Effects of Steroids and Vitamin D₃ on the Permeability of Liposomal Bilayer Membranes

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Abstract: Molecular interactions between L-α-dipalmitoylphosphatidylcholine (DPPC) and steroids differing in the number of double bonds in a monolayer and bilayer were examined by measurement of surface pressure, permeability and microviscosity. The steroids used were 7-dehydrocholesterol (7-DHC), cholesterol (Chol) and β-cholestanol (β-Chol) along with Vitamin D₃ (VD₃) having an opened steroid B-ring. The limiting molecular area for the steroids in the DPPC-steroid mixed monolayer increased with the number of double bonds in the steroid ring in the order β-Chol<Chol<7-DHC. Liposomes were formed with steroids at steroid mole fractions less than 0.5 and VD₃ less than 0.3. The permeability of the liposomes containing steroids decreased with the number of double bonds in the steroid ring and steroid limiting molecular area. That of liposomes containing VD₃ with limiting molecular area exceeding that of steroids was higher than for liposomes containing steroids. The effects of steroids or VD₃ on the permeability of liposomal bilayer membranes cannot be adequately explained based on the number of double bonds or limiting molecular area, although Demel et al. have shown a correlation between the effects of sterols on the permeability of liposomes and molecular area of sterols in monolayers. The permeability of liposomal bilayer membranes containing steroids or VD₃ was found in this study straightly decrease with microviscosity near the bilayer center. The permeability of liposomal bilayer membranes may thus be considered to related to microviscosity near the bilayer center, which can be evaluated based on fluorescence polarization determined with the fluorescent probe, 12AS.

Key words: liposomal bilayer membrane, steroid, vitamin D₃, permeability of membrane, microviscosity

1 Introduction

Many successful experimental achievements have been reported on the medical applications of liposomes, consisting of lipid bilayer membrane, as a drug carrier for the purpose of reduction of drug toxicity or targeting of drugs to specific cells⁽¹⁾. Many studies, however, have been done from the pharmacological viewpoint, only a few studies⁽²⁾ have been done on the physicochemical properties of liposomes and lipid monolayers. Kruyff et al.⁽³⁾ have shown that cholesterol reduces the permeability of liposomal bilayer membranes while epicholesterol does not affect the membrane permeability. Demel et al.⁽⁴⁾ have studied on the
structural requirements of sterols for the interaction with lecithin at the air-water interface and the effect of sterol structure on the permeability of liposomes: the interaction of lecithin with sterols is dependent on the planar sterol nucleus and the intact side chain of sterol; the effects of sterols on the permeability properties of liposomes are in agreement with the effects on the mean molecular area measured in monolayers. In our previous paper, it has been found that the interaction between (hydrophobic groups of) phospholipid and steroids in the monolayer and bilayer membranes increases with decreasing the number of double bonds in the steroid molecules.

In this paper, the effect of double bonds in the steroid ring and molecular area on the interaction between phospholipid and steroids will be reported in detail in terms of permeability and microviscosity of liposomes. The microviscosities of liposomes at the three different membrane regions are measured by three kinds of fluorescent probes, and a correlation between the microviscosity and the permeability will be discussed.

2 Experimental Section

2-1 Materials

Phospholipid L-α-dipalmitoylphosphatidylcholine (DPPC, 99.6% pure) was supplied from Nippon Oil and Fats Co., Ltd., and was used without further purification.

Steroids and Vitamin D₃ Cholesterol (Chol, 99.9% pure), 7-dehydrocholesterol (7-DHC, 98% pure) and 9,10-secocholesta-5,7,10(19)-trien-3β-ol (VD₃, 99% pure) were purchased from Sigma Chemical Co., and were used without further purification. β-Cholestanol (β-Chol) was purchased from Sigma Chemical Co. and recrystallized from ethanol and analyzed the purity by differential scanning calorimetry. The molecular structures of these compounds are shown in Fig.1.

Charged Materials Dicetyl phosphate (DCP, 99.6% pure) was purchased from Sigma Chemical Co., and was used without further purification.

Fluorescent Probes 2-(9-Anthroyloxy) palmitic acid (2AP, 98% pure), 7-(9-anthroyloxy) stearic acid (7AS, 98% pure), 12-(9-anthroyloxy) stearic acid (12AS, 98% pure) and 1,6-diphenyl-1,3,5-hexatriene (DPH, 98% pure) were purchased from Sigma Chemical Co., and were used without further purification. Water used in this study was deionized and twice distilled. Its resistivity was about 18.0 MΩ. Chloroform (99.0% pure) from Wako Pure Chemical Ind., Ltd. was used after distillation. Glucose was purchased from Nippon Rikagakuyakuhin Co., Ltd. Phosphate-buffered saline (PBS) whose pH is 7.4 was purchased from Nissui Pharmaceutical Co., Ltd. All other chemicals were commercial products of reagent grade.

2-2 Method

2-2-1 Measurement of Surface Pressure

The surface pressures of DPPC/sterol or VD₃ mixed monolayers at the air-water interface were determined by a Wilhelmy plate method using a surface pressure meter of type HBM-A (Kyowa Interface Science Co., Ltd.) with a bar made of Teflon. The mixtures of DPPC and steroids or VD₃ in various molar ratios were dissolved in chloroform; the total lipid concentration was 1.5 × 10⁻³ mol/L. After the lipid-chloroform solution was spread on the water without causing surface disturbance using a microsyringe, the system was allowed to stand for 15 min. The speed of compression on the monolayer was 20 mm/min, considering the adsorption equilibrium of lipids at the surface.

2-2-2 Preparation of Liposomes

Liposomes, which were reversed-phase evaporation vesicles (REV), were prepared by a conventional technique similar to the method of Szoka and Papahadjopoulos. Briefly, DPPC, steroid or VD₃, and DCP were dissolved in a mixture of diethyl ether and ethanol. A water in oil (W/O) emulsion was formed by sonication (bath type; Branson B-222). After the solvent of the emulsion

Fig.1 Molecular Orientational Structures of Steroids and VD₃ at the Air-water Interface.
was removed using a rotary evaporator, gel formed. Finally, REV was obtained after being shaken on a vortex mixer for several times. Liposomes were extruded at 55°C through a polycarbonate membrane filter of 0.22 μm pore size according to the method of Olson et al.9).  

2-2-3 Quantification of DPPC  
The concentration of DPPC was determined by enzymatic assay using a phospholipids-B-test Wako (Wako Pure Chemical Ind., Ltd.).  

2-2-4 Measurement of Permeability of Bilayer Membranes  
The time course of glucose leakage from the liposomes was determined. Briefly, glucose as an aqueous model material was entrapped into liposomes and the unentrapped glucose was separated from the liposomes using a dialysis technique. The liposomes were dialyzed in a cellophane tube (Union Carbide Co., Chicago, IL) with 1 L of saline for 3 h at about 5°C; the saline was changed three times during the dialysis. This method and conditions are commonly used to separate the unentrapped materials. Then, the amount of glucose entrapped in the liposomes was determined. Glucose was extracted into a water phase from the liposomes, according to the procedure of Bligh-Dyer10), and then assayed by the phenol-sulfuric acid method11) using a spectrophotometer (type MPS-2000, Shimadzu Co., Ltd.).  

2-2-5 Measurement of Microviscosity of Bilayer Membranes  
Liposomes containing fluorescent probes, either 2AP, 7AS, 12AS or DPH, in their membranes and enclosing PBS in their internal water phase, were prepared. The total lipid concentration was 1 × 10⁻² mol/L. The molar ratio of lipids to probe was 300 : 1 (for 2AP, 7AS, or 12AS) or 250 : 1 (for DPH). The microviscosity of liposomes was determined by fluorescence polarization (P), which can be calculated according to the following equation:

\[ P = \frac{(I_p - G I_v)}{(I_p + G I_v)} \]

where \( I_p \) and \( I_v \) are the fluorescence intensities of the emitted light polarized parallel and vertically to the exciting light, respectively, and \( G \) is the grating correction factor12). The fluorescence intensities of \( I_p \) and \( I_v \) were measured at 35°C with a spectrofluorophotometer (type RF-540, Shimadzu Co., Ltd.), and excitation and emission wavelengths were 350 and 450 nm, respectively. When DPH was used as a fluorescent probe, the excitation and emission wavelengths were 360 and 430 nm, respectively, and the experiments were carried out at 5–60°C.  

All experiments except for the measurement with DPH were carried out at 35°C.  

3 Results and Discussion  
3-1 DPPC/Steroids or VD₃ Mixed Monolayers  
It has been found9) that the interaction between DPPC and steroids depends on the number of side chain and the existence of a double bond in a side chain of steroid molecule. In this study, in order to investigate the effect of planar structure of steroid ring, surface pressure-molecular area curves of DPPC/7-DHC or VD₃ mixed monolayers at the molar ratio of 5 : 5 were measured, and the limiting area and collapse pressure of 7-DHC and VD₃ obtained from the surface pressure-molecular area curves were listed in Table 1.  

In Table 1, the values9) for Chol and β-Chol are also listed, where 7-DHC, Chol and β-Chol have two, one and zero double bond in the steroid B-ring, respectively, VD₃ has a planar structure in spite of the steroid ring being opened. The limiting molecular area for the steroids in the DPPC-steroid mixed monolayer increased with the number of double bonds in the steroid ring in the order β-Chol < Chol < 7-DHC. Similar phenomena have been found for icosapolyenoic acids without ring structure13). The limiting molecular area of VD₃ was larger than that of steroids. It has been found9,14) that the limiting molecular areas depend on the orientation of molecules at the air-water interface. Considering the limiting areas of steroids and VD₃ obtained in this study, the

<table>
<thead>
<tr>
<th></th>
<th>VD₃</th>
<th>7-DHC</th>
<th>Chol9)</th>
<th>β-Chol9)</th>
</tr>
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<td>limiting molecular area (Å²/molecule)</td>
<td>51.0</td>
<td>44.4</td>
<td>43.9</td>
<td>43.3</td>
</tr>
<tr>
<td>collapse pressure (mN/m)</td>
<td>33</td>
<td>45</td>
<td>47</td>
<td>48</td>
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</tbody>
</table>

Table 1 Limiting Molecular Area and Collapse Pressure for Steroid or VD₃ (DPPC : steroid or VD₃=5 : 5 mixed monolayer).
Fig. 2 Effect of the Amount of Added 7-DHC (■) or VD₃ (●) on the DPPC Liposome Formation at 35°C.

The concentration of DPPC is 1×10⁻² mol/L.

3.2 Effect of 7-DHC and VD₃ on Formation of Liposomes

The effect of the mole fraction of 7-DHC or VD₃ on the formation of liposomes was firstly examined. Figure 2 shows the percentages of DPPC in bilayer membranes containing 7-DHC or VD₃. The DPPC amount at 100% on the ordinate axis means that all the DPPC is used to form liposomes. In the case of 7-DHC, up to the addition of 0.5 mole fraction of 7-DHC all the DPPC was able to contribute to the formation of liposomes. In the case of VD₃, up to the addition of 0.3 mole fraction of VD₃ all the DPPC was able to contribute to the formation of liposomes. Beyond these additive amounts, not all the DPPC was utilized. It has been found that the liposomes containing Chol or β-Chol with mole fractions less than 0.5 can be formed. It seems that the number of double bonds in a steroid ring and the limiting area of steroids do not affect the liposome formation. Whereas the planar structure of a molecule is likely to affect the liposome formation.

3.3 Permeability of Bilayer Membranes

In order to investigate the effects of double bonds in the steroid ring and of the planar structure of a molecule on the stability of liposomes, the permeability of bilayer membranes was measured. In this measurement liposomes with a mole fraction of 0.3 of steroids or VD₃ were used, because of the ability to form liposomes as indicated in Fig. 2. Figure 3 represents the time course of glucose leakage from liposomes.

The permeability of bilayer membranes decreased by the addition of steroids or VD₃. The order of permeability of liposomes is 7-DHC < Chol < β-Chol < VD₃, namely the order of stability of bilayer membrane is VD₃ < β-Chol < Chol < 7-DHC. The permeability of bilayer membranes decreased with increasing the number of double bonds in the steroid ring and increasing the limiting area of steroids. The planar structure of steroids having double bonds in the steroid ring is likely to affect the permeability of bilayer membranes. The relationship between the permeability of liposomes and the molecular area of steroids included in the liposomes is consistent with the result reported by Demel et al. However, the permeability of liposomes containing VD₃ cannot be adequately explained based on the limiting molecular area or the number of double bonds in the steroid ring: the permeability of liposomes containing VD₃ is higher than that of 7-DHC in spite of the larger limiting area. Thus we measured the microviscosity of liposomes containing steroids or VD₃ by using a fluorescent probe DPH, where DPH exists hydrophobic region in
the lipid bilayer and is able to evaluate the microviscosity around DPH. Fluorescence polarization is correlated to microviscosity near the fluorescent probes\textsuperscript{16,17}, which is calculated using the Perrin-Weber’s equation\textsuperscript{18}. Microviscosity increases with increasing fluorescence polarization.

3.4 Effect of Steroids or VD\textsubscript{3} on Microviscosity of Bilayer Membranes

The results are shown in Fig. 4.

As is evident from Fig. 4, the fluorescence polarization of DPH in the DPPC bilayer membranes without steroid suddenly decreases above 42°C, which agrees with the phase transition temperature of DPPC. The fluorescence polarization of DPH in the lipid bilayer membranes containing steroid or VD\textsubscript{3} also decreases above the phase transition temperatures of the lipid. The change in the fluorescence polarization of DPH is dependent on the kind of compounds contained in the DPPC bilayer membranes: the order of the fluorescence polarization above the phase transition temperature is 7-DHC > Chol > β-Chol > VD\textsubscript{3}, which is consistent with the order of the permeability of lipid bilayer. The lipid bilayer containing VD\textsubscript{3} indicates the lowest fluorescence polarization (namely, lowest microviscosity) and the highest permeability. It is found that the microviscosity of lipid bilayer is related to the permeability of bilayer membrane.

Next, the relationship between the permeability and the microviscosity of bilayer membranes was investigated in detail by using fluorescent probes 2AP, 7AS and 12AS. It is generally known\textsuperscript{16,17} that the fluorescence polarization of 2AP in the bilayer membranes indicates the microviscosity in the region near the hydrophilic group of bilayer membranes (in the region close to the membrane surface), 7AS indicates the region near the hydrophobic group of bilayer membranes and 12AS can probe the region near the bilayer center, respectively. Figure 5 shows the relationship between the permeability of bilayer membranes to glucose (after a 72 hours incubation) and the microviscosity of bilayer membranes.

It is found that the permeability of bilayer membranes decreases with increasing the microviscosity. Furthermore, it is noted that the permeability straightly decreases with microviscosity of lipid bilayer membranes, which can be evaluated based on fluorescence polarization determined with the fluorescent probe, 12AS. The permeability of liposomal bilayer membranes may thus be considered to related to microviscosity near the bilayer center rather than near the membrane surface.

4 Conclusion

The permeability of liposomes was decreased by adding steroids or VD\textsubscript{3} in the lipid bilayers. The permeability of liposomes containing steroids decreases with increasing the number of double bonds in the steroid ring and increasing the limiting molecular area of steroids, while the permeability of liposomes containing VD\textsubscript{3}, whose
limiting molecular area is larger than steroids, was higher than that of the liposomes containing steroids, indicating that the permeability of liposomal bilayer membranes cannot be adequately explained based on the number of double bonds or limiting molecular area. Whereas the permeability of liposomes containing steroids or VD₃ straightly decreased with microviscosity near the bilayer center. It is concluded that the permeability of liposomal bilayer membrane is related to microviscosity near the bilayer center, which can be evaluated based on fluorescence polarization determined with the fluorescent probe, 12AS.

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References
[報文] リポソーム二分子膜の透過性に及ぼす
ステロイドとビタミン D₃ の影響

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単分子膜および二分子膜における L-α-ジパラミトイルホスファチジルコリン (DPPC) と二重結合の数の異なるいくつかのステロイドとの相互作用を表面圧、膜透過性および微視的粘性を測定することにより検討した。本研究で用いたステロイドは 7-デヒドロコレステロール (7-DHC), コレスステロール (Chol), β-コレスタノール (β-Chol) であり、その他にステロイド B-環が開裂した構造を持つビタミン D₃ (VD₃) も使用した。

DPPC-ステロイド混合単分子膜におけるステロイドの極限分子占有面積は、ステロイドリング内に二重結合が増えるにつれて増加し、その順序は β-Chol<Chol<7-DHC であった。次に、リポソーム形成能については、ステロイドのモル分率 0.5 以下、VD₃ のモル分率 0.3 以下では加えた DPPC は全てリポソームを形成した。ステロイドを含んだリポソームの膜透過性はステロイド環中の二重結合の数が増えるにつれて減少し、またステロイドの極限分子占有面積が増えるにつれて減少した。一方、VD₃ を含んだリポソームの場合には、VD₃ の極限分子占有面積はステロイドよりも大きいにもかかわらず、膜透過性はステロイドを含んだリポソームの膜透過性よりも高かった。すなわち、Demel らは単分子間中で測定されるステロイドの分子占有面積とリポソームの膜透過性に及ぼすステロイドの影響との関係性を示しているのではあるが、リポソーム二分子膜の透過性に及ぼすステロイドあるいは VD₃ の影響は二重結合の数や極限分子占有面積では充分には説明できないことが示された。しかしながら本研究において、ステロイドあるいは VD₃ を含んだリポソーム二分子膜の透過性は二分子膜中心付近の微視的粘性が増加するにつれて直線的に減少することが認められた。すなわちリポソーム二分子膜の透過性は、栄光プローブ 12 AS を使った栄光方向の測定により求められる二分子膜中心付近の微視的粘性とよい関連性があるものと考えられた。


[報文] キトサン誘導体を用いた水銀 (Ⅱ) イオンのイオン浮遊

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キトサン誘導体（キトパール）を使用した Hg²⁺ イオン浮遊はキトサン誘導体を吸着剤とし、凝集剤としてポリアクリルアミド系 (PAA), 浮遊剤としてヘキサデシルトリメチルアンモニウムクロライド (HTAC) を用いて行った。Hg²⁺ イオンの浮遊率は Hg²⁺ イオン濃度が 2.03 × 10⁻⁷ mol·m⁻³, キトサン誘導体の系内濃度は 1000 ppm, PAA および HTAC はそれぞれ 10 ppm を添加した最適条件のもとで、窒素ガスの微細な気泡を送り込むことによって、最大 100% 近い浮遊率を示し、さらに、pH 4-10 の広い範囲にわたって 98% の浮遊率が認められた。Hg²⁺ イオンはキトサン誘導体分子と強く配位結合されていると考えられる。それゆえ、Hg²⁺ イオン浮遊率の機構は吸着剤、凝集剤、浮遊剤の複合体形成とその相互的な作用が推察できる。

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