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^{13}C-Nuclear Magnetic Resonance Spectroscopic Studies on Saturated, Mono-, Di- and Polyunsaturated Fatty Acids, Phospho- and Sphingolipids

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Summary: Complete and unequivocal carbon-13 magnetic resonance (cmr) data have been obtained for the following biologically relevant lipids: the fatty acids palmitic, stearic, oleic, linoleic, α-linolenic and arachidonic acid; the phospholipids 1-stearoyl-2-linoleoyl-3-glycerophosphorylcholine, 1,2-distearoyl-3-glycerophosphorylcholine, [*N*-$^{13}CH_3$-*choline*]phosphatidylcholine, [*N*-$^{13}CH_3$-*choline*]sphingomyelin; sphinganine (dihydrosphingosine), 4*t*-sphingenine (sphingosine) and 3-dehydrosphinganine. Accurate assignments of resonance lines were ascertained using synthetic compounds labelled with appr. 90% carbon-13 in specific positions of the respective molecule and substituted differentially. The parameters determining the chemicals shifts were analyzed.

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^{13}C-Kernmagnetische Resonanzspektroskopie von gesättigten, mono-, di- und hochungesättigten Fettsäuren sowie von Phospho- und Sphingolipiden

Zusammenfassung: Die vollständige ^{13}C-magnetische Resonanz-(cmr)-Analyse folgender biologisch relevanter Verbindungen wurde durchgeführt: der Fettsäuren Palmitin-, Stearin-, Öl-, Linol-, α-Linolen- und Arachidonsäure; der Lecithine 1-Stearoyl-2-linoleoyl-3-glycerophosphorylcholin, 1,2-Distearoyl-3-glycerophosphorylcholin, [*N*-$^{13}CH_3$-*cholin*]Phosphatidylcholin, [*N*-$^{13}CH_3$-*cholin*]-Sphingomyelin und der langkettigen Basen Sphinganin (Dihydrosphingosin), 4*t*-Sphingenin (Sphingosin) und 3-Dehydrosphinganin. Die sichere Zuordnung der Resonanzlinien wurde durch Verbindungen ermöglicht, die in spezifischen Positionen mit ca. 90% ^{13}C durch Totalsynthese signiert wurden oder deren chemische Verbindungen durch differenzierte Substitution verändert wurden. Die Parameter, die diese chemische Verschiebung bestimmen, wurden analysiert.

Increasing interest has arisen in elucidating the role of lipids and proteins in membrane structure and function, both ultimately depending on the physicochemical behaviour of these membrane components. Since all complex lipids present in biological membranes are amphiphilic compounds, their hydrophobic and hydrophilic moieties contribute to their properties and functions.

Different physical techniques are being adapted to the study of the properties of purified lipids, model

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Abbreviations:

cmr = carbon-13 nuclear magnetic resonance.
TMS = tetramethylsilane.

Enzyme:

Phospholipase C, phosphatidylcholine choline-phosphohydrolase (EC 3.1.4.3).

membranes and natural membranes. The monolayer technique[1] has been applied to pure phospholipids with defined acyl groups in positions 1 and 2 of the glycerol molecule[2,3] and the relationship between fatty acid structures (degree of unsaturation and chain length) and phase transitions depending on the temperature defined[3]. The importance of these phase transitions for biological functions (growth, respiration and β-galactoside transport) has been demonstrated with an *E. coli* auxotroph depending on exogenous fatty acids[4].

The spin labelling technique of lipids led to an important elucidation of the molecular arrangement an motion of simple and complex lipids[5–12] in model membrane systems. The nitroxide label introduced into the molecules represents a bulky group which leads to a perturbation of the arrangement of the lipid molecules when present in the lipid phase. ESR spectra of the nitroxide radical then permit conclusions to be drawn regarding the motion and environment of these labels. However, the reaction of the radicals in the neighbourhood of highly unsaturated fatty acids prone to autoxidation makes this method questionable. The orientation of spin labelled molecules in interphases has been studied recently by the monolayer technique[13]. These studies suggest a reconsideration of the structural arrangement of these molecules proposed for model membranes. Spin labelled molecules have been used for incorporation studies in biological membranes[14–18]. The possible reduction of the spin label and the hazard of nitroxide radicals in the environment of the polyunsaturated fatty acids of the phospholipids prone to autoxidation could be drawbacks of this method.

Limitations also exist for the use of fluorescence probes in the study of biological membranes. The most frequently used labels are the anthroyl group, as esters of hydroxystearic acid, or the dansyl group (dimethylamino naphthalene sulfonyl group)[19–22]. Anthroyl stearic acid cannot be incorporated biosynthetically into biological membranes because the ester bond is hydrolyzed before the molecule passes through the plasma membrane. This has been demonstrated with *O*-anthroyl-[12-^{3}H]hydroxystearic acid in tissue culture. The plasma membrane of cells in tissue culture is impermeable to the non hydrolyzable anthroyl ether of 12-hydroxystearate*. Most spin label and fluorescence studies were concerned with the molecular mobility around the transition point at which the liquid phase changes into the liquid crystalline and into the crystalline phase.

Carbon-13 nuclear magnetic resonance spectroscopy (cmr) appears to be a potent technique for

[1] Gaines, G. L., Jr. (1966) in Insoluble Monolayers at Liquid-Gas Interphases (Prigogine, I., ed.) pp. 208–280, Intersci. Publishers, New York.

[2] Deenen, L. L. M. van, Houtsmuller, U. M. T., Haas, G. H. de & Mulder, E. (1962) *J. Pharm. Pharmacol.* **14**, 429–444.

[3] Stoffel, W. & Pruss, H. D. (1969) *this J.* **350**, 1385–1393.

[4] Overath, P., Schairer, H. U. & Stoffel, W. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **67**, 606–612.

[5] Hamilton, C. L. & McConnell, H. M. (1968) in Structural Chemistry and Molecular Biology (Rich, A., Davidson, N., eds.) pp. 115–149, W. H. Freeman & Co., San Francisco.

[6] Hubbel, W. L. & McConnell, H. M. (1969) *Proc. Nat. Acad. Sci. U.S.A.* **63**, 16–22.

[7] Hubbell, W. L. & McConnell, H. M. (1971) *J. Amer. Chem. Soc.* **93**, 314–326.

[8] Seelig, J. (1970) *J. Amer. Chem. Soc.* **92**, 3881–3887.

[9] Seelig, J. (1971) *J. Amer. Chem. Soc.* **93**, 5017–5022.

[10] Kornberg, R. D. & McConnell, H. M. (1971) *Biochemistry* **10**, 1111–1120.

[11] Devaux, P. & McConnell, H. M. (1972) *J. Amer. Chem. Soc.* **94**, 4475–4481.

[12] Hubbell, W. L., Metcalfe, J. C., Metcalfe, S. M. & McConnell, H. M. (1970) *Biochim. Biophys. Acta* **219**, 415–427.

[13] Tinoco, J., Gosh, D. & Keith, A. D. (1972) *Biochim. Biophys. Acta* **274**, 279–285.

[14] Keith, A. D., Waggoner, A. S. & Griffith, O. H. (1968) *Proc. Nat. Acad. Sci. U.S.A.* **61**, 819–826.

[15] Tourtelotte, M. E., Branton, D. & Keith, A. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **66**, 909–916.

[16] Stanacev, N. Z., Stühne-Sekalec, L., Schreier-Muccillo, S. & Smith, I. C. P. (1972) *Biochem. Biophys. Res. Commun.* **46**, 114–119.

[17] Nakamura, M. & Ohnishi, S. (1972) *Biochem. Biophys. Res. Commun.* **46**, 926–932.

[18] Colbeau, A., Vignais, P. M. & Piette, L. H. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1495–1503.

[19] Waggoner, A. S. & Stryer, L. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **67**, 579–589.

[20] Gulik-Krzywicki, T., Shechter, E., Iwatsubo, M., Ranck, J. L. & Luzzati, V. (1970) *Biochim. Biophys. Acta* **219**, 1–10.

[21] Shechter, E., Gulik-Krzywicki, T., Azerad, R. & Gros, C. (1971) *Biochim. Biophys. Acta* **241**, 431–442.

[22] Shechter, E., Gulik-Krzywicki, T. & Kaback, H. R. (1972) *Biochim. Biophys. Acta* **274**, 466–477.

* Stoffel, W., unpubl.

the study of the molecular arrangement and motion of lipid molecules of simple and complex lipids in solution, model membranes and particularly of biological membranes and their components. Spectral accumulation or Fourier transform analysis of the free induction decay and proton decoupling also allows the measurement of carbon chemical shifts of rather large molecules. The relatively large amounts of sample required and the often difficult interpretation of cmr spectra regarding the assignment of peaks, however, seem to be drawbacks of this method. A number of methods have been designed to overcome particularly the latter problem, such as selective proton decoupling[23,24] and off-resonance decoupling[25,26]. Also the Fourier transform technique makes these studies more practicable.

Lipids of mammalian membrane systems are characterized by phospholipids with a high content of mono-, di- and polyunsaturated fatty acids and differ thereby from bacterial lipids with saturated and monoenoic acids. Furthermore sphingolipids are typical of mammalian membranes and rather unusual in bacteria. The isothermic conditions required for the mammalian cell lend these components particular importance. Previous studies in this laboratory were concerned with the chemistry and metabolism of unsaturated fatty acids. Our more recent studies are aimed at contributing to the understanding of the molecular arrangement and mobility of these compounds in model and biological membranes.

The limitations which are inherent to any perturbation probe led us to choose carbon-13 as a probe which may be incorporated into membranes without perturbation of the lipid or protein phase. A number of fatty acids, phospho- and sphingolipids (Tables 1 and 2) enriched at particular carbon atoms with 88 to 90% carbon-13 allow the accurate assignment of the chemical shifts (resonances) of the carbon atoms of these compounds. Another valuable advantage of carbon-13 enrichment in a particular compound of interest is the increase of sensitivity. In summary, this approach allows studies of the molecular organisation and motion of these compounds in solution, model membranes and natural membranes.

We have completed the chemical synthesis of a number of fatty acids, phospho- and sphingolipids of biological significance which, because they are highly labelled, will also overcome the problem of small amounts of membrane material available for cmr spectroscopy. Saturated ($C_{16:0}$, $C_{18:0}$), monoenoic ($C_{18:1}$) and dienoic ($C_{18:2}$) fatty acids with ^{13}C in positions 1, 3, 8 and 14, [*N*-$^{13}CH_3$-*choline*]phosphatidylcholine and sphingomyelin were synthesized. For model membranes 1-stearoyl-2-linoleoyl- and 1,2-distearoyl-3-glycerophosphorylcholine and sphinganines were prepared and characterized. In this communication we would like to report the complete cmr analysis of these fatty acids, complex phospho- and sphingolipids and of long chain bases. Table 1 summarizes the carbon chemical shifts of stearic, oleic, linoleic, α-linolenic and arachidonic acid.

The carbon-13 label in [14-^{13}C]linoleic acid unequivocally allows the determination of the chemical shift of this C atom of 27.0 ppm, Fig. 1. The same is true for oleic acid ^{13}C-enriched in C-8 and C-11. Therefore the signal at 27 ppm in the spectra of α-linolenic and arachidonic acid must be assigned to C-8 of linolenic and C-4 and C-16 of arachidonic acid. It can be concluded that carbon atoms in juxtapositions pointing to the carboxy and methyl ends of *cis*-olefinic bonds are characterized by this resonance. The terminal double bond of all fatty acids of the linolenic acid group leaves only the penultimate CH_2 and terminal CH_3-group with their typical chemical shifts, although a slightly chemical shift to higher fields (20.4 ppm) can be observed for C-17 of α-linolenic acid.

The methylene groups interrupting the olefinic bond system exhibit constant chemical shifts of 25.5 ppm. Other typical resonances such aus that of the carboxy group at 180 ppm and the α and β-carbon atom at 34.0 and 24.5 ppm agree well with those measured by Metcalfe *et al.*[27–29] for dipalmitoyl-

[23] Dorman, D. E., Angyal, S. J. & Roberts, J. D. (1970) *J. Amer. Chem. Soc.* **92**, 1351–1354.

[24] Dorman, D. E. & Roberts, J. D. (1972) *J. Amer. Chem. Soc.* **92**, 1355–1361.

[25] Reich, H. J., Jautelat, M., Hesse, M. T., Weigert, F. J. & Roberts, J. D. (1969) *J. Amer. Chem. Soc.* **91**, 7445–7454.

[26] Johnson, F. L. & Tate, M. E. (1969) *Can. J. Chem.* **47**, 63–73.

[27] Metcalfe, J. C., Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K. & Partington, P. (1971) *Nature (London)* **233**, 199–201.

[28] Lee, A. G., Birdsall, N. J. M., Levine, Y. K. & Metcalfe, J. C. (1972) *Biochim. Biophys. Acta* **255**, 43–56.

[29] Levine, Y. K., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1972) *Biochemistry* **11**, 1416–1421.

Table 1. Carbon chemical shifts* of stearic, oleic, linoleic, α-linolenic and arachidonic acid.

Carbon atom	Acids stearic	oleic	linoleic	α-linolenic	arachidonic
1	180.7	180.0	180.0	180.0	180.0
2	34.4	33.9	33.9	33.2	33.2
3	25.1	24.5	24.4	24.4	24.3
4					26.3
5		29.0 – 29.6	28.9 – 29.4	28.9 – 29.4	127 – 130
6					
7					25
8		27.0	27.0	27.0	127 – 130
9	29.7	129.4	129.7	127 – 131	
10		129.7	129.6		25.5
11		27.0	25.5	25.4	127 – 130
12			127.9	127 – 131	
13		29.0 – 29.6	127.5		25.5
14			27.0	25.4	127 – 130
15	29.5		29.1	127 – 131	
16	32.3	31.8	31.4		27.1
17	23.1	22.6	22.4	20.4	29.2
18	14.2	14.1	14.0	14.1	31.4
19					22.4
20					14.0

* The chemical shifts are referenced to TMS.

Table 2. Carbon-13 chemical shifts of derivatives of long chain sphingosine bases.

Carbon atom	Triacetyl-4*t*-sphingenine	Diacetyl-3-dehydro-sphinganine	*N*-Acetyl-3-dehydro-sphinganine	Sphinganine
1	62.3	62.8	63	63
2	50.6	57.4	60.9	50 – 51
3	73.4	204.6	206.4	69.4
4	136.7	39.6	40.0	–
5	124.5	23.2	23.5	23 – 24
6	32.1			
7				
8				
9				
10				
11	29.5 – 28.9	29.5 – 28.9	29.6 – 29.4	29.7 – 28.8
12				
13				
14				
15				
16	31.8	31.8	31.8	31.9
17	22.5	22.5	22.5	22.5
18	14.0	14.0	14.0	14.0

$CH_3-C(=O)-O-$ (20.2, 169.2) $CH_3-C(=O)-NH-$ (22.8, 168.5)

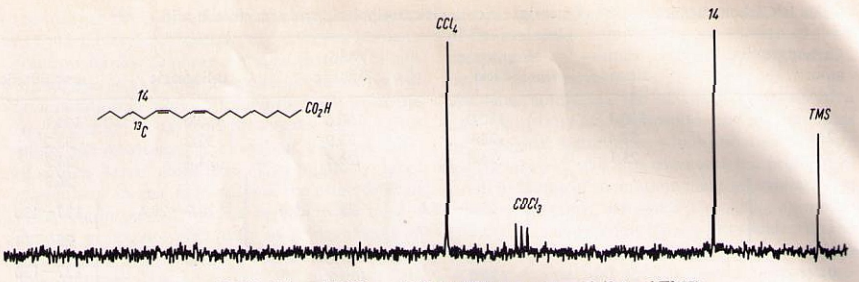

Fig. 1. cmr-Spectrum of [14-^{13}C]linoleic acid (80 mg in 2 ml CCl_4/0.5 ml $CDCl_3$/0.1 ml TMS). Transients (1000) were accumulated with an acquisition time of 1 sec.

lecithin. It should be noted that the resonance of C-2, which generally appears around 34 ppm, splits up in a doublet at 32.8 and 35.2 ppm with a coupling constant of 56.5 Hz due to the carbon 13 enrichment in the carboxyl group, Fig. 2.

Except for the carboxy carbon, identical chemical shifts were observed when the labelled fatty acids were incorporated into phosphatidylcholine (Fig. 3a/b) and sphingomyelin. This yielded sharp resonances at 173 ppm when present in the ester bond of the phospholipids and amide bond of the ceramide part of sphingomyelin. The carbon atoms of the N-$(CH_3)_3$ group showed the characteristic shift of 54.0 ppm as shown in Fig. 4, which represents that of the cmr spectrum of lecithin with a ^{13}C label in the CH_3 group of the choline moiety. This value is in good agreement with the value reported before[27–29]. An identical resonance line is observed in sphingomyelin labelled with ^{13}C in the methyl group of choline.

In order to resolve the resonance lines of the glycerol- and choline methylene groups cmr spectra of lecithin and its diglyceride moiety obtained by phospholipase C hydrolysis were compared, Fig. 5a, b. In the cmr spectrum of dilinoloylglycerol (Fig. 5b) the resonances at 54 ppm (N-CH_3 group of choline), 57.2 and 59.0 ppm of the lecithin spectrum (Fig. 5a) have disappeared. Resonances at 62 ppm, 61 ppm and 72 ppm are attributed to the carbon atoms of glycerol. In order to further assign the three chemical shifts an off-resonance experiment was carried out. The resonance line at 72 ppm was split into a doublet and each line at 61 and 62 ppm into a triplet. Therefore, the chemical shift at 72 ppm represents that of carbon atom 2 of the glycerol backbone and at 61 and 62 ppm those of C-1 and C-3 respectively.

Table 2 summarizes the chemical shifts of the sphingosine bases. The resonances of the carbon skeleton from C-6 to C-15 appear at 28.8 to 29.7

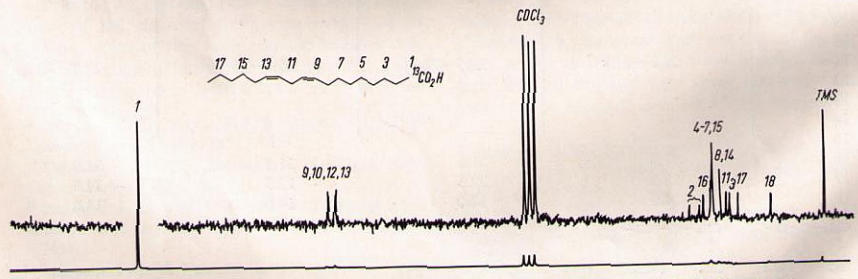

Fig. 2. cmr-Spectrum of [1-^{13}C]linoleic acid, 8000 transients, 80 mg in 2 ml CCl_4/0.5 ml $CDCl_3$/0.1 ml TMS.

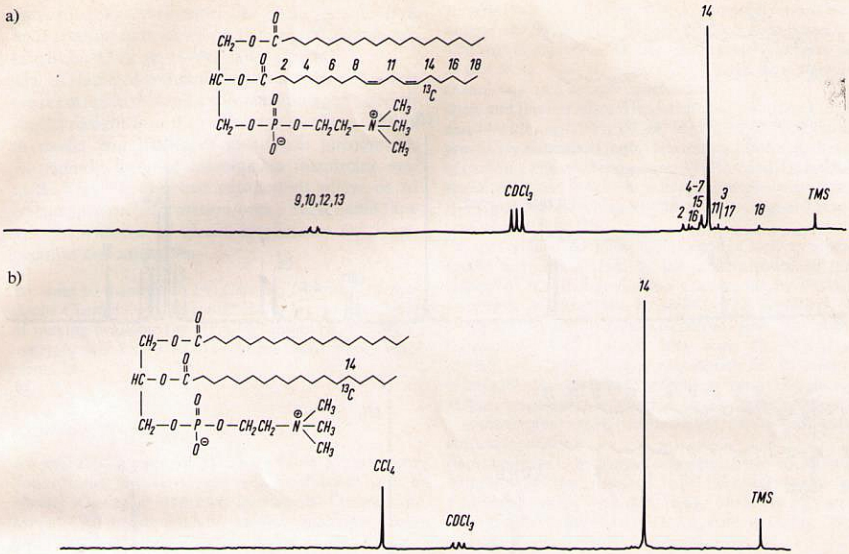

Fig. 3. cmr-spectra of a) 1-stearoyl-2-[14-^{13}C]linoleoyl-3-glycerophosphorylcholine (240 mg in 2.5 m*l* $CDCl_3$/0.1 m*l* TMS), 8190 transients; b) 1-stearoyl-2-[14-^{13}C]stearoyl-3-glycerophosphorylcholine (200 mg in 2.0 m*l* CCl_4/0.5 m*l* $CDCl_3$/0.1 m*l* TMS), 10000 transients.

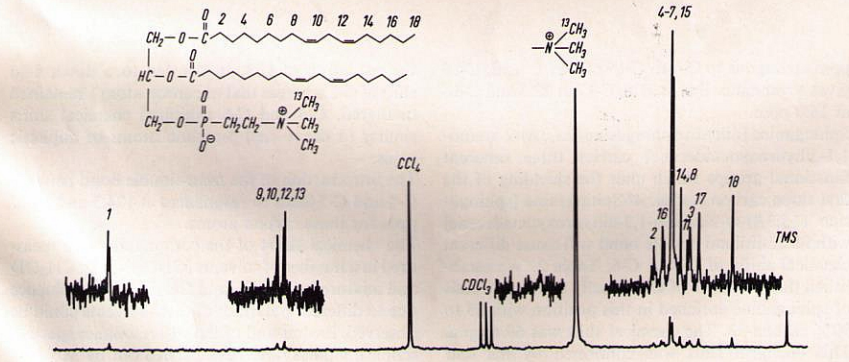

Fig. 4. cmr-Spectrum of [*N*-$^{13}CH_3$-*choline*]phosphatidylcholine (240 mg in 2.0 m*l* CCl_4/0.5 m*l* $CDCl_3$/0.1 m*l* TMS), 32000 transients.

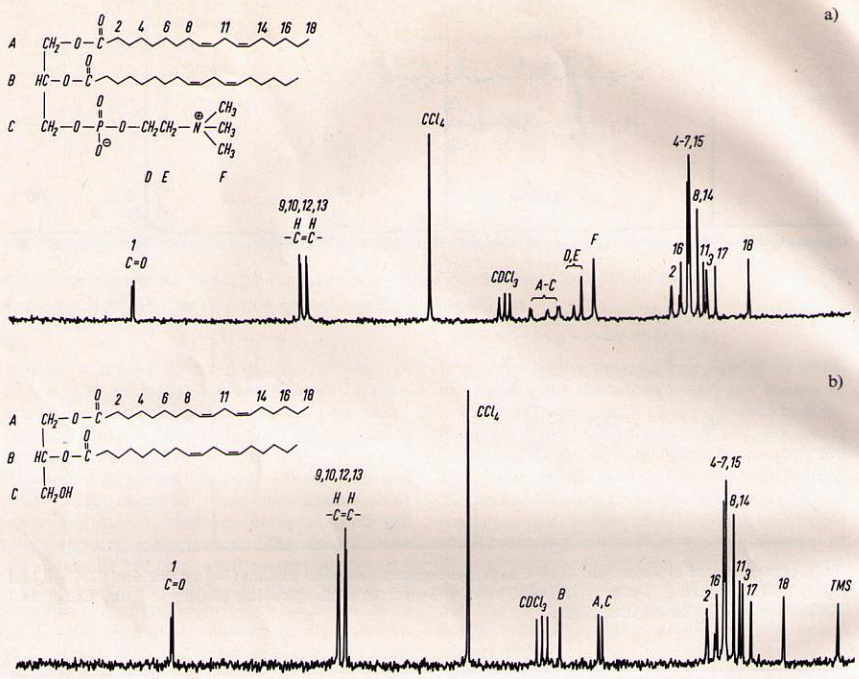

Fig. 5. cmr-spectrum of dilinoleoylphosphatidylcholine (a) and dilinoleoylglycerol (b). (300 mg in 2.0 m*l* CCl_4/0.5 m*l* $CDCl_3$/0.1 m*l* TMS).

ppm analogous to C-4 to C-15 of stearic acid, C-16 gives a resonance line at 31.8, C-17 at 22.5 and C-18 at 14.0 ppm.

Sphinganine [dihydrosphingosine (2*S*,3*R*)-2-amino-1,3-dihydroxyoctadecane] carries three different functional groups which alter the shielding of the first three carbon nuclei. 4*t*-Sphingenine [sphingosine (2*S*,3*R*)-4*t*-2-amino-1,3-dihydroxyoctadecene] with its additional double bond will cause different chemical shifts of C-3 to C-6, Table 2. We established the resonance of C-3 directly by the synthesis of sphinganine enriched in this position with 85 to 90% carbon-13. The chemical shift was 69.4 ppm. This chemical shift was confirmed by the cmr spectrum of *N*-acetyl-3-dehydrosphinganine (3-keto). The resonance line shifted to 205 ppm due to the oxidation of C-3. It also led to a down field shift of C-2 whereas that of carbon atom 1 remained unaltered. C-4 and C-5 exhibited chemical shifts similar to the α- and β-carbon atoms of aliphatic acids.

The introduction of the *trans*-double bond between C-4 and C-5 leads to resonances at 124.5 and 136.7 ppm for these carbon atoms.

The chemical shifts of the compounds were measured in a number of solvents ($CDCl_3$, CCl_4, CH_3OD and mixtures of $CDCl_3$ and CH_3OD). No influence due to different polarities of these solvents could be observed. Proton and carbon-13 resonance spectroscopical studies have been carried out by Metcalfe *et al.*[27–29] on lipids of sonicated lecithin, sarcoplasmic reticulum and acholeplasma membranes

and valuable information has been derived from their spectral data based on the natural abundance of carbon-13 or on [1-^{13}C]palmitic acid.

The ^{13}C labelled compounds, the cmr data of which are reported in this and a subsequent paper*, permit further insight into the molecular events occurring in model and biological membrane phenomena, particularly those of mammalian membrane systems. The ^{13}C enriched compounds allow us to obtain spectral information over a reasonable time interval which is of special importance for T_1 relaxation measurements.

We want to thank Prof. Dr. *Günther*, Institut für Organische Chemie der Universität Köln, for his generosity of making available the HFX-90 Multinuclear Spectrometer, Bruker-Physik AG Forchheim, for our measurements.

Experimentals

All spectra were recorded on a HFX-90 Multinuclear Spectrometer (Bruker-Physik AG, Forchheim) with a Nicolet Computer (Nicolet Instrument Corporation Model 290) to accumulate the free induction decay (interval 1.0 sec, impulse width 8.0 μsec). The Fourier transformation of the resulting impulse interferograms gives the ^{13}C NMR magnitude spectra.

The ^{13}C chemical shifts were measured on samples in 10 mm tubes in the internal lockmode (deuterium frequency of $CDCl_3$) with complete proton decoupling. The digital frequency sweep spectrometer operates at 22.628 MHz. The ^{13}C resonance of TMS (tetramethylsilane) was used as internal standard.

Oleic and linoleic acids were labelled in positions 1, 3, 8 and 14 with appr. 90% of ^{13}C according to procedures previously elaborated in this laboratory for the synthesis of carbon-14 labelled compounds[30,31]. Catalytic hydrogenation yielded the corresponding labelled stearic acid. [1-^{13}C]palmitic acid was obtained by a Grignard reaction with 90% $^{13}CO_2$.

^{13}C-methyl-labelled phosphatidylcholine and sphingomyelin were synthesized by the quaternization of the respective *N,N*-dimethylamino compounds by $^{13}CH_3I$ essentially as described before[32]. The acylation of 1-stearoyl-3-glycerophosphorylcholine with the chlorides of the ^{13}C labelled fatty acids ([1-^{13}C]$C_{18:0}$, [3-^{13}C]$C_{18:2}$, [14-^{13}C]$C_{18:2}$) was carried out in chloroform with dimethylaminopyridine as base. The purity of the compounds was checked by combined gas-liquid chromatography/mass spectroscopy and thin-layer chromatography.

Phospholipase C hydrolysis was carried out on 500 mg dilinoleoyllecithin dissolved in 10 m*l* of ethyl ether in a biphasic system with *Bac. cereus* filtrate (5 m*l*) and 5 m*l* borate buffer 0.1M, pH 7.4, 0.001M $CaCl_2$. The reaction was completed overnight.

* Stoffel, W., Zierenberg, O. & Tunggal, B. D., in preparation.

[30] Stoffel, W. (1964) *Liebigs Ann. Chem.* **673**, 26–36.
[31] Stoffel, W. (1965) *J. Amer. Oil Chem. Soc.* **42**, 583–587.
[32] Stoffel, W., LeKim, D. & Tschung, T. S. (1971) *this J.* **352**, 1058–1064.