsic interfacial permeability coefficient for the free solute and is constant for constant barrier conditions and independent of the total solute in the aqueous phase [18]. Because of the extremely small water/liposome partition coefficient for cholesterol, the following limiting equation holds:

$$C_F = \frac{C_T}{V_L} K \qquad \text{eqn. 3}$$

where V_L is the volume fraction of the liposomes and K is a partition constant. Since it is clear that dilution of the system will not affect the C_T/V_L ratio, C_F is independent of dilution. As our data shows no dependence of P upon dilution, it follows from Eqns. 2 and 3 that the free solute delivery mechanism is not a significant pathway.

The lower P value for sitosterol ($2P \ll 0.001$) indicates that unloading must occur in an environment which is still aqueous or hydrophilic. If the intact liposome had carried the solute into the membrane, one would expect similar P-values for cholesterol and sitosterol. It is interesting to note that the difference in the P-values between these sterols is similar to that observed in micelle delivery of these sterols to oil droplets [19]. In the above-mentioned study, a system in which the solute was transported from an aqueous polysorbate 80 solution to hexadecane droplets, the permeability coefficient

of sitosterol was 4–8-times smaller than that of cholesterol. This may be an indication that the rate limiting step, namely the interfacial barrier, is similar in the micellar and in the liposome system. The importance of the interfacial barrier for limiting the transport rate of some organic solutes across interfaces was first observed and described in detail by Bikhazi and Higuchi [19]. Although the nature of this barrier is to date not fully understood at the molecular level, our data suggest a concerted two-step process involving a 'collision complex' followed by a transfer of solute through a thin hydrophilic layer into the silastic membrane surface. Had the mechanism, for example, involved direct coalescence of the liposome with the silicone membrane interface, the permeability coefficients for cholesterol and sitosterol would be expected to be the same, since the process involves transfer from one hydrophobic environment to another. Further work is being pursued to gain more insight into the molecular characteristics of this interfacial barrier.

The results of this study provide insight as to the possible mechanism of liposome delivery of hydrophobic drugs to cell membranes. Although this simple experimental system cannot differentiate all of the possible mechanisms of solute transfer, this method can discriminate between facilitated transfer of drug by adsorption of the liposomes at the membrane surface and simple diffusion of drug to the membrane. As mentioned above, a simple diffusion mechanism, i.e., diffusion of free solute, can be ruled out because the permeability coefficient did not increase after dilution of the liposome suspension. The lower permeability coefficients obtained for sitosterol as compared to cholesterol indicate that a hydrophilic interfacial barrier is involved in the sterol delivery.

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