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# DIRECT OBSERVATION OF MOLECULAR ORDERING OF CHOLESTEROL IN HUMAN ERYTHROCYTE MEMBRANES **

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**The first observation of the orientation and order of cholesterol in a natural membrane is reported. The $^2H$-NMR spectrum of [2,2,3,4,4,6-$^2H_6$]cholesterol incorporated into human erythrocyte ghosts demonstrates that the orientation and anisotropic motion of cholesterol is very similar in natural and model membranes.**

Cholesterol is a major component of many biological membranes and has been investigated extensively. The previous studies have dealt mainly with the influence of cholesterol on other membrane components [1–3], on probe molecules [4,5] or on a bulk membrane property, such as the lipid phase transition [6]. Some information on the orientation or mobility of cholesterol in model membranes has come from neutron diffraction [7], $^1$H-NMR [8], or from $^2$H-NMR studies of specifically deuterated cholesterol in egg phosphatidylcholine (egg PC) [9,10] and dimyristoylphosphatidylcholine (DMPC) [11]. Deuterium NMR is particularly valuable in the study of anisotropic systems such as membranes [12], because the quadrupolar splitting of the $^2$H-NMR spectrum of a molecule embedded in a liquid-crystalline phase depends on both the orientation and the angular fluctuations of C-$^2$H bonds with respect to the director of the motion. In specifically-deuterated cholesterol, the rigid sterol rings ensure that a C-$^2$H bond on the ring system maintains a constant geometry with respect to the axis of motional averaging. Therefore the $^2$H-NMR spectrum of [2,2,3,4,4,6-$^2H_6$]cholesterol in DMPC [11] or egg PC [9] allows a determination of the position of the motional axis, and the degree of ordering, of the steroid backbone of cholesterol in the membrane.

We have extended this method to a natural membrane by incorporating [2,2,3,4,4,6-$^2H_6$]cholesterol into the membranes of human erythrocytes. The erythrocyte membrane is known to have approx. 27% of its total lipid weight as cholesterol [13], and this cholesterol can be exchanged with that from sonicated cholesterol/PC vesicles [14].

Ghosts of human erythrocytes were prepared by standard methods [13]. Vesicles were prepared by drying 20 mg of [2,2,3,4,4,6-$^2H_6$]cholesterol (see Refs. 9 and 11 for synthesis) and 40 mg of dipalmitoylphosphatidylcholine (DPPC) from chloroform. The mixture was sonicated in 150 ml of a KCl/Tris buffer at pH 7.4 and centrifuged at $35\,000 \times g$ for 30 min [14]. The buffer, containing the sonicated vesicles, was added to a suspension of 200 mg of ghosts in 200 ml of KCl/Tris buffer, and the mixture was stirred gently for 2 h at 37°C. The membranes were recovered by centrifugation ($35\,000 \times g$), washed with 50 ml of buffer and recentrifuged. The washing was repeated twice more with buffer, once with distilled water, and the ghosts were lyophilized. The resulting dry

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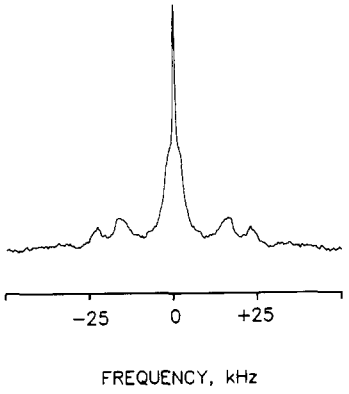


Fig. 1. $^2$H-NMR spectrum of [2,2,3,4,4,6-$^2H_6$]cholesterol in membranes of human erythrocytes.

TABLE I

QUADRUPOLAR SPLITTINGS OF [2,2,3,4,4,6-$^2H_6$]-CHOLESTEROL IN DMPC AND ERYTHROCYTE GHOSTS [a]

| Deuterium-labeled carbon position | Quadrupolar splitting (kHz) | |
|---|---|---|
| | in DMPC [b] | in ghosts |
| [6-$^2$H] | 3.4 | 4.5 |
| [4-$^2$H]$_{eq}$ | 32.0 | 34.6 |
| [2-$^2$H]$_{eq}$ | 34.2 | |
| [2,4-$^2H_2$]$_{ax}$ | 48.2 | 47.0 |
| [3-$^2$H] | 51.5 | 49.0 |

[a] Both samples at 25°C.
[b] Cholesterol: DMPC = 3:7 molar ratio.

ghosts were rehydrated with 1.5 ml of deuterium-depleted water.

The $^2$H-NMR spectra were acquired on a Bruker CXP-300 spectrometer operating at 46.063 MHz. The quadrupolar echo sequence [15], with full phase cycling of 90° (4.0 $\mu$s) pulses was used. Spectra were acquired on resonance with 500 kHz spectral windows, $1.5 \cdot 10^6$ scans and a 50 ms recycle time.

The $^2$H-NMR spectrum of [2,2,3,4,4,6-$^2H_6$]cholesterol is shown in Fig. 1. It is characteristic of axially symmetric motions in a liquid crystalline phase. It is consistent with that seen for [2,2,3,4,4,6-$^2H_6$]cholesterol in DMPC at similar cholesterol/DMPC ratios [11]; the quadrupolar splittings were assigned by comparison with the earlier results (Table I).

In order to ensure that the observed signal was from cholesterol in the ghosts, and not from small vesicles adhering to the membrane surface, a control incubation using perdeuterated dipalmitoylphosphatidylcholine (DPPC-$d_{62}$) and unlabelled cholesterol was performed. The $^2$H-NMR spectrum of the control showed only a very small signal from DPPC-$d_{62}$, indicating that the washing was successful in removing residual vesicles and that the labelled cholesterol was in the erythrocyte bilayer.

The quadrupolar splittings observed for [2,2,3,4,4,6-$^2H_6$]cholesterol in ghosts were used to

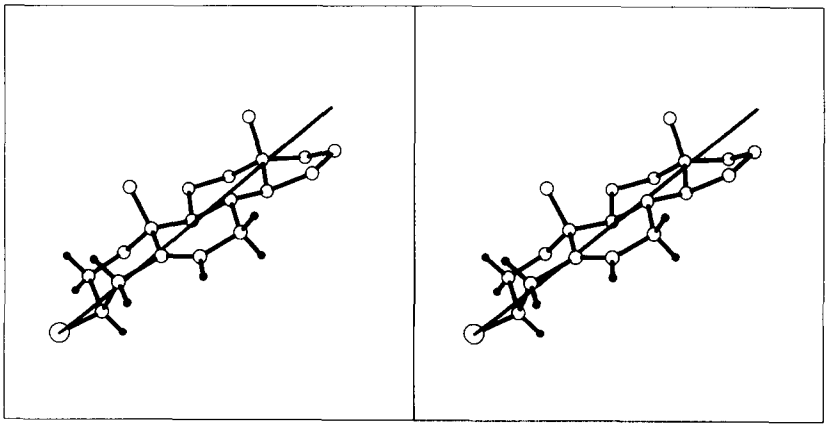

Fig. 2. Stereo plot of the axis of motional averaging for cholesterol in membranes of human erythrocytes. The small black circles are the deuterium labels (from Ref. 9).

calculate the axis of motional averaging of cholesterol (see Ref. 9 for details). The calculated axis lies along the sterol backbone and makes an angle of $\theta = 81° \pm 2°$ with the $C_3$-$^2H$ bond vector. The $\theta(C_3\text{-}^2H)$ angle is quite close to the $84° \pm 2°$ and $79° \pm 2°$ found for cholesterol in DMPC [11] and in egg PC [9], respectively, indicating a very similar orientation for cholesterol in model and natural membranes. The orientation of the axis of motional averaging is shown in the stereo plot of cholesterol [9] (Fig. 2).

Once the axis of motion is known, the determination of the molecular order parameter, $S_{mol}$, of the steroid skeleton is straightforward. The $S_{mol}$ defines the ordering of the rigid sterol rings in ghosts. A value of $S_{mol}$ of $0.76 \pm 0.05$ is calculated for cholesterol in ghosts which, within experimental error, is the same as that found for cholesterol in DMPC ($S_{mol} = 0.80 \pm 0.03$ at similar cholesterol to DMPC ratios). This is particularly interesting and indicates that the anisotropic motion of the cholesterol molecule is the same in natural and model membranes. Because the erythrocyte membrane contains a large number of different lipid classes and a high proportion of protein, the similarity in $S_{mol}$ suggests that the whole body motions of cholesterol are essentially independent of the composition of the matrix.

Finally, it is worth noting that the observed similarity between the model and natural membrane results indicates that studies of cholesterol in model membrane systems can be extrapolated to the biological membrane. This may prove particularly useful in studying the action of polyene antibiotics which are known to form complexes with cholesterol [16].

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