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^{13}C-NMR DETECTION OF LIPID POLYMORPHISM IN MODEL AND BIOLOGICAL MEMBRANES

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Summary

1. The application of the ^{13}C-NMR technique to the study of lipid polymorphism is described for various model and biological membranes.

2. The ^{13}C-NMR line-width of various resonances of the lipid molecule are sensitive to the bilayer ⇄ hexagonal and the bilayer ⇄ 'isotropic' phase transition. The latter transition in some cases is accompanied by the occurrence of lipidic particles as detected by freeze-fracturing. Thus, specific ^{13}C-labeling experiments allow the study of the individual phase behaviour of lipids in mixed lipid systems.

3. In diet experiments using rats, the choline group of phosphatidylcholine present in erythrocyte, endoplasmic and sarcoplasmic reticulum membranes could be specifically ^{13}C-labeled. The ^{13}C line-widths of the resonance from the erythrocyte are typical for a lamellar arrangement of the membrane lipids. In strong contrast, the line-width observed at 37°C for the endoplasmic and sarcoplasmic reticulum membranes is much smaller, typical of the isotropic phases observed in model membranes. In isolated rat liver microsomes and liver slices, the ^{13}C line-width is strongly temperature dependent. At lower temperatures the line-widths strongly increase towards values typical of lipids in a bilayer structure.

Introduction

^{31}P-NMR, in combination with freeze-fracture electron microscopy, has recently been extensively applied in studies of the polymorphic phase behaviour of membrane lipids and in investigations on the structure of the lipid

part of biological membranes (for review see Refs. 1 and 2). The line-shape of the ^{31}P-NMR spectrum is sensitive to the structural organization of the lipids, allowing identification of lipids in the bilayer phase, the hexagonal H_{II} phase and phases in which the phospholipids can undergo isotropic motion on the NMR time scale (see Fig. 1 of Ref. 3). An advantage of ^{31}P-NMR compared to X-ray analysis the classical technique for phase identification, is that it can give quantitative information on the phase behaviour of mixed systems in which different phases coexist [1,2]. However, in most cases it is not possible to decide from the ^{31}P-NMR spectrum which lipid is present in which phase. Furthermore, since in ^{31}P-NMR the motional properties of the ^{31}P atom are used to obtain structural information, the possibility that in some systems changes in local order in the phosphate region of the lipid molecule can give rise to changes in the ^{31}P-NMR spectrum cannot be entirely excluded. Another disadvantage of the ^{31}P-NMR technique as applied to biological membranes is that the ^{31}P-NMR spectrum of other phosphorus-containing molecules can obscure the spectrum arising from the phospholipids. With these points in mind we report in this paper the application of ^{13}C-NMR, employing both naturally occurring and specifically enriched compounds, to the study of the lipid polymorphism in model and biological membranes. The results are compared with ^{31}P-NMR measurements made on the same systems.

Experimental Procedure

Materials. Egg phosphatidylcholine was isolated from hen eggs and 3-(*O*-α-D-glucopyranosyl)-1,2-diacyl glycerol (monoglucosyl diacyl glycerol) was isolated from membranes of *Acholeplasma laidlawii* cells grown on a medium supplemented with elaidic acid [4]. 1,2-Dioleoyl-*sn*-glycero-3-phosphorylcholine (18 : 1_c/18 : 1_c-phosphatidylcholine) and 1,2-dioleoyl-*sn*-glycero-3-phosphorylethanolamine (18 : 1_c/18 : 1_c-phosphatidylethanolamine) were synthesized as described before [26]. [4-^{13}C]Cholesterol was obtained from Merck, Sharpe and Dohme (Munich, F.R.G.) and unlabelled cholesterol from Fluka (Buchs, Switzerland). Dioleoylphosphatidyl[*N*-($^{13}CH_3$)$_3$]choline and [*N*-($^{13}CH_3$)$_3$]-choline were synthesized as described elsewhere [5].

Lipid dispersions. Unless otherwise indicated, aqueous dispersions of lipids were prepared by dispersing at 30°C a dry film of 50—100 μmol lipid (in the case of natural abundance, ^{13}C- or ^{31}P-NMR) or 5—10 μmol ^{13}C-labelled lipid in 1.3 ml of 10% ^2H_2O containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.0) buffer.

Diet experiments and isolation of biological membranes. Rats were fed a choline-deficient diet supplemented with [*N*-(*Me*$_3$-^{13}C,^{14}C)]choline according to the method of Arvidson et al. [6] as described before [7]. To 150 g choline-deficient diet (I.C.N. Pharmaceuticals Inc., Cleveland) 4 mmol of [*N*-($^{13}CH_3$)$_3$]-choline and 20 μCi [*N*-($^{14}CH_3$)]choline (Radiochemical Centre, Amersham, England) were added. This was fed to a male Wistar rat for 10 days (15 g daily diet, excess water). In control experiments for ^{31}P-NMR, the choline-deficient diet was supplemented with unlabelled choline. The intact liver and the isolated liver microsomes of these rats showed ^{31}P-NMR spectra identical to those

reported previously [8]. Each rat was killed after 11 days and the liver was removed and immediately placed in ice-cold Krebs-Ringer type C [9] buffer saturated with carbogen gas (95% O_2 and 5% CO_2), in which phosphate was replaced by isotonic Tris. For NMR experiments, the liver was cut into slices of approx. 3 × 8 mm in size. Two of four slices were transferred to a 10 mm NMR tube and 0.5 ml of the above buffer containing 10% ^2H_2O was added. The tube was flushed with carbogen gas, sealed and immediately used for the NMR experiment.

Liver microsomes, sarcoplasmic reticulum (hind leg muscle) and erythrocyte membranes were isolated as described before [8]. The final pellets were suspended in 150 mM NaCl, 10 mM Tris-HCl (pH 7.0) containing 10% ^2H_2O (10—20 mg membrane protein per ml). To quantify the fraction of [*N*-(*Me*$_3$-^{13}C,^{14}C)]choline incorporated in the liver lipids, a Folch extraction [10] was performed on part of the homogenized liver. 60% of the ^{14}C radioactivity was recovered in the chloroform phase. Thin-layer chromatography of the chloroform extract on silica gel G (chloroform/methanol/ammonia/H_2O, 90 : 54 : 5.5 : 15.5) revealed that 87% of the radioactivity was present in phosphatidylcholine and 13% in sphingomyelin. Using the same techniques, it was found for microsomes that 88% of the radioactivity was present in the chloroform phase, 95% of which was present in phosphatidylcholine. For sarcoplasmic reticulum and erythrocyte membranes 90 and 96% of the radioactivity was in the chloroform phase, respectively, 94 and 90% of which was present in phosphatidylcholine. By comparing the specific ^{14}C radioactivity of the choline used in the diet with the specific activities in the isolated phosphatidylcholines it could be calculated that the ^{13}C enrichment was 90, 32 and 26%, respectively. for endoplasmic reticulum, sarcoplasmic reticulum and erythrocyte membranes [7]. Thus, for all membrane preparations studied, the main source of the N-methyl-^{13}C resonance was phosphatidylcholine.

Nuclear magnetic resonance (NMR). ^{13}C-NMR measurements were performed at 90.5 MHz on a Bruker WS 360 spectrometer as described before [11]. Chemical shifts were measured with respect to external 1.4 dioxane and are presented relative to the chemical shift of trimethylsilane (chemical shift dioxane 67.4 ppm downfield of trimethylsilane). For ^{31}P-NMR measurements a Bruker WH 90 operating at 36.4 MHz was used under conditions as described elsewhere [12]. Data accumulation times for model membranes were 20—60 min, for biological membranes 10—20 min (^{13}C) and 30 min (^{31}P).

Analytical methods. Phosphate was determined according to the method of Fiske and Subbarow [12], protein according to the method of Lowry et al. [13] and ^{14}C radioactivity by liquid scintillation counting using standard procedures.

Results and Discussion

Model membranes

In Fig. 1A the natural abundance 90.5 MHz ^{13}C-NMR spectrum of 18 : 1$_c$/18 : 1$_c$-phosphatidylcholine bilayers is shown at various temperatures. Comparison with previously published ^{13}C-NMR spectra [14—20] reveals that the resonances at 130, 55 and 14 ppm originate from the olefinic carbon atoms,

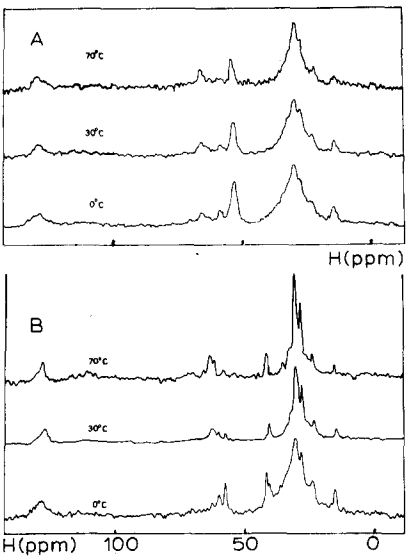

Fig. 1. 90.5 MHz ^{13}C-NMR spectra of aqueous dispersions of 18 : 1_c/18 : 1_c-phosphatidylcholine (A) and 18 : 1_c/18 : 1_c-phosphatidylethanolamine (B) at various temperatures.

the N-($^{13}CH_3$)$_3$ carbons and the terminal chain methyl carbons, respectively. In the 50—70 ppm range, partially resolved resonances of the polar part of the molecule are found whereas the main chain -(CH_2)- resonances are found at about 31 ppm. The important observation is that over a wide temperature range, the line-widths of the various resonances only gradually decrease with increasing temperature (for data on the choline carbons see Table I) due to a decreased order and/or an increased motion of the various atoms at higher temperatures. For 18 : 1_c/18 : 1_c-phosphatidylethanolamine a sudden, more dramatic decrease in line-width occurs for the chain resonances in the 0—30°C temperature interval (Fig. 1B). Above 30°C, the line-widths are considerably smaller as compared to the 18 : 1_c/18 : 1_c-phosphatidylcholine system. This must be caused by the bilayer $\rightleftarrows$ hexagonal H_{II} phase transition which occurs at approx. 10°C [21]. In considering the nature of the motion causing the decreased line-widths, it should be pointed out that ESR, ^{2}H-NMR and ^{31}P-NMR [22—24] studies strongly indicate that the local motion and order of the various atoms in the lipid molecule are similar in the bilayer and hexagonal H_{II} phase. Therefore, in analogy with the ^{31}P-NMR results [24,25], it can be suggested that the observed line narrowing must be caused by fast lateral diffusion of the entire lipid molecule around the aqueous channels of the hexagonal H_{II} phase which (partially) averages the various ^{1}H-^{13}C dipolar interactions. For a theoretical treatment of this motional averaging the reader is referred to the appendix in Ref. 25.

In a number of different mixed lipid system in which one of the lipids in isolated form adopts the bilayer and the other adopts the hexagonal H_{II} phase, a new phase is observed which was characterized by an isotropic ^{31}P-NMR signal [26]. In several of these mixtures, freeze-fracturing has revealed the presence of approx. 100-Å-sized particles and pits which are associated with the lipid

TABLE I

N-($^{13}CH_3$)$_3$ LINE-WIDTHS OF DIFFERENT PHOSPHOLIPID STRUCTURES

	Temperature (°C)	Line-width (Hz) N-($^{13}CH_3$)$_3$ resonance	Phospholipid phase as detected by ^{31}P-NMR
Model membranes			
18:1$_c$/18:1$_c$-phosphatidylcholine	0	234	Bilayer
	30	220	Bilayer
	70	190	Bilayer
18:1$_c$/18:1$_c$-phosphatidylcholine/	30	220	Bilayer (Ref. 29)
18:1$_c$/18:1$_c$-phosphatidylethanolamine (1:1)	30 *	64	Isotropic (Ref. 29)
Egg phosphatidylcholine/monoglucosyldiglyceride (1:1)	30	200	Bilayer (Ref. 26)
	30 **	50	Isotropic (Ref. 26)
18:1$_c$/18:1-phosphatidylcholine/	0	180	Bilayer (Ref. 26)
18:1$_c$/18:1$_c$-phosphatidylethanolamine/ cholesterol (3:1:2)	0 **	75	Partly Isotropic (Ref. 26)
Total liver lipids	2	200	Bilayer
	37	180	Bilayer
Isolated biological membranes:			
Rat erythrocyte membrane	37	150	Bilayer ***
Rat sarcoplasmic reticulum membranes	37	40	Isotropic (broad)† (Ref. 7)
Rat liver microsomes	37	34	Isotropic (Ref. 8)

* Dispersion heated for 10 min at 90°C (Ref. 29).

** Dispersion heated for 10 min at 60°C (Ref. 26).

*** Spectrum similar to that of human erythrocyte membranes published before [3].

† The ^{31}P-NMR signal of sarcoplasmic reticulum membranes, although clearly lacking the typical bilayer shape, is broader than the isotropic signal observed for the microsomes [7].

bilayer [26—28]. These 'lipidic particles' most likely represent inverted micelles sandwiched in between the two monolayers of the lipid bilayer.

Dispersing an equimolar mixture of 18 : 1$_c$/18 : 1$_c$-phosphatidylcholine and 18 : 1$_c$/18 : 1$_c$-phosphatidylethanolamine in buffer at 30°C results in the formation of a lamellar phase due to the bilayer-stabilizing effect of phosphatidylcholine [29]. The ^{13}C-NMR spectrum is shown in Fig. 2A. The spectrum strongly resembles the spectrum of lamellar 18 : 1$_c$/18 : 1$_c$-phosphatidylcholine at that temperature (compare Fig. 1A and Table I), and has much broader resonances than the spectrum of 18 : 1$_c$/18 : 1$_c$-phosphatidylethanolamine in the hexagonal H_{II} phase at 30°C (compare Fig. 1B). By increasing the temperature, an isotropic component grows in the ^{31}P-NMR spectrum such that at 90°C the spectrum only consists of a narrow isotropic signal (results identical to those shown in Fig. 6 of Ref. 29). The bilayer $\rightleftarrows$ isotropic transition in these model membranes systems often shows a pronounced hysteresis [29], as when the mixture is cooled down to the starting temperature of 30°C the isotropic ^{31}P-NMR signal remains unchanged. The ^{13}C-NMR spectrum depicted in Fig. 2B shows that in such a situation virtually all resonances are markedly narrower demonstrating that in the isotropic phase the entire lipid molecule experiences more motion than in the bilayer phase (see also Table I). This argues against the possibility that the effects noted in the ^{31}P-NMR spectrum

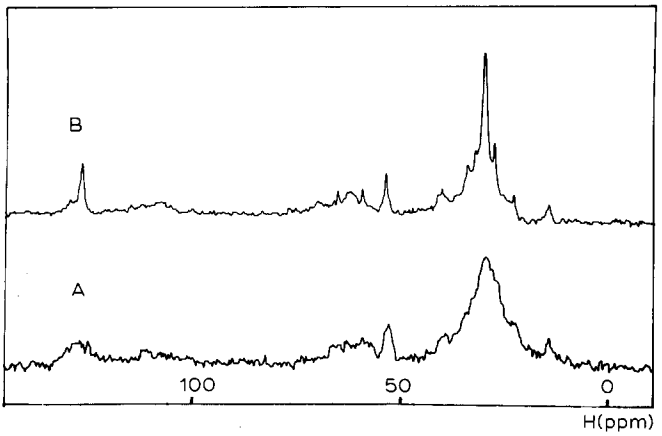

Fig. 2. (A) 90.5 MHz ^{13}C-NMR spectra of an aqueous dispersion of an equimolar mixture of 18 : 1_c/ 18 : 1_c-phosphatidylcholine and 18 : 1_c/18 : 1_c-phosphatidylethanolamine, at 30°C. (B) Same sample recorded at 30°C after being heated until 90°C for 10 min inducing the formation of the isotropic phase.

could be caused by a change in local order or orientation in the phosphate region of the lipid molecule. Very similar ^{13}C-NMR results were obtained for the temperature-induced bilayer → isotropic transition in an equimolar mixture of egg phosphatidylcholine and monoglucosyl diacyl glycerol. This transition which is accompanied by an isotropic ^{31}P-NMR signal and the appearance of lipidic particles [26] results in a dramatic narrowing of all observable ^{13}C resonances. The data on the N-($^{13}CH_3$)$_3$ resonance are presented in Table I.

The great potential of the ^{13}C-NMR technique is that by using ^{13}C-labelled lipids one is able to follow the individual behaviour of different lipids in a mixed system. This is illustrated in Fig. 3 for a mixture of 18 : 1_c/18 : 1_c -phosphatidylethanolamine/dioleoylphosphatidyl[*N*-($^{13}CH_3$)$_3$]choline/[4-^{13}C]cholesterol (3 : 1 : 2). Dispersing the mixed lipid film in buffer at 0°C results in the formation of a lamellar phase as demonstrated by ^{31}P-NMR and freeze-fracturing [26]. The ^{13}C-NMR spectrum shows only one resolved resonance from the N-($^{13}CH_3$)$_3$ carbons (Fig. 3A) with a line-width of 180 Hz (Table I) which is slightly less than in the case of the other phosphatidylcholine bilayers, probably reflecting an increased motion or decreased order of the choline group due to the spacing effect of cholesterol as indicated by earlier NMR studies [20,24]. By comparing the intensity of this resonance with that of a choline solution of known concentration under conditions of gated decoupling with 20-s waiting times between subsequent pulses, it could be concluded that 90–100% of these carbon atoms are observed in the spectrum. Similarly, virtually all carbon atoms associated with N-($^{13}CH_3$)$_3$-labelled lysophosphatidylcholine incorporated in sarcoplasmatic reticulum membranes are observed [33]. This demonstrates that with the high-resolution NMR spectrometer used also in large bilayer systems virtually all of the N-methyl carbons are observed. In agreement with previous observations [11] no well resolved 4-^{13}C signal is visible from the cholesterol molecules due to the rigid nature of the cholesterol ring system. Heating the sample to 60°C and subsequently cooling it down to 0°C results in the appearance of an isotropic ^{31}P-NMR signal, being approx. 30% of the total

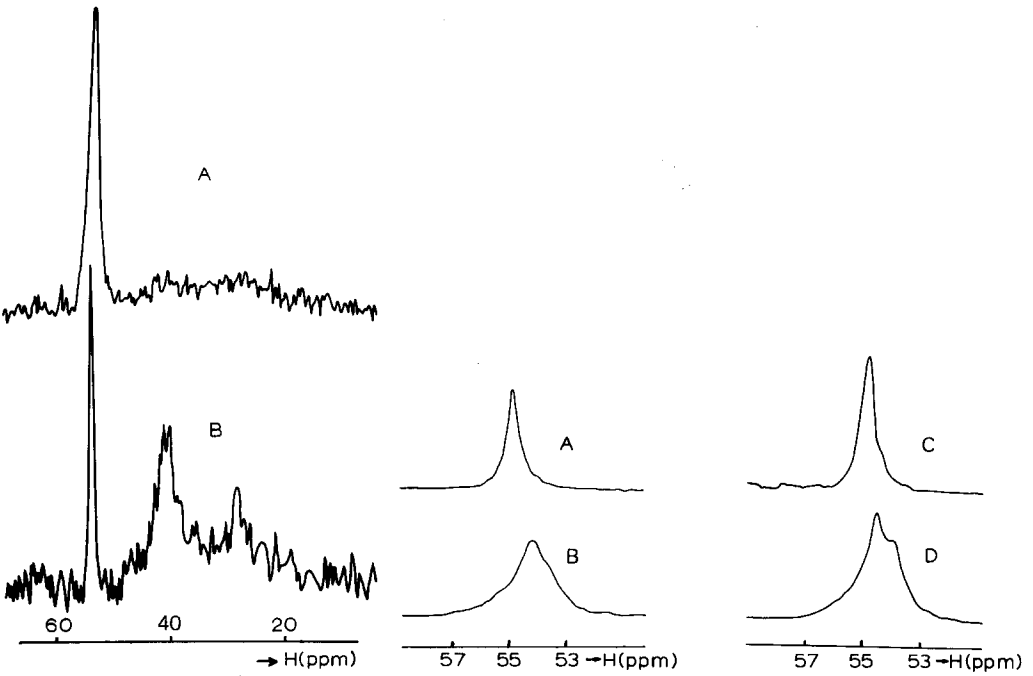

Fig. 3. 90.5 MHz ^{13}C-NMR spectra of an aqueous dispersion of 18 : 1_c/18 : 1_c-phosphatidylethanolamine/18 : 1_c/18 : 1_c-phosphatidylcholine/cholesterol (3 : 1 : 2) at 0°C (A) and at 0°C after being heated to 60°C for 10 min inducing the isotropic phase (B). The sample consisted of 66 μmol 18 : 1_c/18 : 1_c-phosphatidylethanolamine, 15 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine, 7 μmol dioleoylphosphatidyl-[*N*-($^{13}CH_3$)$_3$]choline and 44 μmol [4-^{13}C]cholesterol dispersed in 1.3 ml of the buffer.

Fig. 4. 90.5 MHz ^{13}C-NMR spectra of rat liver microsomes at 37°C (A) and 2°C (B) and rat liver slices at 37°C (C) and 2°C (D). The rat liver slices and microsomes were obtained from rats fed the [*N*-($^{13}CH_3$)$_3$]-choline-supplemented diet as described in Experimental Procedure.

^{31}P-NMR signal and the presence of lipidic particles [26]. In the ^{13}C-NMR spectrum, a corresponding significant line narrowing of the choline signal is observed (Fig. 3B, Table I). In addition, well defined resonances are now observed at 40 and 28 ppm. The large signal at 40 ppm may be assigned to [4-^{13}C]cholesterol (see Ref. 11) whereas the small signal at 28 ppm arises from naturally abundant ^{13}C in the fatty acyl chains. This clearly demonstrates that in the isotropic phase of this particular lipid mixture, cholesterol also can undergo considerably more motion than in the lipid bilayer system. It should be emphasized that these and other NMR data do not provide direct information on the exact structure of these isotropic phases.

Biological membranes

At present only the bilayer structure of biological membranes is well documented. Nevertheless, NMR studies recently indicated that isotropic motion of membrane phospholipids does occur in various membrane preparations. For instance in rat, beef and rabbit liver microsomes (vesiculated fragments of the endoplasmic reticulum) a major fraction of the phospholipids can undergo isotropic motion at 37°C [8,30,31]. At 4°C the 'bilayer' shaped ^{31}P-NMR spec-

trum is observed [8,30,31]. This temperature dependent bilayer ⇄ isotropic transition also appears to occur in part of the membrane phospholipids in liver slices and intact perfused liver [31]. Furthermore, early ^{1}H-NMR [32] and recent ^{31}P-NMR [7] studies demonstrate occurrence of isotropic motion of part of the phospholipids in the sarcoplasmic reticulum membrane. The isotropic motion might arise from (transitory) non-bilayer phases but the possibility that lateral diffusion along curved membrane surfaces produces isotropic motion cannot be excluded.

In order to apply the ^{13}C-NMR technique to biological membranes, specific ^{13}C labelling is required to improve the sensitivity so that the spectrum can be recorded in a reasonably short time. Since the line-width of the N-($^{13}CH_3$)$_3$ resonance is sensitive to the bilayer-isotropic transition in model membranes (Table I) it was decided to incorporate [*N*-($^{13}CH_3$)$_3$]choline in various membranes of rats. As described in Experimental Procedure, a good incorporation of this labelled compound could be obtained in phosphatidylcholine present in the erythrocyte, liver microsomal and sarcoplasmic reticulum membrane. The ^{13}C-NMR spectra of these membranes showed only the presence of a strong N-($^{13}CH_3$)$_3$ resonance (see Fig. 4A for the spectrum of the microsomes at 37°C). The line-widths of the N-($^{13}CH_3$)$_3$ resonance of the various membranes at 37°C are presented in Table I. For the rat erythrocyte in which ^{31}P-NMR demonstrates bilayer structure, a broad slightly asymmetrical ^{13}C resonance is observed with a line-width close to that of the cholesterol-containing phosphatidylcholine/phosphatidylethanolamine mixture in the bilayer phase. In strong contrast, the microsomes and the sarcoplasmic reticulum membranes

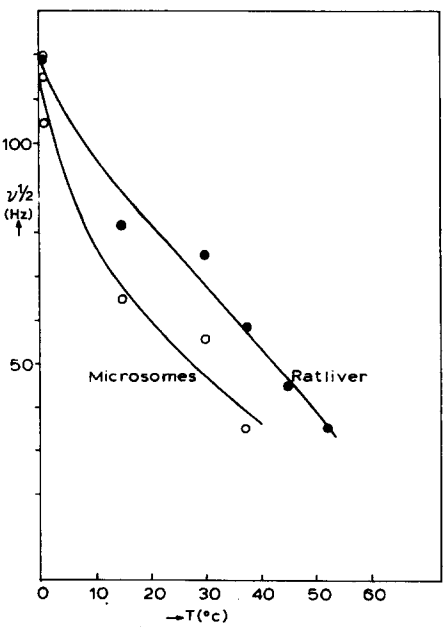

Fig. 5. Temperature dependence of the line-width of the N-($^{13}CH_3$)$_3$ resonance in rat liver microsomes and rat liver slices obtained from rats fed an [*N*-($^{13}CH_3$)$_3$]choline-supplemented diet. Line-widths were determined as the width at half-length of the highest peak in the spectrum.

exhibit a narrow symmetrical resonance with a line-width typical of that of the isotropic phase(s) observed in the model membrane systems. This strongly supports the ^{31}P-NMR data on these membranes which suggests that at physiological temperatures the phospholipids can undergo isotropic motion. In equally good agreement with the ^{31}P-NMR data [8,30,31] at 2°C the ^{13}C resonance of the microsomes is markedly broadened (Fig. 4B). The temperature dependence of the line-width is shown in Fig. 5. The line-width change was fully reversible and displayed no hysteresis.

Since microsomal membranes are derived from the endoplasmic reticulum membrane network in the liver cell, ^{13}C-NMR measurements were also performed on liver slices. As detailed in Experimental Procedure, 60% of the ^{13}C label is incorporated in the liver lipids, the great majority in the phosphatidylcholine fraction. The remaining 40% of the label is present as water soluble compound(s). In the ^{13}C-NMR spectrum of the liver slices only an N-($^{13}CH_3$)$_3$ resonance is observed (Fig. 4). The spectra at 37 and 2°C strongly resemble the spectra of the isolated microsomes at these temperatures in that the signal is much narrower and more symmetrical at 37 than at 2°C. In the spectrum at 2°C (Fig. 4D) there is clearly a narrow signal present on top of the broader asymmetrical signal. This most likely originates from free [*N*-($^{13}CH_3$)$_3$]choline still present in the liver since the diet was given to the rats until the day of removal of the liver in order to ensure a maximal incorporation of [^{13}C]choline in the endoplasmic reticulum. In the 37°C spectrum (Fig. 4C) only one signal is observed which might be due to the much narrower underlying signal from the membrane phospholipids. The temperature dependence of the line-width change in the liver slices resembles that of the isolated microsomes (Fig. 5) and also was fully reversible and showed no hysteresis. Extraction of the total lipids from the liver and dispersing them in buffer results in the formation of bilayers as detected by ^{31}P-NMR (results identical to those shown in Ref. 31). In full agreement, the line-width of the N-($^{13}CH_3$)$_3$ resonance of this dispersion was 200 and 180 Hz at 2 and 37°C, respectively. These data support and extend previous ^{31}P-NMR experiments on isolated liver microsomes [8,30] and liver slices [31] in that at the physiological temperature a significant fraction of the membrane phospholipids (including phosphatidylcholine) of the endoplasmic reticulum undergo isotropic motion on the NMR scale. At present, no evidence is available concerning the exact nature of this isotropic motion.

Conclusions

1. The line-widths of the various resonance resolved in the ^{13}C-NMR spectrum of an 18 : 1$_c$/18 : 1$_c$-phosphatidylethanolamine dispersion are sensitive to the bilayer → hexagonal H_{II} phase transition.

2. In lipid mixtures displaying isotropic ^{13}C-NMR signals and lipidic particles as detected by freeze-fracturing, ^{13}C-NMR line-widths are greatly reduced indicating that the entire lipid molecule can undergo isotropic motion.

3. Specific ^{13}C labelling allows the detection of the bilayer → isotropic phase transition of individual lipids in mixed lipid systems.

4. The line-width of the N-($^{13}CH_3$)$_3$ resonance of rat erythrocyte membranes is consistent with a lamellar organization of the membrane lipids.

5. Line-widths of the N-($^{13}CH_3$)$_3$ resonance in rat liver, rat liver microsomes and sarcoplasmic reticulum membranes at 37°C are similar to those observed for the isotropic phase(s) observed in model membrane systems.

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