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PROTON MAGNETIC RESONANCE STUDY OF CHOLESTEROL TRANSFER BETWEEN EGG YOLK LECITHIN VESICLES

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Summary

The intensity of the proton magnetic resonance signal of the $(CH_2)_n$ chain in phospholipids of sonicated lecithins is sensitive to the cholesterol content in the resulting vesicles. In the present study this signal has been used to monitor transfer of cholesterol between phospholipid vesicles. Vesicles prepared from pure egg yolk lecithin were mixed with vesicles that contained equimolar amounts of cholesterol and lecithin, and the time evolution of the $(CH_2)_n$ signal intensity was followed. The results show that a homogenous distribution of cholesterol among vesicles is reached after about 4 h at 37°C and 60 h at 4°C. In order to determine the mechanism of the cholesterol transfer process, experiments were performed over a 2.5-fold range of vesicles concentrations. The accuracy of the kinetic results was not sufficient however to decide on the order of the reaction with respect to vesicle concentration. Simultaneous observation of the choline proton resonance in the presence of Eu^{+3} and Pr^{+3} indicates that fusion between vesicles does not occur during cholesterol transfer.

Introduction

Exchange of cholesterol between red blood cells or ghosts and plasma or between plasma and lipoproteins has been established using radioactive tracer techniques. It was shown that it is possible to remove up to about 50% of the cholesterol from red blood cells thereby altering their membrane properties. It is possible to remove the cholesterol by interacting the red blood cells either with cholesterol-depleted plasma or with phospholipid vesicles [1—6]. A similar procedure was used to alter the cholesterol level in lymphocytes [7].

Suspensions of lecithin vesicles produced by ultrasonic irradiation usually

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give relatively well resolved proton NMR spectra. The main features of these spectra are a strong sharp signal from the choline $N^+(CH_3)_3$ protons, an intense rather wider peak from the $(CH_2)_n$ chain, a weak peak due to the terminal CH_3 and a few other weak signals belonging to other protons of the lecithin molecules [8]. (See trace a, Fig. 1).

It is known that vesicles prepared from egg yolk lecithin are impermeable to ions [9]. This fact has been used in NMR spectroscopy to distinguish between internal and external vesicles surfaces using paramagnetic ions (e.g. Mn^{2+}, Pr^{3+}, Eu^{3+}) [10]. The signal that is predominantly affected by the paramagnetic ions is the $N^+(CH_3)_3$ choline group. The effect on the rest of the spectrum is too small to be observed. However, the shape of the $(CH_2)_n$ signal is very sensitive to the cholesterol contents; its relative peak height in the spectrum is significantly reduced upon addition of cholesterol [8]. In the present work, we have used these properties of the proton magnetic resonance spectrum to study cholesterol transfer between vesicles and to check whether fusion of vesicles occurs at the same time.

Materials and Methods

Egg lecithin grade I dissolved in a methanol/chloroform mixture within sealed ampoules was purchased from Lipid Products (South Nutfield NR Redhill, SY). Thin layer chromatography showed a single spot, so that the material was used with no further purification. The cholesterol (CH-Ln) was purchased from Sigma Chemical Co., St. Louis, Mo., $PrCl_3$ and $EuCl_3$ were purchased from the Research Chemical Nuclear Corporation of America.

A dry film of the lipid was produced by evaporating the solvent with a stream of Argon and subsequent vacuum pumping. Whenever vesicles with cholesterol were prepared, weighted amounts of cholesterol were added to the original solution of egg lecithin. The lipids were then suspended in 1—5 ml of pure 99.9% ^2H_2O or in solutions of $3 \cdot 10^{-2}$ M $PrCl_3$ and $7 \cdot 10^{-2}$ M $EuCl_3$. Vesicles were produced by ultrasonic radiation with a Braunsonic 300S sonicator, using a metal tip immersed in the suspension. Sonication was carried out to optical clarity, under argon, in glass tubes within an ice water bath. The sonicated suspension was centrifuged at $20\,000 \times g$ for 30 min at 4°C. Usually three phases could be distinguished after centrifugation, a middle phase and two smaller surrounding phases. The vesicles of only the bulk middle phase were studied in our experiments. PMR experiments were conducted on a Brucker 90 MHz instrument. Spectra were obtained by a CW technique, recordered every 80 s. The recording time was 15 s. A baseline was chosen systematically for all spectra, and from it the CH_2 signal intensity was measured.

Results and Discussion

In Fig. 1, the effect of cholesterol on the PMR spectrum of the egg yolk lecithin vesicles is demonstrated. Trace a is the spectrum of vesicles prepared from a dispersion of 50 mg/ml lecithin in ^2H_2O. Traces b and c were obtained from vesicles composed of lecithin and cholesterol at molar ratios 2 : 1 and 1 : 1 respectively. It may be seen that cholesterol produces a considerable reduction

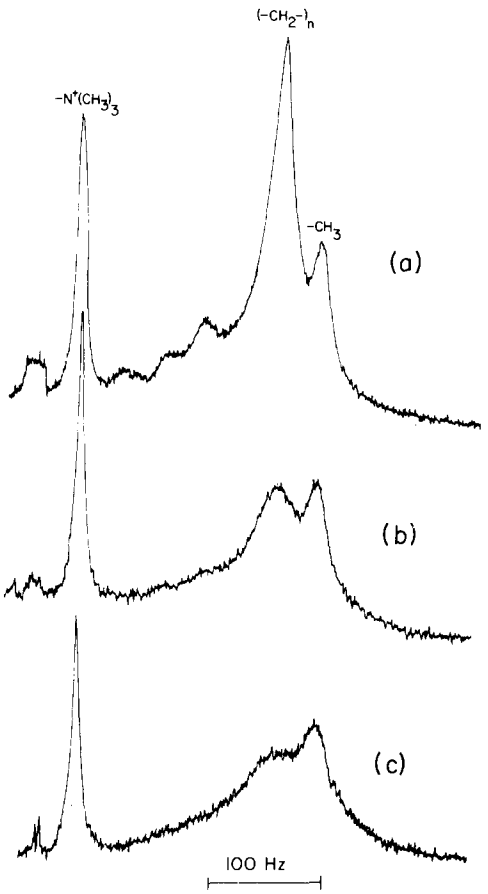

Fig. 1. Spectra of vesicles in ^2H_2O composed of (a) 50 mg/ml egg lecithin, (b) 50 mg/ml lecithin and 13 mg/ml cholesterol (2 : 1 molar ratio), and (c) 50 mg/ml lecithin and 26 mg/ml cholesterol (1 : 1 molar ratio).

in the relative height of the $(CH_2)_n$ peak. Thus, this peak can be used to monitor the cholesterol content of vesicles. In practice, the exchange of cholesterol between phospholipid vesicles was studied by mixing equal amounts of two types of vesicles suspensions: (i) pure egg yolk lecithin vesicles and (ii) vesicles consisting of lecithin cholesterol in molar ratio 1 : 1. The exchange of cholesterol was studied by monitoring the NMR spectrum of the mixture as a function of time after mixing. In Figs. 2 and 3 are shown such spectra recorded respectively at 37 and 4°C. In these particular mixtures, suspension i was prepared by sonication in the presence of $3 \cdot 10^{-2}$ M $PrCl_3$ and suspension ii in the presence of $7 \cdot 10^{-2}$ M $EuCl_3$. The paramagnetic ions were added in order to check whether fusion of vesicles occurs during cholesterol exchange. This problem will be discussed below. It should be stressed however, that the paramagnetic ions have no effect on the kinetic studies and similar time evolution of the $(CH_2)_n$ signal was observed in mixtures not containing paramagnetic ions.

The spectral feature of the $(CH_2)_n$ peak immediately after mixing can be visualized as a superposition of trace a and c of Fig. 1. As time proceeds the

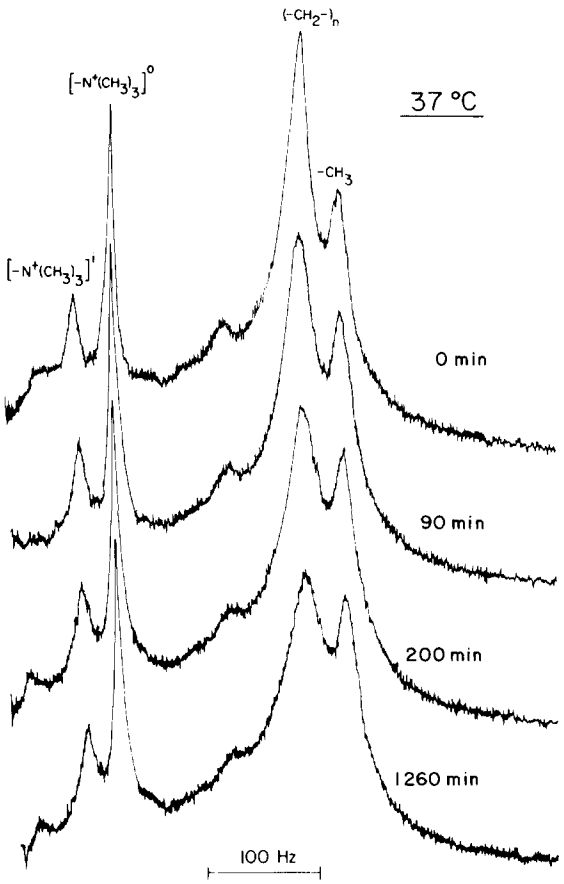

Fig. 2. Spectra of a mixture of equal volumes of vesicles with the following constituents. (1) 50 mg/ml lecithin sonicated in the presence of $3 \cdot 10^{-2}$ M $PrCl_3$ and (2) 50 mg/ml lecithin and 26 mg/ml cholesterol (1 : 1 molar ratio), sonicated in the presence of $7 \cdot 10^{-2}$ M $EuCl_3$. The mixture was incubated at 37°C and spectra recorded at different times after mixing, as indicated.

intensity of this peak is reduced reflecting redistribution of cholesterol among the vesicles. The final shape of this peak resembles that of trace b of Fig. 1, i.e. the spectrum of vesicles consisting of egg lecithin and cholesterol in molar ratio 2 : 1, indicating that the cholesterol was distributed equally among the vesicles. It takes about 4 h to complete this process at 37°C and about 60 h at 4°C. It may, however, be significant that the time taken for completion of the cholesterol transfer (4 h at 37°C) is similar to that found previously by Smith and Green [11] for cholesterol 'flip-flop' in phospholipid vesicles, suggesting that as the cholesterol transfer proceeds, this reaction might become a limiting step.

There are several ways by which cholesterol transfer between vesicles can occur. (i) Equilibrium of cholesterol between the membrane bilayer and solvent water, (ii) transfer induced by direct collision between vesicles. This latter process might be accompanied by fusion of the vesicles. In principle, it should be possible to distinguish between i and ii by determining the order of the transfer reaction with respect to vesicle concentration. We assume that the $(CH_2)_n$ signal

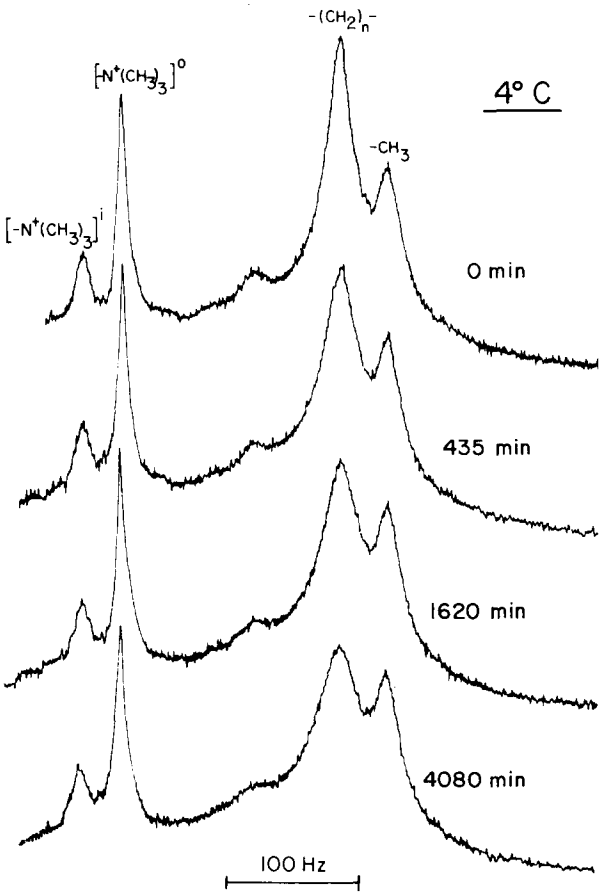

Fig. 3. Spectra of a similar mixture as in Fig. 4 incubated at 4°C and recorded at different times after mixing, as indicated.

intensity a short time after mixing, is proportional to the concentration of the cholesterol free vesicles that did not react. Kinetic studies were performed at four different vesicles concentrations. The various solutions were prepared by dilution of the most concentrated one, by as much as a factor of 2.5. Fig. 4 shows the normalized intensity of the observed $(CH_2)_n$ peak, minus the intensity of the corresponding peak due to the vesicles containing 1 : 1 lecithin cholesterol, plotted against time for the first 20 min of the process. Thus, the ordinate is proportional to the concentration of the cholesterol-free vesicles in the beginning of the reaction. The initial rate of decrease of the $(CH)_n$ peak taken from Fig. 4 are summarized in Table I, for the first stages of the transfer process. These slopes should follow approximately the relation:

$$(dA/dt)_{init.} \approx k(A)^n_{init.} \quad (1)$$

where A represents the vesicle concentration and n is the order of the reaction, with respect to vesicle concentration. Thus, a log-log plot of $(dA/dt)_{init.}$ versus A should yield n. Such a plot is shown in Fig. 5, where the bars represent the uncertainties of the kinetic results (see Table I). As may be seen, these uncer-

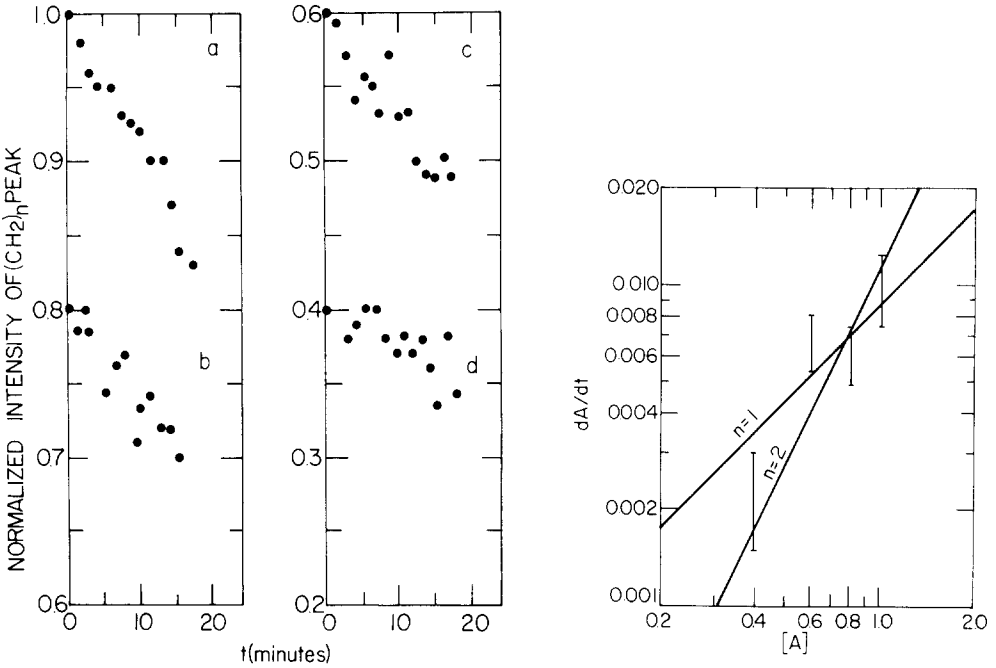

Fig. 4. Initial decrease of the $(CH_2)_n$ peak upon mixing equal amounts of lecithin vesicles with vesicles consisting of 1 : 1 lecithin/cholesterol. The total lecithin concentrations are: (a) 50, (b) 40, (c) 30, (d) 20 mg/ml. The ordinate, A, corresponds to the CH_2 peak intensity of the cholesterol-free vesicles and normalized to unity for the initial intensity of solution a.

Fig. 5. Log-log plot of the initial slopes given in Table I versus the initial concentrations of vesicles.

tainties are too large to decide on the value of n, and thus, distinguish between the two mechanisms of cholesterol transfer. However, due to low cholesterol solubility in water, we believe that the second mechanism is more plausible. We therefore proceeded to study the problem of whether the cholesterol transfer is accompanied by vesicle fusion.

Several methods to study vesicle fusion were proposed previously [12—16]. We have found it convenient to use paramagnetic shift reagents for that purpose. It has been shown that Pr^{3+} in solution interacts with the surface mem-

TABLE I

INITIAL SLOPES FOR THE DECREASE OF THE CH_2 PEAK IN 1 : 1 MIXTURES OF LECITHIN VESICLES

The concentrations correspond to total egg lecithin in mg/ml. The initial slopes are taken from Fig. 4. The two figures in each case correspond to the largest and smallest slopes that enclose the experimental points.

Concentration	Initial slope
50	0.0075, 0.0125
40	0.005, 0.0075
30	0.0055, 0.008
20	0.0015, 0.003

brane yielding an upfield shift of the choline peak while Eu^{3+} causes a downfield shift. In Fig. 6 are shown NMR spectra of egg yolk lecithin vesicles prepared by sonication in the presence of various lanthanide ions. Spectrum a corresponds to a suspension prepared in $3 \cdot 10^{-2}$ M $PrCl_3$ solution. Since in this solution, both the inner and outer surfaces of the vesicles are exposed to the same concentration of the paramagnetic ion, a single upfield shifted choline peak is obtained. Trace b is a result of a similar experiment with $7 \cdot 10^{-2}$ M $EuCl_3$, where a single downfield shifted choline peak is observed. Spectrum c of this figure has been obtained from a mixture of equal amounts of the two vesicle preparations. Three choline signals are expected to be seen in the mixture; a strong signal from the choline group of the outer vesicle layers belonging to both vesicular population and affected by the mixed environment of Eu^{3+} and Pr^{3+}, and two signals from the inner vesicle layers, one shifted upfield belonging

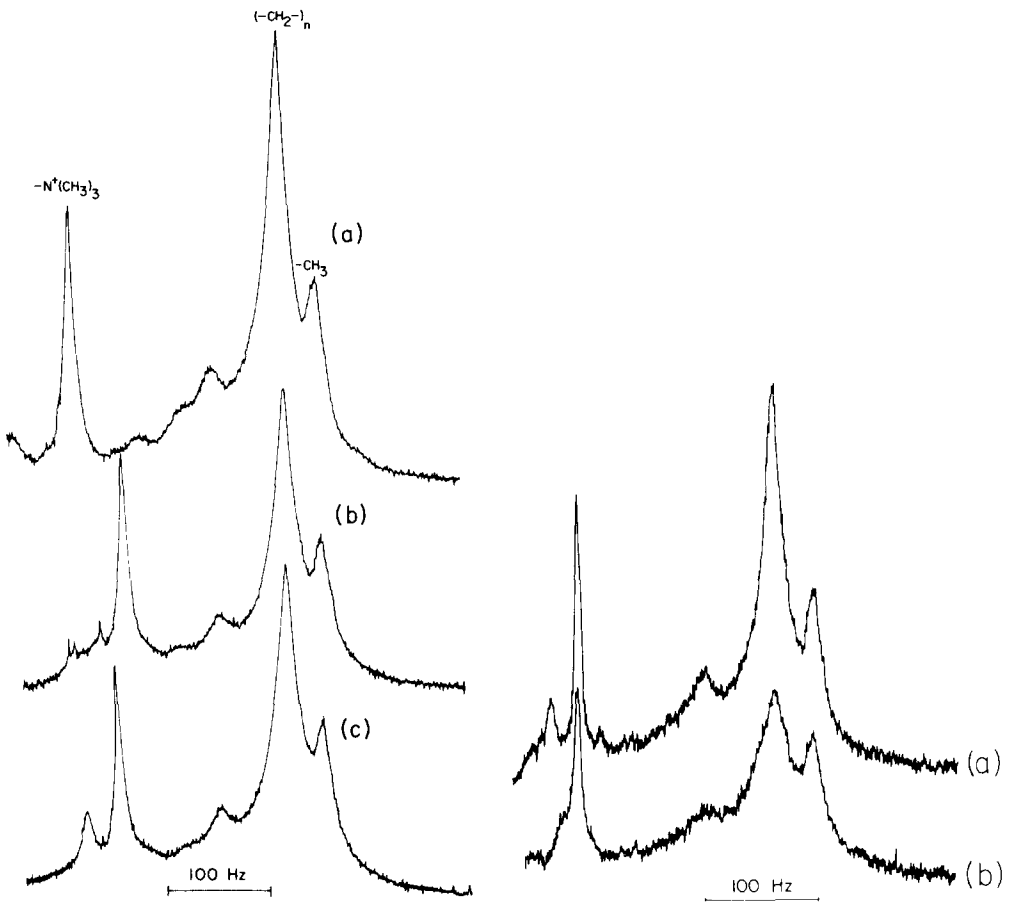

Fig. 6. Spectra of vesicles prepared from a dispersion of 50 mg/ml egg lecithin in ^2H_2O (a) sonicated in the presence of $3 \cdot 10^{-2}$ M $PrCl_3$, (b) sonicated in the presence of $7 \cdot 10^{-2}$ M $EuCl_3$, and (c) a 1 : 1 mixture of a and b.

Fig. 7. (a) Spectrum of a mixed vesicle preparation as in trace c of Fig. 6. (b) Spectrum of the same mixed preparation after incubation at 37°C with oleic acid to induce fusion.

to a vesicular population loaded with Pr^{3+} and the other shifted downfield belonging to the vesicles loaded with Eu^{3+}. The upfield peak reflecting the inner layer of vesicles with Pr^{3+} can be clearly distinguished in our experimental spectrum: the downfield signal belonging to the vesicles with Eu^{3+} is not resolved in the spectrum from the central intense peak. If fusion would occur the ionic environment within all the vesicles would be mixed and tend to become similar to that of the bathing medium, resulting in a collapse of the choline spectrum into a single peak. This is, e.g., the case when fusogenic material such as oleic acid [16] is added to the suspension, as shown in Fig. 7. The spectrum as shown in Fig. 6c remained unchanged for days indicating that no fusion occurs in these preparations. As may be seen from Figs. 2 and 3, no change in the $N^+(CH)_3$ proton spectra occurred during the time when cholesterol transfer was maintained.

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