ABSTRACT
The aim of this review is to demonstrate the usefulness of the detergent sodium cholate in order to obtain highly resolved $^{31}$P NMR spectra of phospholipids (PLs) and, accordingly, a maximum of information from complex mixtures. $^{31}$P NMR spectra of PLs are characterized by broad resonances in water. Detergents normally possess one polar and one apolar moiety and form vesicles (in the same way as PLs) when they are dissolved in water. Sodium cholate forms very small micelles consisting of just four molecules. Therefore, sodium cholate is the detergent of choice in comparison to other common detergents that are characterized by higher aggregation numbers. The resulting ‘mixed micelles’ between PL and sodium cholate yield highly resolved $^{31}$P NMR spectra. Using this approach, beside the differentiation of all major individual PL classes the estimation of the rough fatty acid composition of PL mixtures is possible.

BACKGROUND
Beside proteins, lipids and particularly phospholipids (PLs) are the main constituents of biological membranes in animals, plants and microorganisms. PLs are defined as apolar compounds that can be extracted with organic solvents from biological tissues or body fluids. Their amphiphilic character - caused by the hydrophilic head group and the hydrophobic fatty acid residues - is responsible for the typical PL bilayer arrangement, which characterizes the plasma membrane of cells, cellular vesicles and membranes of cellular organelles. The structures, physical properties, as well as functions of cell membranes are effected to a large extent by their lipid components, in terms of both, the lipid class and the molecular composition of each lipid class. Compositional aspects of the PLs are responsible for maintaining the membrane in a functionally-active state. For example, the degree of unsaturation of the fatty acid residues contributes to membrane fluidity and is one of the primary determinants of membrane physical properties (1). However, PLs do not only have structural relevance in cells. They directly participate in numerous cellular processes with high physiological impact: they may act as intercellular messenger molecules and are involved in the pathogenesis of many diseases. For instance, diabetes, arteriosclerosis and hyperlipidemia are caused - or at least accompanied - by alterations in the lipid composition of the related cells or tissues. Beside lipid pattern changes, dysfunctions in the synthesis of lipid second messengers are also involved in some pathologies. Therefore, PL research on the level of cellular and model systems has advanced significantly in the last decades, generating the need for fast, reliable and informative analytical techniques. PL analysis is traditionally based on chromatographic and/or mass spectrometric methods (1). $^{31}$P nuclear magnetic resonance (NMR) spectroscopy is an convenient tool in the analysis of the PL composition of a given mixture. In that way individual PL classes can be easily differentiated and quantitative information about the relative moieties of PL in complex mixtures is readily available by integration (2).

THE METHOD OF CHOICE TO OBTAIN HIGHLY RESOLVED $^{31}$P NMR SPECTRA OF PHOSPHOLIPIDS
$^{31}$P NMR spectra provide detailed information about the PL composition of a lipid mixture in a less laborious way than chromatographic methods. Unfortunately, due to the comparably low sensitivity of NMR, PL amounts in the 0.1 mg range are required (2). However, when lipids are extracted from cell cultures or tissues, such amounts are normally easily available. The special advantage of $^{31}$P NMR is the simultaneous detection of all metabolites containing at least one $^{31}$P nucleus in a single experiment. $^{31}$P NMR spectra can be acquired by using very simple pulse sequences: The sample preparation has a much higher impact on spectral quality than the NMR device or the field strength of the available magnet. $^{31}$P NMR spectra of PLs in natural membranes and of synthetic PLs in model bilayer membranes are generally characterized by broad resonances with line-widths in the kilohertz range (2). Although the analysis of the shape of such broad resonances may provide important physicochemical information on bilayer structure (crystalline, lamellar, hexagonal, cubic and micellar phases), this technique is not the method of choice for individual component analysis of a given mixture. Fig. 1 illustrates the effect

Figure 1. $^{31}$P NMR spectra of total organic bovine brain extract a) was recorded in chloroform and b) was recorded in aqueous sodium cholate to avoid bilayer formation (assignments of individual PL classes are indicated). Abbreviations: LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PS: phosphatidylserine; SM: sphingomyelin

See text for details.
of the solvent on the achievable line-widths of $^{31}$P NMR spectra of phospholipids. Trace 1 (a) represents the $^{31}$P NMR spectrum of an organic bovine brain extract in chloroform. This spectrum is obviously dominated by two broad, less informative resonances. In the lower spectrum (b) the detergent sodium cholate was used for the solubilization of the same organic brain extract. This leads to a much higher resolved $^{31}$P NMR spectrum with moderate line-widths allowing the differentiation of the individual PL species (3, 4, 5). Historically the application of detergents and particularly sodium cholate grew out of the observation covered in Nouri-Sorkhabi et al. (6).

Nevertheless, there is still an ongoing discussion regarding which solvent system works best with PLs (7). In very early NMR reports, chloroform/methanol in a ratio 2:1 (v/v) was used and acceptable line-widths of PLs could be obtained (8). In more recent reports, mixtures of chloroform, methanol and a very small quantity of aqueous ethylene-diamine-tetraacetate (EDTA) (1:25/8/1 v/v/v) were used to remove paramagnetic species (7). In the authors opinion, however, detergents are superior in comparison to solvent mixtures because of their amphiphilic properties (5).

In Fig. 2 three different kinds of micelles are shown which can be formed in solvents of different polarities. PLs give in water ‘conventional’ micelles with the hydrophobic heads of the phospholipids pointing to the outside of the micelle ring (a). In contrast, inverse micelles with the heads pointing to the inside of the micelle ring are formed in an organic solvent (b). ‘Mixed micelles’ are formed when PLs are dissolved in aqueous detergent (c). The polar and apolar moieties of detergents lead to the formation of vesicles (in the same way as aqueous lipids) when they are dissolved in water (9). Since PLs will enter these detergent micelles at an appropriate lipid/detergent ratio, the formation of large PL vesicles normally occurring in water is suppressed.

Table 1 compares the properties of various types of detergents. From the right column it is obvious that sodium cholate exhibits the smallest aggregation number consisting of just four individual molecules (9). Consequently, sodium cholate produces very small micelles. The resulting “mixed micelles” between PL and sodium cholate are very small and yield highly resolved $^{31}$P NMR spectra. The achievable resolution of $^{31}$P NMR spectra of PL in aqueous cholate is shown in Fig. 3. All physiologically relevant PL classes can be detected simultaneously without major resolution problems (Trace 3 (a)). Just the resonances of phosphatidylethanolamine (PE) show a slight overlap that can, however, be improved by altering the pH or the temperature (2). Please note that the resonances of phosphatidylinositol (PI), and especially phosphatidic acid (PA), are broadened. This is caused by the acidity of these lipids and their capacity to bind paramagnetic ions, leading to moderate to severe line-broadening (2). Furthermore, the use of the detergent enables the monitoring of the fatty acid composition and the position of the fatty acid residue in lysophospholipids (LPL) (Trace 3 (b)) (2, 5, 10). It should be noted that $^{31}$P spectra of LPL give besides, for instance, 2-LPC always a minor resonance of 1-LPC. This isomerization is caused by the migration of the fatty acid residue from the sn-1 into the sn-2 position and vice versa. The mechanism of this migration that occurs exclusively in detergent is still unknown, although some speculations exist (8, 11).

**Table 1. Comparison of selected properties of different detergents.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW (g/mol)</th>
<th>CMC (µM)</th>
<th>Aggregation number</th>
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<td>C&lt;sub&gt;12&lt;/sub&gt;E&lt;sub&gt;8&lt;/sub&gt;</td>
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<td>1.3</td>
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<td>638</td>
<td>2.1</td>
<td>280</td>
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<td>720</td>
<td>2.3</td>
<td>150</td>
</tr>
<tr>
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<td>1200-7100</td>
<td>62-101</td>
</tr>
<tr>
<td>Cetylpyridinium chloride (CP)</td>
<td>522</td>
<td>19000-25000</td>
<td>90</td>
</tr>
<tr>
<td>C6-chelate (PAM)</td>
<td>509</td>
<td>580</td>
<td>63</td>
</tr>
<tr>
<td>N-dodecyl-phosphocholine (DCPC)</td>
<td>352</td>
<td>1100</td>
<td>50-60</td>
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<tr>
<td>Deoxycholic acid</td>
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<td>22</td>
</tr>
<tr>
<td>Taurodeoxycholic acid</td>
<td>500</td>
<td>1300</td>
<td>20</td>
</tr>
<tr>
<td>Glycerophosphate acid</td>
<td>466</td>
<td>1500</td>
<td>6</td>
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<tr>
<td>Sodium cholate</td>
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<td>10000</td>
<td>4</td>
</tr>
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**APPLICATIONS IN BIOMEDICAL SCIENCE AND DETERMINATION OF ENZYME ACTIVITIES**

High resolution $^{31}$P NMR has already been used in many studies of biofluids, including blood from cancer patients (7) and serum or synovial fluid from rheumatic patients (12) as well as in studies of pancreatic juice from patients with pancreatic dysfunction (13, 14). Special emphasis is regularly given to the determinations of the PL/LPL ratios (it allows to draw conclusions on the activity of the enzyme phospholipase A<sub>2</sub> (PLA<sub>2</sub>) since all individual PLs as well as the corresponding LPL can be differentiated in a single spectrum (8). Mechanistic investigations of phospholipases often require kinetic studies in the presence of complex PL mixtures. Therefore, monitoring the hydrolysis of individual PL in complex...
mixture would be particularly laborious by "traditional" methods of lipid analysis, whereas this can be done easily by $^{31}$P NMR. In another recent work, it was also shown that $^{31}$P NMR investigations are not only useful for the determination of phospholipase activities but also for kinases: the phosphorylation investigations are not only useful for the determination of alterations of the lipid pattern. Although not very sensitive, NMR does not require radioactive labelling (15).

CONCLUSIONS

There is considerable interest in lipid analysis and it is expected that this interest will continuously grow since an increasing number of diseases are recognized to be correlated with alterations of the lipid pattern. Although not very sensitive, NMR provides the considerable advantages that (a) all PL classes can be differentiated (b) no major sample workup is required and (c) imurities is tolerated. In the authors’ opinions the detergent sodium cholate is the best choice to obtain highly resolved $^{31}$P NMR spectra because the formation of large PL vesicles is prevented.

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