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Effect of fatty acyl chain length of phosphatidylcholine on their transfer from liposomes to erythrocytes and transverse diffusion in the membranes inferred by TEMPO-phosphatidylcholine spin probes

Akira Tamura [a], Katsumi Yoshikawa [a], Tatsuzo Fujii [a,*], Kazuo Ohki [b], Yoshinori Nozawa [b] and Yoshio Sumida [c]

[a] *Department of Biochemistry and* [c] *Department of Pharmaceutical Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, and* [b] *Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi 40, Gifu 500 (Japan)*

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TEMPO-phosphatidylcholine (PC) spin probes which have homologous saturated acyl chains of 10, 12, 14 and 16 carbon atoms, were synthesized as analogues of PC. Transfer of TEMPO-PCs from liposomal membrane to the ghost membrane of human erythrocyte and transverse diffusion of TEMPO-PCs within the membrane of intact erythrocytes were determined by measurement of spontaneous increase and decrease in signal amplitude of an anisotropic triplet spectrum, due to dilution of the label by natural phospholipid of the membrane and reduction of the label by the cytoplasmic content of the erythrocyte, respectively. TEMPO-PC molecules in TEMPO-PC liposomes, except dipalmitoyl TEMPO-PC, were rapidly incorporated into the ghost membrane by incubation at 37°C; the PC having shorter acyl chains was transferred faster. The cytoplasmic content of the erythrocyte rapidly reduced the nitroxide radical of the spin probe. The central peak height of ESR signal was once increased by incorporation of TEMPO-PC into the erythrocyte membrane and then was spontaneously decreased during further incubation at 37°C. This decrease indicates that PC molecules traverse from the outer to the inner layer of the membrane lipid bilayer. The decrease of signal amplitude was faster with PC of shorter acyl chain. These findings suggest that both transfer between membranes and transverse diffusion in the membrane may be favored to the PC species with shorter acyl chains.

Introduction

Incorporation of amphiphilic molecules into biological membrane and their transverse diffusion in lipid bilayer of the membrane are of vital importance in understanding the interaction of various amphiphilic drugs with membranes and their effects on cellular functions.

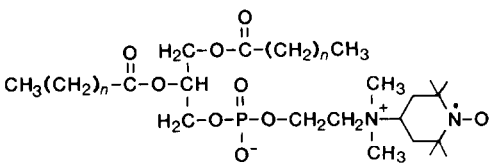
Spontaneous transfer of lipids such as cholesterol [1], phosphatidylcholine (PC) bearing medium fatty acyl chains [2,3] and lysoPC species [4] to the artificial and biological membranes has been widely observed, and there is increasing evidence that these molecules can be passively transferred between membranes as soluble monomers diffusing through the aqueous medium [5,6].

From the studies using tools such as phospholipases [7,8] and electron spin probes [9,10], it has been concluded that phospholipids

* To whom correspondence should be addressed at: Department of Biochemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan.

incorporated into model and biological membranes can be subject to transmembrane movement. Such movement of lipids in biomembranes is significantly faster than that in artificial lipid membranes such as liposomes [11], probably due to the presence of membrane-penetrating proteins in biomembranes [12].

In the present study, the effect of acyl chain length of exogeneously-added PC on the rate of transfer of lipid from the liposomal membrane to the human erythrocyte membrane and the rate of transverse diffusion from the outer to the inner layer of the lipid bilayer of the erythrocyte membrane was determined, using the ESR technique with the following spin-labeled PC having homologous acyl chains of 10–16 carbon atoms:



Materials and Methods

Erythrocytes and ghosts. Fresh human blood from normal donors was used throughout the experiments. Several milliliters of blood were drawn into a glass syringe containing heparin. The erythrocytes were washed three times with isotonic phosphate-buffered saline (PBS, 10 mM phosphate, 140.5 mM NaCl, pH 7.4) and then resuspended in phosphate-buffered saline to give a hematocrit value of 20%. Resealed ghosts were prepared by the method of Steck and Kant [13].

TEMPO-PC. Didecanoyl ($C_{10:0}$)-, dilauroyl ($C_{12:0}$)-, dimyristoyl ($C_{14:0}$)- and dipalmitoyl ($C_{16:0}$)-*sn*-glycerophosphocholine were purchased from Sigma Co. Each PC was converted to phosphatidic acid by the action of phospholipase D (from *Streptomyces chromofuscus*, Boehringer Mannheim GDH). Preparation of 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO)-choline and condensation of phosphatidic acid and TEMPO-choline were carried out according to Kornberg and McConnell [11]. Dried samples of each lipid were dispersed in phosphate-buffered saline containing 10 mM glucose with a sonicator equipped with a microprobe (Branson, Model 185) at 30 watts for 3 min at room temperature.

Determination of the TEMPO-PC incorporated from liposomes into the erythrocyte. One volume of the dispersion of 25 μM TEMPO-PC was added to one volume of the ghost suspension equivalent to a hematocrit value of 20% suspension of original washed erythrocytes at 4°C. An aliquot of the mixture was immediately transferred to a glass capillary (0.85 mm in diameter and 75 mm in length), and the ESR spectra were recorded at 37°C or 25°C during 2 h, using a JEOL FE-1X ESR spectrometer equipped with a variable temperature control unit. For most experiments the central peak height of the derivative spectrum was used as a measure of the relative spin-label concentration.

Determination of TEMPO-PC traversed from the outer layer to the inner layer of erythrocyte membrane. One volume of the dispersion of 25 μM TEMPO-PC was added to one volume of the erythrocyte suspension (hematocrit value 20%), and the mixture was incubated at 37°C for an appropriate time. After the incubation, an aliquot of the mixture was transferred to the glass capillary described above and then centrifuged at 11 000 $\times$ g for 5 min at 5°C. A part of the supernatant was cut off and ESR spectrum of the packed erythrocytes was measured at 5°C. The nitroxide radical of TEMPO-PC exposed on the inner surface of the erythrocyte membrane was rapidly reduced at 37°C by the cytoplasmic content of the cells [9]. Thus, the amplitude of the ESR signal immediately after incorporation of TEMPO-PC represents the amount of PC located in the outer layer of the membrane, and the decrease of the amplitude during incubation at 37°C appears to correspond to the amount of PC traversed from the outer to the inner layer of the bilayer.

Lipid analyses. Total lipids of the erythrocytes which had been treated with 12.5 μM of each TEMPO-PC at 37°C for 3 h were extracted with chloroform/methanol [14]. Total cholesterol content was determined by the method of Zlatkis and Zak [15] and amount of each phospholipid was determined after fractionation by two-dimensional thin-layer chromatography with chloroform/methanol/7 M ammonia (90 : 54 : 11, v/v) and chloroform/methanol/acetic acid/water (30 : 15 : 4 : 2, v/v). This solvent system could sep-

arate the TEMPO-PC from the natural phospholipids contained in the erythrocyte membrane. Lipid phosphorus was measured by the method of Bartlett [16]. The amount of the TEMPO-PC incorporated into the erythrocytes was determined by measuring the decrease in the amount of the TEMPO-PC in the supernatant of the incubation mixture [3,17].

Results

Transfer of TEMPO-PC from the liposomal membrane to the erythrocyte ghost membrane

The ESR spectra of $C_{10:0}$-PC probe before and after mixing with ghosts are shown in Figs. 1a and 1b, respectively. In the liposomal dispersion, the PC spin probe yielded a spectrum composed of a narrow triplet and an exchange-broadened signal (Fig. 1a). Mixing the PC spin probe with ghosts, however, produced a new resonance pattern (Fig. 1b) quite different from the spectrum of the TEMPO-PC liposomes per se. This new spectral pattern is attributed to the spin probes that are incorporated into the membrane lipid bilayer and then diluted by the surrounding phospholipids [9,11].

The relative amount of each TEMPO-PC transferred from the liposomal membrane to the erythrocyte membrane was obtained by an estimate of the height (H) of the midfield peak, shown in Fig. 1b, as an estimate of the relative spin-probe concentration in the latter membrane. The transfer experiment was carried out using

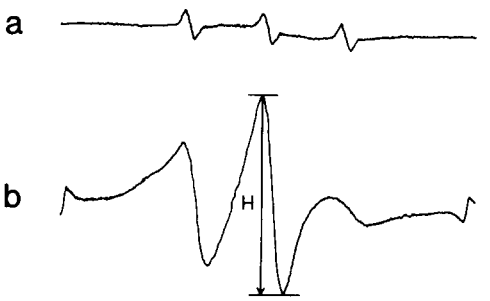


Fig. 1. ESR spectra of TEMPO-dilauroylphosphatidylcholine before (a) and after (b) mixing with erythrocyte ghost membranes.

erythrocyte ghosts because the cytoplasmic content of intact erythrocyte can reduce the nitroxide radical of TEMPO-PC rapidly, as described later. Fig. 2 shows the results of transfer experiments. By incubation with ghosts at 25°C, the peak height of $C_{10:0}$-PC probe reached a maximum level within 2 min, and the shape of spectrum and the signal intensity were consistent over 2 h. In contrast to the $C_{10:0}$-PC probe, spontaneous increase of the ESR signal was observed with $C_{12:0}$-PC and $C_{14:0}$-PC probe. The transfer of $C_{12:0}$-PC approached the maximum level in 20 min while the transfer of $C_{14:0}$-PC was much slower at 25°C. When the mixture was incubated at 37°C, as shown in Fig. 2b, the maximum incorporation of $C_{12:0}$-PC probe into the membrane occurred within 2 min as against in 20 min at 25°C, and the transfer of $C_{14:0}$-PC reached approximately the maximum level in 60 min, while $C_{16:0}$-PC was scarcely incorporated during 2 h incubation.

TABLE I

DETERMINATION OF THE LIPID CONTENT OF THE TEMPO-PC-TREATED ERYTHROCYTES

5 ml of washed-erythrocyte suspension (Ht 20%) were mixed with the same volume of 25 μM $C_{10:0}$-, $C_{12:0}$-, or $C_{14:0}$-TEMPO-PC at 37°C for 3 h. The amounts of cholesterol and each phospholipid of the cells were determined, as described in Methods. The data represent the means ± S.D. obtained from three independent experiments.

Treatment of erythrocytes with	Lipid content (μmol/10^{10} cells)				
	Phospholipid				Total cholesterol
	Phosphatidylcholine	Phosphatidylethanolamine	Sphingomyelin	Phosphatidylserine	
None (control)	1.06 ± 0.03	1.07 ± 0.01	1.06 ± 0.05	0.47 ± 0.09	3.24 ± 0.15
$C_{10:0}$-TEMPO-PC	1.04 ± 0.03	1.07 ± 0.04	1.02 ± 0.05	0.47 ± 0.02	3.23 ± 0.31
$C_{12:0}$-TEMPO-PC	1.07 ± 0.03	1.06 ± 0.03	1.01 ± 0.06	0.45 ± 0.12	3.30 ± 0.24
$C_{14:0}$-TEMPO-PC	1.06 ± 0.02	1.06 ± 0.02	1.01 ± 0.05	0.44 ± 0.12	3.25 ± 0.22

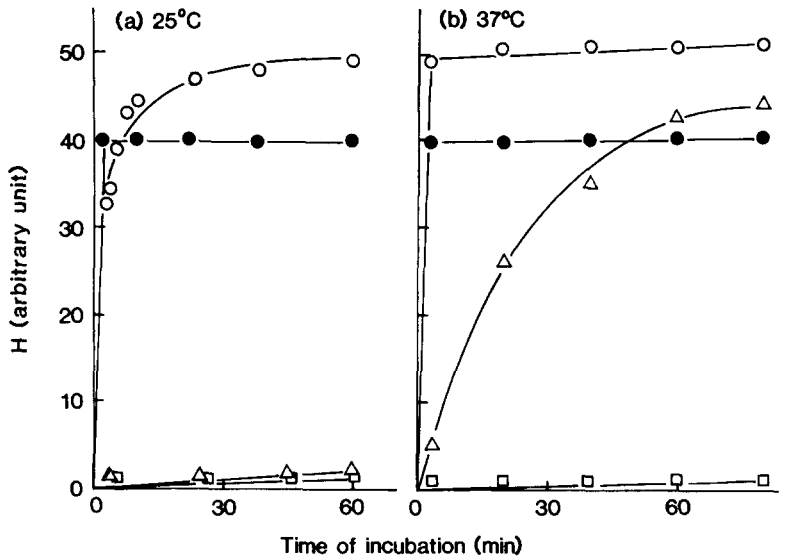


Fig. 2. Incorporation of TEMPO-PC into erythrocyte ghost membrane from liposomal dispersion. Erythrocyte ghosts were incubated with 25 μM of $C_{10:0}$-(●), $C_{12:0}$-(○), $C_{14:0}$-(△) and $C_{16:0}$-PC (□) liposomes at 25°C (a) and 37°C (b). Ordinate indicates the height of central peak shown in Fig. 1 with arbitrary unit.

Transverse diffusion of the TEMPO-PC from the outer to the inner layer of the erythrocyte membrane

As reported by Rousselet et al. [9], the nitroxide radical of TEMPO-PC located on the inner surface of the membrane of intact human erythrocyte is rapidly reduced by reducing agents present in the cytoplasma at 37°C. We applied this naturally occurring reducing agents to determine the transverse diffusion of PC spin probe. At first, we confirmed the kinetics of reduction of TEMPO-choline with the hemolysate (Fig. 3). After the erythrocyte suspensions (hematocrit value 70, 10 and 1%) were hemolyzed by sonication, they were mixed with TEMPO-choline and incubated at 37°C. The decrease of the signal amplitude depended on the hemolysate concentration; the lysate of 70% hematocrit showed a rapid decline of the ESR signal within 15 min while the 1% lysate induced no significant decrease of the signal amplitude. When the mixture of 70% lysate and TEMPO-choline was incubated at 5°C, the decrease of signal intensity was suppressed and its half-time became 20 min. Neither hemoglobin nor NADPH reduced the nitroxide radical of TEMPO-choline, while 2.5 mM glutathione (which is considered to be the physiological concentration [18]) reduced it significantly (data not shown). When the TEMPO-choline moiety of the TEMPO-PC located in the inner layer of the bilayer is exposed to the cytoplasmic content of the erythrocyte at 37°C, the nitroxide radical of the TEMPO-PC appears to be reduced immediately. Thus, the decrease of signal amplitude by incubation may reflect translocation of TEMPO-PC from the outer to the inner layer of the bilayer, since no reduction of TEMPO-PC located in the outer layer of the bilayer occurs under the present experimental condition where the hemolysis percentage of all the experiments was less than 0.5%.

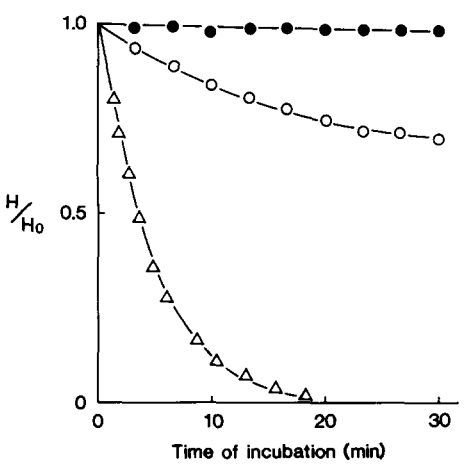


Fig. 3. Kinetics of reduction of TEMPO-choline by hemolysate. Hemolysates prepared from 1% (●), 10% (○) and 70% (△) erythrocyte suspension were incubated at 37°C for indicated time. H_0 was obtained from the spectrum of TEMPO-choline without hemolysate and H was the peak height after each incubation time.

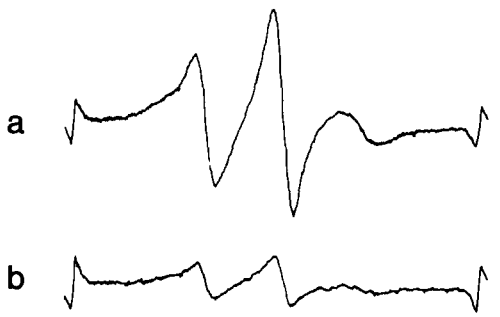


Fig. 4. ESR spectra of TEMPO-dilauroylphosphatidylcholine incorporated into the membrane of intact erythrocytes. Spectra taken immediately after incorporation (a) and after a 2-h incubation period at 37°C (b).

Before investigating the transverse diffusion of each TEMPO-PC, we examined whether treatment of erythrocytes with TEMPO-PC affects the membrane organization of the cell membrane or not. After the erythrocytes were treated with each TEMPO-PC at 37°C for 3 h, the amount of TEMPO-PC incorporated into the cell membrane and the contents of cholesterol and of each phospholipid of the cells were determined. When the cells were treated with 12.5 μM (125 nmol/10^{10} cells) of $C_{10:0}$-, $C_{12:0}$- or $C_{14:0}$-PC probe, 30, 75 or 96 nmol of TEMPO-PC per 10^{10} cells, respectively, were incorporated into the membrane. Both cholesterol and phospholipid contents of these cells treated with each TEMPO-PC were almost the same as those of the untreated cells (Table I).

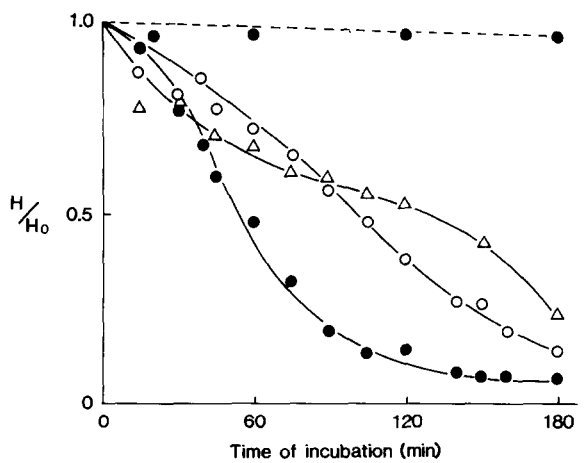


Fig. 5. Transverse diffusion of TEMPO-PC from outer to inner layer of membrane lipid bilayer of intact erythrocytes. Erythrocytes were incubated with $C_{10:0}$- (●), $C_{12:0}$- (○) and $C_{14:0}$-PC (△) at 37°C (solid line) or 5°C (dotted line) for the indicated time. H_0 was obtained from the spectrum after incubation for 10 min with $C_{10:0}$- and $C_{12:0}$-PC, and for 60 min with $C_{14:0}$-PC.

Fig. 4 shows the ESR spectra taken immediately after incorporation of $C_{10:0}$-PC probe into the erythrocyte (a) and after 2 h incubation of the erythrocyte at 37°C (b). No change in the ESR line shapes composed of three peaks was observed during the incubation, whereas the signal amplitude was markedly decreased.

The diffusing rate of TEMPO-PC from the outer to the inner layer after an appropriate incubation time depended on the acyl chain length of the PC molecules. In Fig. 5, the peak amplitude of ESR spectrum for the labeled erythrocytes was plotted as a function of incubation time. The transverse diffusion of $C_{10:0}$-PC probe was the fastest among three types of TEMPO-PC tested and its half-time was about 54 min. In contrast to $C_{10:0}$-PC probe, both $C_{12:0}$-PC probe and $C_{14:0}$-PC probe diffused slowly and their half-times were about 100 and 130 min, respectively. When the erythrocytes labeled with $C_{10:0}$-PC were incubated at 5°C (also shown in Fig. 5, dotted line) the signal intensity did not change up to 3 h, indicating no transverse diffusion at this temperature.

Discussion

In this study, we demonstrate the influence of chain length of PC series labeled with nitroxide on the choline moiety (TEMPO-PC) upon the rate of transfer between liposomal and erythrocyte ghost membranes, and on the rate of transverse diffusion of the TEMPO-PC molecules in the membrane lipid bilayer of intact erythrocytes.

The ESR technique was employed here, because when probes such as radioisotope-labeled lipids were used to examine the transfer between membranes, the molecules incorporated into the acceptor membrane must be separated from the molecules in the donor membrane [2–6], while the ESR technique has a great advantage not to require this separation step [9–11]. Spontaneous transfer of the phospholipids bearing short acyl chains between liposomal membrane and erythrocyte ghost membrane has been observed to occur [2,3]. And the rate of transfer was affected by the chain length of

the TEMPO-PCs (Fig. 2); it increased in the order of $C_{14:0} < C_{12:0} < C_{10:0}$-TEMPO-PC. But no transfer of $C_{16:0}$-PC probe was observed, even though it was incubated at 37°C at least for 2 h. These findings suggest that the relative rate of transfer between membranes becomes higher as the chain length of the lipids decreases. These results agree very closely with those reported most recently by Ferrell et al. [19] and by us [20].

There are many reports describing that cholesterol or phospholipid depletion of erythrocyte membrane was induced by incubation of the cells with PC liposome. For example, when the cells were incubated at 37°C for 48 h with dipalmitoylPC (which is believed to be hardly incorporated into the membrane) the membrane cholesterol content decreased by about 60% [21]. On the other hand, by incubating the cells at 37°C with a high concentration (720 μM) of dimyristoylPC, vesicles which contain membrane phospholipids and proteins were released from the membrane, and as a result the phospholipid content of the membrane decreased [22]. In the present study, however, neither cholesterol nor phospholipid depletion in the erythrocyte membrane occurred when the cells were incubated with each TEMPO-PC liposome (Table I). The difference between the results obtained here and in previous reports cited above may be due to the following reasons; (1) a very low concentration of TEMPO-PC (12.5 μM) was used here, (2) almost all the TEMPO-PC used here can easily penetrate into the membrane within a short time period, and (3) the incorporated TEMPO-PC percentage of the total membrane phospholipid was only 0.8, 1.9 and 2.5% for $C_{10:0}$-, $C_{12:0}$- and $C_{14:0}$-TEMPO-PC, respectively.

The phospholipids having short acyl chains are selectively incorporated into the outer layer of the lipid bilayer of human erythrocyte membrane [17,23]. From the results (using phospholipases and the ESR technique) it has been confirmed that the phospholipids incorporated into the membrane can translocate between outer and inner layer of the lipid bilayer in both artificial and biological membranes [7–10]. Ascorbate as a reducing agent of the spin probe has been used to detect the distribution of spin-labeled lipids between the bilayer halves [10,11]. In the present experiment, we applied the cytoplasmic content (probably the reduced form of glutathione) of intact erythrocytes which can reduce the TEMPO-PC exposed on the inner surface of the membrane, for measurement of the amount of TEMPO-PC traversed from the outer to the inner layer of the membrane bilayer [9]. The signal amplitude of each TEMPO-PC decreased spontaneously with increasing time of incubation at 37°C, indicating transverse diffusion of labeled PC (Fig. 5). It is noted that the rate of diffusion of PC from the outer to the inner layer of the bilayer was greatest with $C_{10:0}$-PC and followed by that of $C_{12:0}$-PC and $C_{14:0}$-PC. Thus, it was suggested that the transverse diffusion as well as transfer between membranes as described above, may be more favorable to the PC species with shorter acyl chains. The similar tendency was found also with ^{14}C-labeled PC and lysoPC species, as reported by us [20].

The rate of transverse diffusion of $C_{14:0}$-TEMPO-PC exhibited a biphasic pattern. We can not yet explain this phenomenon clearly. Both $C_{10:0}$- and $C_{12:0}$-TEMPO-PC were incorporated into the membrane within 1 min at 37°C, whereas $C_{14:0}$-TEMPO-PC required about 60 min for its maximal incorporation (Fig. 2). Therefore, the measurement of transverse diffusion of $C_{14:0}$-TEMPO-PC had to start after 60 min preincubation. This delay might affect the rate of transverse diffusion.

A large difference in the transfer rate was observed between TEMPO-PC used in the present study and ^{14}C-labeled PC reported previously [2,3]. The time periods required to reach the maximum incorporation of $C_{12:0}$-PC and $C_{14:0}$-PC were approximately 10 and 60 min with TEMPO-PC, respectively, while ^{14}C-labeled $C_{12:0}$-PC and $C_{14:0}$-PC required more than 30 min and 12 h to reach the plateau. From the findings [5,6] that (1) the lipid transfer takes place by diffusion of the monomers through the aqueous phase of the suspending medium and (2) the rate of transfer is associated with desorption of lipid from the donor bilayer in the aqueous phase, the difference between TEMPO-PC and ^{14}C-labeled PC molecules would be considered to be due to a possible perturbation of the liposomal membrane (donor membrane) by the nitroxide group in the choline moiety.

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