31P-NMR STUDIES ON MEMBRANE PHOSPHOLIPIDS IN MICROSOMES, RAT LIVER SLICES AND INTACT PERFUSED RAT LIVER

B. DE KRUIJFF [a,*], A. RIETVELD [a] and P.R. CULLIS [b]

[a] *Institute of Molecular Biology and Department of Biochemistry, State University of Utrecht, Transitorium 3, Padualaan 8, 'De Uithof', Utrecht 3508 TB (The Netherlands) and [b] Department of Biochemistry, University of British Columbia, Vancouver, B.C. (Canada)*

Summary

1. The 36.4 and 81 MHz 31P-NMR spectra of isolated rat liver microsomes, rat liver slices and perfused rat liver have been recorded in the 4—40°C temperature range.

2. In isolated microsomes at 37°C the majority of the phospholipids undergo isotropic motion, whereas at 4°C most of the phospholipids give rise to typical 'bilayer' spectra.

3. Isolated hydrated rat liver microsomal phosphatidylethanolamine is organised in the hexagonal H_{II} phase above 7°C.

4. The Mn^{2+} permeability of the microsomal membrane is strongly temperature dependent. At 37°C Mn^{2+} addition eliminates the entire 31P-NMR spectrum, demonstrating that all phospholipids interact with Mn^{2+}. At 4°C a 43% reduction in signal intensity is observed, indicating that at this temperature 43% of the phospholipids are located in the outer monolayer of a lipid bilayer.

5. In liver slices incubated in oxygenated Krebs-Ringer buffer at 4°C there is a rapid decrease in ATP level such that within 20 min almost all ATP is degraded. In these ATP-depleted liver slices at 4°C, virtually all phospholipids in all membranes have 31P-NMR spectra indicating bilayer structure. At 37°C approx. 14% of the phospholipids undergo isotropic motion.

6. Preliminary experiments on perfused rat liver show stable ATP levels for 4 h at 37°C. The spectra, furthermore, suggest similar behaviour for the membrane phospholipids to that observed in the liver slices.

---

* Present address: Department of Biochemistry, University of British Columbia, Vancouver, B.C., Canada.

7. The possible sources of the observed isotropic motion of the membrane phospholipids are discussed.

Introduction

The preference of certain membrane lipids for non-bilayer arrangements on hydration is well documented [1]. In particular, ^{31}P-NMR techniques have proven to be most useful in detecting the structural organisation of the membrane lipids [1].

However, at present there is little evidence that lipids in biological membranes adopt structures other than bilayers. For instance, erythrocyte membranes show a bilayer type of ^{31}P-NMR spectrum for virtually all the phospholipids [2]. Recently however, it was demonstrated that for beef and rat liver microsomes (small vesiculated fragments originating primarily from the endoplasmic reticulum in the liver cell) a fraction of the membrane phospholipids at $37°C$ undergoes isotropic motion which does not originate from vesicle tumbling [3]. Similar data were obtained for rabbit microsomes by Stier et al. [4], who also demonstrated a striking temperature dependence of the ^{31}P-NMR spectrum, which shows a bilayer shape at low ($8°C$) temperatures.

In this paper we study further the ^{31}P-NMR characteristics of isolated rat liver microsomes. In order to investigate whether isotropic motion of membrane phospholipids occurs in the membrane network of the intact endoplasmic reticulum at $37°C$, ^{31}P-NMR measurements were performed on liver slices and intact perfused liver.

Materials and Methods

Experimental
Preparation of liver slices and microsomes. 1-day fasted adult male Wistar rats were killed and the livers were removed and immediately washed in ice-cold Krebs-Ringer type C [5] buffer saturated with carbogen gas (95% O_2, 5% CO_2), in which phosphate was replaced by Tris, and subsequently cut into slices of approx. 3×8 mm in size. 2—4 slices were transferred to a 10 mm NMR tube containing 0.5 ml of the above buffer plus 10% ^2H_2O. Additional carbogene gas was flushed into the NMR tube, which was then hermetically sealed, after which the NMR experiment was immediately performed (within 10 min of killing the rat). In some experiments employing the high field spectrometer, the liver slices were put in a 15 mm tube through which carbogene gas was bubbled during the NMR experiment. Microsomes were isolated as described above [3]. The final pellet was suspended in 150 mM NaCl/10 mM Tris-HCl (pH 7.0)/10% ^2H_2O (50—75 mg microsomal protein per ml) unless otherwise indicated.

Perfusion of rat liver. 1-day-fasted juvenile (100 g) male Wistar rats were anaesthetized with nembutal and within 5 min a polyethylene cannula was inserted into the portal vein, through which 10% ^2H_2O containing Krebs-Ringer type C buffer saturated with carbogen gas was pumped at a rate of 5 ml/min. While perfusing, the liver was dissected from the surrounding tissue and placed in a flat-bottomed 20 mm diameter NMR tube already filled with buffer. The per-

fusion liquid, which freely leaves the liver, was pumped back out of the top of the NMR tube into a 150 ml reservoir through which carbogene gas was constantly bubbled. In this way the perfusion liquid was kept recycling through the liver.

Lipid extraction and preparation of lipid dispersions. To quantify which fraction of the phosphorus atoms in liver slices was present in phospholipids, a Folch extraction [7] was performed on homogenised liver slices. The extraction was repeated two more times, thereby extracting an additional 5—10% of phospholipids, after which the phosphorus content of the different lower chloroform and upper water/methanol phases was determined. The recovery of phosphorus ranged from 90 to 100%. The fraction of phospholipid phosphorus was calculated from the amount of phosphorus in the combined chloroform layers relative to the total amount of phosphorus in the sample prior to extraction.

For ^{31}P-NMR experiments, total lipids of liver were isolated from a homogenate according to Bligh and Dyer [6]. Phosphatidylcholine and phosphatidylethanolamine were isolated from the Bligh and Dyer [6] extract of rat liver microsomes by means of silica gel column chromatography. Both lipids were more than 99% pure as judged from TLC. Lipid dispersions were prepared by dispersing 50—100 μmol phospholipid in 1 ml 10% ^{2}H$_{2}$O/150 mM NaCl/10 mM Tris-HCl (pH 7.0) buffer as described before [8].

NMR. ^{31}P-NMR measurements were performed under conditions of proton decoupling at 36.4 and 81 MHz as described before [8]. With the 36.4 MHz spectrometer, which was equipped with a 10 mm probe, 5000—10 000 transients in the case of liver slices or 10 000—20 000 transients in the case of microsomes were accumulated using 45° radio frequency pulses with a 0.17 s interpulse time. The dead-time of this spectrometer was 100 μs. The spectrum of liver slices was also recorded with this spectrometer, with identical results at 4°C and 37°C using gated decoupling with 2 s waiting times between subsequent 90° pulses. The 81 MHz spectrometer was used with 15 and 20 mm ^{31}P probes, providing a much better sensitivity. Total accumulation time was typically 2—5 min with a pulse rate of 0.3 s using 90° radio frequency pulses. T_1 determinations on microsomes were performed with this spectrometer using the inversion recovery technique. A value of 0.1 s was found at 37°C for the entire spectrum, suggesting that paramagnetic impurities are present in the microsomes, since the T_1 of phospholipids in membranes in general is a few seconds [9].

In spectra of liver slices and microsomes the fraction of the spectrum with the 'bilayer shape' was obtained by computer subtraction from the observed spectrum, the 'bilayer' spectrum (see Results) of the total liver lipids, until a flat base line and only positive peaks were observed. In experiments on the Mn^{2+} permeability of microsomes, peak areas were determined by computer integration of the spectrum with respect to an external trimethyl phosphite standard.

Analytical methods. Protein was determined according to Lowry et al. [10] and phosphorus according to Fiske and Subbarrow [11] after perchloric acid destruction of the sample. The composition of the rat liver phospholipids was determined by two-dimensional TLC as described by Broeckhuyse [12] and

was found to be: phosphatidylcholine (48.5%); phosphatidylserine (7%); phosphatidylethanolamine (26%); cardiolipin (5%) and sphingomyelin (4.5%), which agreed with published data [13]. Incubation of the liver slices at 37°C for 30 min did not affect the phospholipid content or the phospholipid composition of the slices.

Results

Microsomes

In view of the strong temperature dependence of the ^{31}P-NMR spectrum of rabbit liver microsomes [4], it is of interest to determine whether rat liver microsomes exhibit similar behaviour. As shown in Fig. 1A, the ^{31}P-NMR spectrum obtained from rat liver microsomes is relatively narrow and almost symmetrical at 37°C, demonstrating isotropic motion of the membrane phospholipids. In contrast, at 4°C (Fig. 1B) the spectrum has a pronounced 'bilayer' line shape with a high-field peak and a low-field shoulder (compare the spectrum of the total rat liver lipids in Fig. 8). By computer subtraction of the 'bilayer' spectrum of the total rat liver lipids (which is virtually identical to the spectrum of the total microsomal lipids, Ref. 3), an estimate can be obtained on the temperature dependence of the fraction of the signal with a 'bilayer' line shape. As indicated in Fig. 2 at 4°C, ±90% of the spectrum has a 'bilayer' shape and the remaining 10% of the spectral intensity resides in an 'isotropic' component. With increasing temperature the fraction of the spectrum with a 'bilayer' line shape decreases, to the point that at 30°C only 35% of the spectrum is com-
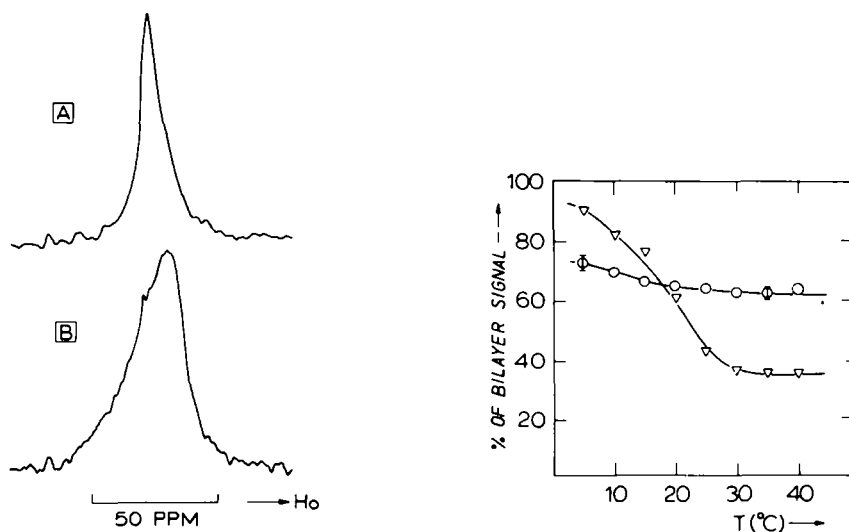

Fig. 1. 36.4 MHz ^{31}P-NMR spectra of rat liver microsomes at 37°C (A) and 4°C (B). A 50 Hz line-broadening was applied to the free induction decays.

Fig. 2. Temperature dependence of the percentage of the [31]P—NMR signal with the 'bilayer' lineshape in the 36.4 MHz spectrum of microsomes (▽———▽) and rat liver slices (○———○). The S.D. in the percentages for rat liver slices are presented as error bars.
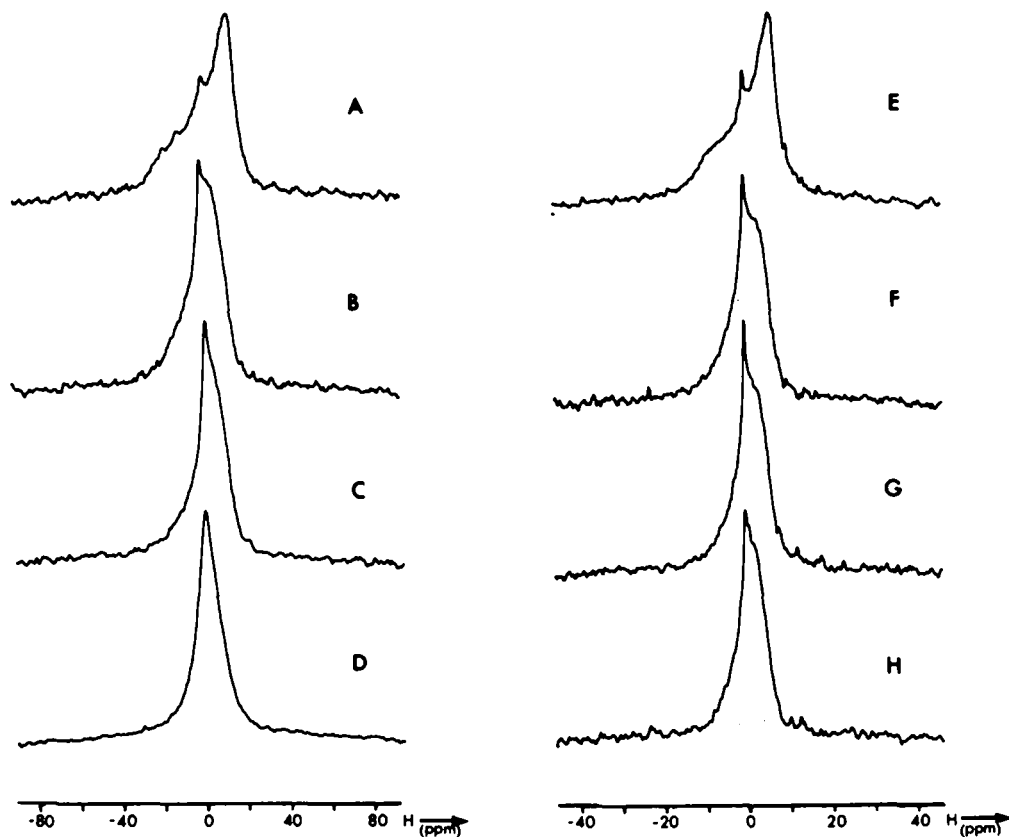
Fig. 3. 81 MHz ^{31}P-NMR spectra of rat liver microsomes dispersed in buffer without (A—D) and with 0.5 mM EDTA (E—H) at 4°C (A and E) and incubated at 37°C for 4.5 (B, F), 10 (C, G) and 30 min (D, H). Line-broadenings of 50 Hz were applied to the free induction decays.

posed of a 'bilayer' component. This fraction remained constant up to 40°C. This temperature-dependent behaviour was reversible, as cooling the microsomes down to 4°C resulted in the same spectrum as obtained originally at 4°C.

The 36.4 MHz ^{31}P-NMR measurements required relatively long data accumulation times (up to 30 min at 37°C). The possibility exists that time-dependent changes in the spectrum occurred during this time (see the next sections). Thus, similar measurements were performed on a more sensitive spectrometer operating at 81 MHz for ^{31}P using larger sample volumes, thereby reducing the data accumulation time to 2.5 min. At 4°C (Fig. 3A) again the typical 'bilayer' spectrum was observed with a small amount (less than 3%) of a very narrow peak, which probably arises from extracellular water-soluble phosphates as the intensity of this component decreases after washing the microsomes. Storing the microsomes at 4°C for up to 4 h did not significantly affect the spectrum. At 37°C the first spectrum (total time at 37°C, 3 min) was much narrower than the 4°C spectrum but was still slightly asymmetrical (Fig. 3B). Incubating the microsomes at 37°C for 30 min resulted in a slight but significant change in the NMR spectrum, which became almost symmetrical (Fig. 3C, D) as was observed
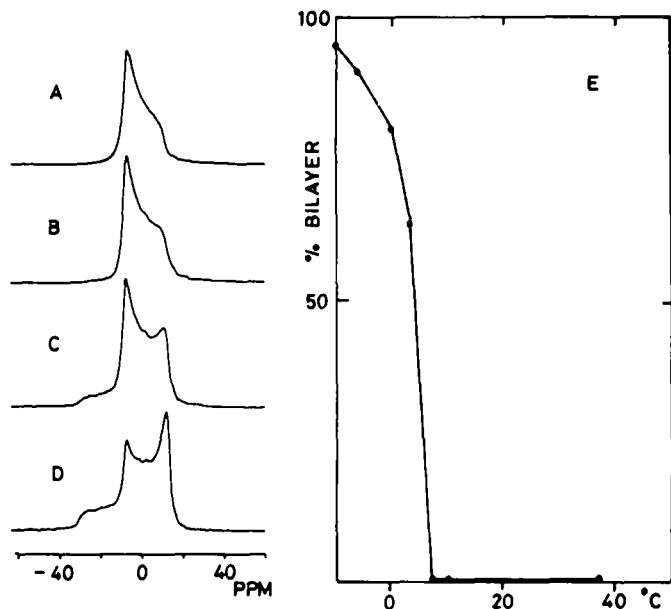
Fig. 4. 81 MHz ^{31}P-NMR spectra of hydrated rat liver microsomal phosphatidylethanolamine at 37°C (A), 7°C (B), 3°C (C) and 0°C (D). In E the percentage of the spectrum with the bilayer lineshape is plotted against the temperature.

at 36.4 MHz (Fig. 1A). Incubation of the microsomes at 37°C for 60 min did not further change the spectrum.

It is possible that divalent cations might be involved in the time dependence of the microsomal ^{31}P-NMR spectrum at 37°C. The measurements were therefore repeated with microsomes dispersed in the same buffer containing 0.5 mM EDTA (Figs. 3E—H). The spectra are very similar to those in the absence of EDTA except that the spectrum is more stable in time.

We conclude that, apart from these small time dependencies in the spectrum at 37°C, a similar temperature dependence of the microsomal spectra is obtained at 81 MHz and at 36.4 MHz.

Since the isotropic motion of the phospholipids could possibly be caused by non-bilayer phospholipid phases in the membrane, we investigated the ^{31}P-NMR line shapes of aqueous dispersions of the two main microsomal phospholipids, e.g., phosphatidylcholine and phosphatidylethanolamine. Microsomal phosphatidylcholine, like all other phosphatidylcholines tested so far [1], organizes itself in extended bilayers as evidenced by the characteristic 'bilayer' line shape (data not shown). In contrast, but in accord with previous data on natural phosphatidylethanolamines [14], the ^{31}P-NMR spectrum of phosphatidylethanolamine at 37°C is typical for that of the hexagonal H_{II} phase (Fig. 4A). Below 7°C the bilayer phase is preferred (Fig. 4). As observed before [14], the hexagonal H_{II} → bilayer transition is remarkably sharp for a naturally-occurring lipid.

Paramagnetic ions such as Mn^{2+} can be used in conjunction with ^{31}P-NMR to discriminate between the two halves of a lipid bilayer, since they dramatically
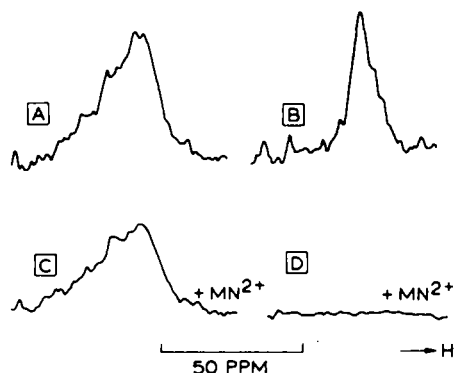
Fig. 5. Effect of Mn^{2+} on the 36.4 MHz ^{31}P-NMR spectra of isolated rat liver microsomes. Spectra A anu B were obtained from microsomes at 4 and 37°C in the absence of Mn^{2+}. Spectra C and D were obtained from microsomes at 4 and 37°C after the addition of 3 mM $MnCl_2$ to the sample. A line-broadening of 50 Hz was applied to the free induction decays.

broaden the signal of those molecules they can approach closely. As shown in Fig. 5, addition of 3 mM $MnCl_2$ to the microsomes at 37°C broadened the total ^{31}P-NMR signal beyond detection, demonstrating that all phosphate groups are exposed to Mn^{2+}. In strong contrast, addition of an identical amount of $MnCl_2$ to the microsomes at 4°C eliminates 43% of the signal. The remaining 57% of the signal still displays the 'bilayer' line shape. Addition of 6 mM $MnCl_2$ did not reduce further the signal intensity, nor was it affected by incubation of the microsomes at 4°C in the presence of 3 mM Mn^{2+} for up to 2 h. However, heating the sample to 37°C and incubating for 10 min and subsequently cooling it to 4°C completely eliminated the signal. This demonstrates that in rat liver microsomes at 4°C, 43% of the phospholipids are exposed to externally added Mn^{2+}.

Liver slices

Fig. 6 shows the 36.4 MHz ^{31}P-NMR spectrum of rat liver slices at 4°C and 37°C. In the 4°C spectrum (total time from removal of the liver from the rat until the midpoint of the data accumulation, 15 min) a broad asymmetrical 'bilayer' component is visible on top of which some much narrower resonances are present. Due to the 50 Hz line-broadening applied to the free induction decay (to increase the signal/noise ratio), these resonances are only partly resolved. That they originate from small water-soluble phosphates is apparent in Fig. 7B. In this spectrum no line-broadening was applied to the free induction decay, which reduces the signal/noise ratio for the 'bilayer' component. However, three narrow resonances are clearly visible. By analogy with ^{31}P-NMR data obtained for hepatocytes [15], we can assign these resonances as follows: the largest peak has the chemical shift position of inorganic phosphate, and the low-field peak the position of a phosphate monoester. The origin of the high-field peak is unknown. The chemical shift position is similar for that of the β-phosphate peak of ATP and ADP. No signal corresponding to the γ-phosphate peak of ATP is present in the spectrum. Incubating the liver slices at 4°C for as long as 105 min did not significantly affect the NMR spectrum (Figs. 6A—D).
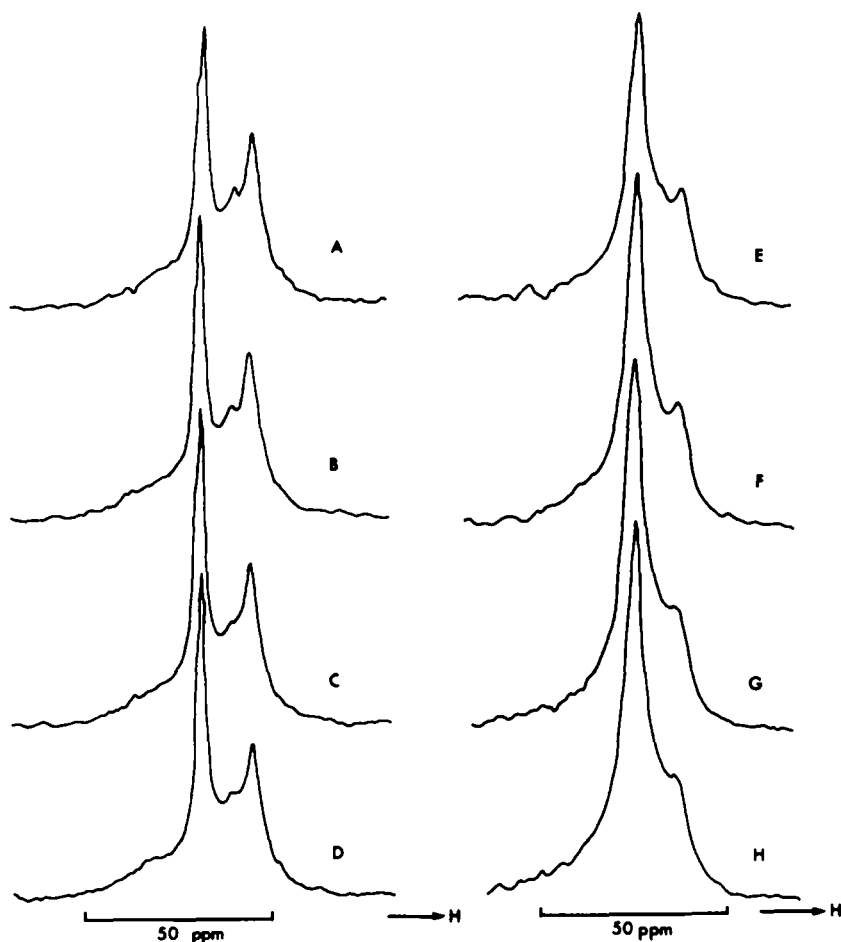
Fig. 6. 36.4 MHz ^{31}P-NMR spectra of rat liver slices incubated at 4 (A—D) and 37°C (E—H) for 15 (A, E), 45 (B, F), 75 (C, G) and 105 min (D, H). A line-broadening of 50 Hz was applied to the free induction decays.
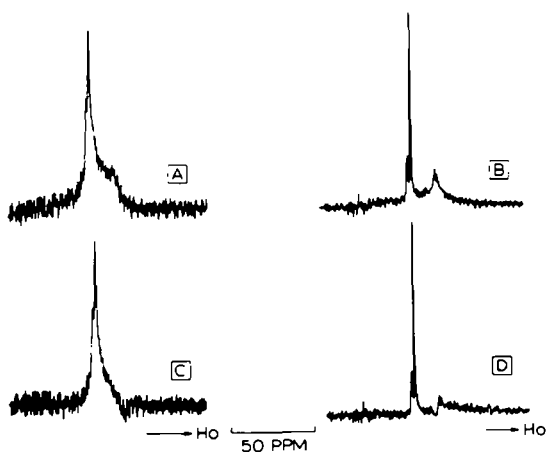

Fig. 7. 36.4 MHz ^{31}P-NMR spectra of rat liver slices at 37°C (A) and 4°C (B). Spectra C and D were obtained after subtraction of the 'bilayer' signal from the rat liver total lipids (see Fig. 8). No line-broadening was applied to the free induction decay.

Fig. 6E shows that the spectrum of liver slices at 37°C (the total time from removal of the liver from the rat until the midpoint of the data accumulation was 15 min) is again composed of a broad asymmetrical 'bilayer' signal upon which a more intense, much broader peak is now superimposed. This is unusual, since in general in the absence of exchange phenomena at higher temperature, NMR signals become sharper due to an increase in motion. In the spectrum recorded under 'high resolution' conditions (Fig. 7A) the broad isotropic peak appears to be composed of the phosphomonoester, the phosphate peak and a broader underlying peak (compare Fig. 7B). The chemical shift position of this peak is very close to that of phospholipids experiencing isotropic motion. Cooling the slices and recording the spectrum at 4°C gave results identical to those shown in Figs. 6A and 7B, showing that the transition in the spectrum is reversible. Incubating the liver slices at 37°C for up to 105 min slightly changed the spectrum (Figs. 6E—H). However, the fraction of signal with the 'bilayer' shape remained virtually constant (66, 67, 65 and 66% for the spectra in Figs. 6E—H, respectively). In view of these time-dependent effects at 37°C, only fresh liver slices were used in further experiments.

As indicated in Experimental, information on the relative amount of the 'bilayer' spectrum could be obtained by subtracting the bilayer signal of the total liver lipids from the spectrum of the liver slices (Fig. 8). Using this procedure it was found that at 4°C, 73 ± 3% (mean ± S.D., six measurements on three different livers) of the signal intensity had the 'bilayer' shape. At 37°C this decreased to 63 ± 2% (mean ± S.D., nine measurements on three different livers). The temperature dependence of the fraction of the signal with the 'bilayer' shape is given in Fig. 2. The main change in the spectrum occurred in the 4—30°C temperature range as was observed for the isolated microsomes. Chemical analysis revealed that 70 ± 4% (mean ± S.D., four experiments) of the phosphate in the rat livers used was present in phospholipids. This strongly suggests that at 4°C all the phospholipids in all the membranes in rat liver slices are organised in extended bilayers. In fact, the spectra in Figs. 8A, C and Figs. 7B, D do show that the spectrum of the liver slices at 4°C only is composed of a 'bilayer' component and the sharp resonances for the various phosphates. However, at 37°C some 10% of the phospholipids appear to have a different dynamic behaviour. In particular, the spectra indicate that these phospholipids undergo isotropic motion (see the difference spectra of Fig. 8F with 50 Hz line-broadening, and Fig. 7C, without line-broadening). Comparing Figs. 8F and 7C with Fig. 1A shows that the broad isotropic peak resembles the microsome spectrum at 37°C.

Very similar 36.4 MHz ^{31}P-NMR spectra were obtained for isolated rat hepatocytes, except that the change in the amount of 'bilayer' component in the spectrum between 4°C and 37°C was 15% (Rietveld, A. and de Kruijff, B., unpublished observations).

In recent ^{31}P-NMR studies on perfused rat liver [16] ^{31}P-NMR signals were obtained from ATP. In our spectra of liver slices no ATP signals could be detected which might be caused by the relatively long accumulation times used and the limited oxygen supply. Therefore, some of the measurements were repeated with the 81 MHz spectrometer using 2 min data accumulation times and passing carbogene gas through the buffer surrounding the slices during the
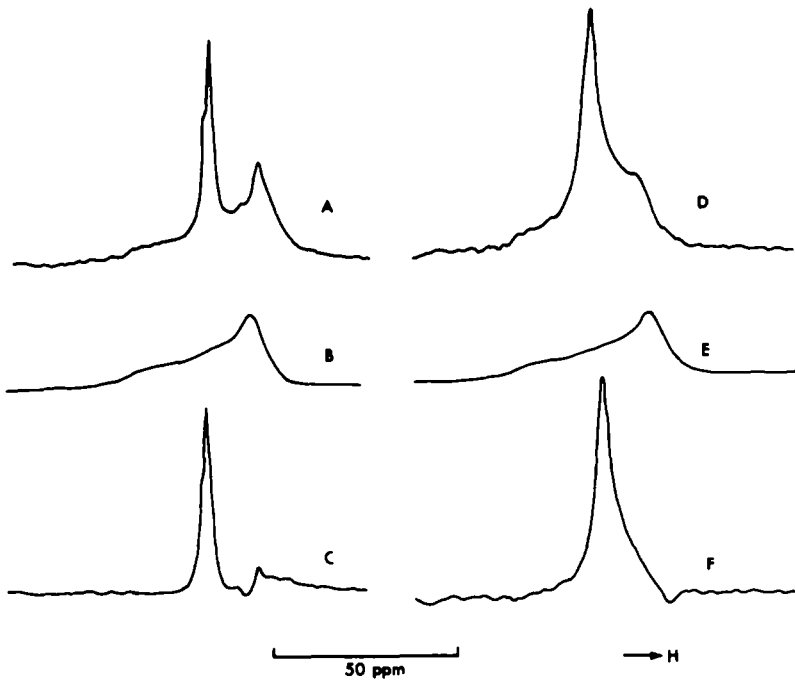
Fig. 8. 36.4 MHz spectra of rat liver slices at 4°C (A) and 37°C (D) and hydrated total liver lipids (B, E). Spectrum C is the difference spectrum of A and B. Spectrum F is the difference spectrum of D and E. A 50 Hz line-broadening was applied to the free induction decays.

NMR experiment. Fig. 9A shows such an experiment for liver slices at 4°C, 3 min after removal of the liver from the rat. Next to the 'bilayer' signal narrow peaks from a phosphomonoester, phosphate, the α- and β-phosphates of ADP and ATP (6 and 13 ppm) and the γ-phosphate of ATP (22 ppm) are resolved (compare with Ref. 16). Incubating the liver slices at 4°C for even as briefly as 5 min caused a dramatic decrease in ATP level (Fig. 9B). Almost no ATP and ADP signals could be detected after 20 min of incubation at 4°C, in agreement with the 36.4 MHz ^{31}P-NMR experiments. The decrease in ATP and ADP signals was accompanied by an increase in the inorganic phosphate signal. The spectrum of the liver slices at 37°C (total time from removal of the liver until the end of the NMR measurement was 5 min) showed the total absence of ATP and ADP signals, a strong phosphate signal on top of a broader 'isotropic' and a 'bilayer' spectrum (Fig. 9C). The temperature dependence of the 81 MHz ^{31}P-NMR spectra of the 'aged' slices (incubated for 20 min at the various temperatures) was very similar to that observed in the 36.4 MHz measurements.

Since the metabolic stability of the liver slices (as judged from the ATP level) was surprisingly low, which is probably caused by an inadequate substrate supply to the liver cells, we decided to investigate the ^{31}P-NMR properties of intact perfused rat liver using the 81 MHz spectrometer.

Intact perfused rat liver

Recently, ^{31}P-NMR measurements were reported on the various metabolite
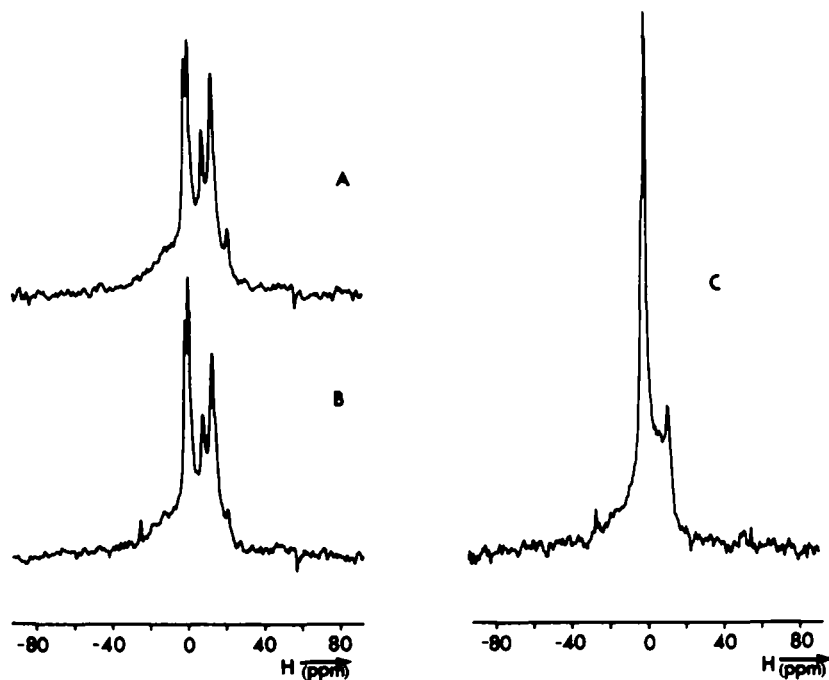
Fig. 9. 81 MHz ^{31}P-NMR spectra of rat liver slices in buffer at 4°C for 3 (A) and 5 min (B) and at 37°C for 5 min (C). Line-broadenings of 50 Hz were applied to the free induction decays.
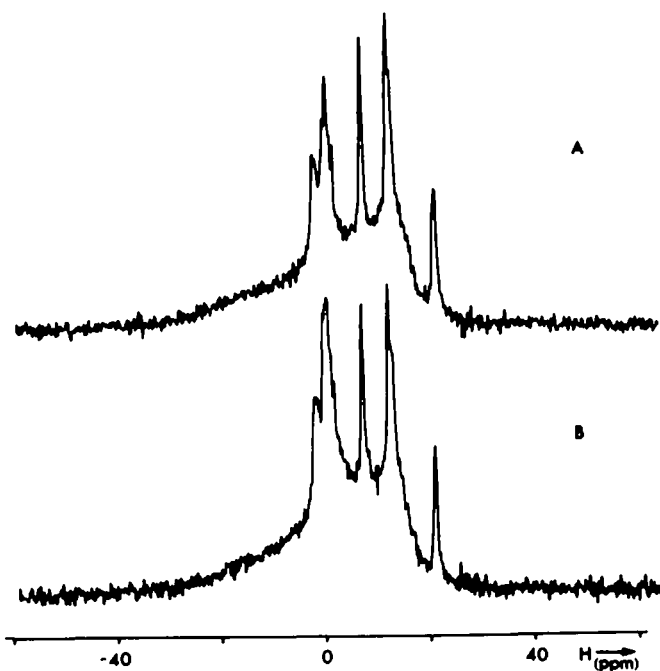

Fig. 10. 81 MHz ^{31}P-NMR spectra of intact perfused rat liver at 4°C (A) and 37°C (B). No line-broadening was applied to the free induction decays. Accumulation time 20 min.

levels in intact perfused rat liver [16]. Using a similar perfusing technique and employing a 20 mm NMR tube, we were also able to monitor the ^{31}P-NMR spectra of intact perfused rat liver. Some preliminary data are presented in Fig. 10. The spectrum at 37°C (Fig. 10B) is again composed of a broad asymmetrical 'bilayer' component on top of which the resonances of the various small molecules observed in the liver slices at 4°C are present. As judged from the intensity of the peak of the γ-phosphate of ATP (22 ppm), the ATP level is considerably higher than in the liver slices. The spectrum of the liver remained unchanged for perfusing the liver for up to 4 h at 37°C, demonstrating the efficiency of the perfusion process in maintaining high ATP levels.

In the spectrum of the perfused liver at 4°C several features can be observed. Firstly, the resonances in the zero ppm region are narrower and less intense as compared to the 37°C situation whereas, as expected, the ATP resonances are slightly broader at the lower temperature. Furthermore, the low-field shoulder of the 'bilayer' spectrum is more pronounced at 4°C as compared to the 37°C spectrum. These preliminary data are consistent with the results obtained on the liver slices in that the great majority of the phospholipids in the various membranes present in the liver are organised in bilayers but are not inconsistent with a small fraction of the phospholipids undergoing isotropic motion at 37°C.

Discussion

The present data confirm and extend a previous ^{31}P-NMR study [3] on rat liver microsomes in that at 37°C a large fraction (approx. 60% as determined using difference spectroscopy, see Fig. 2) of the membrane phospholipids experiences isotropic motion at the NMR time scale. On cooling to 4°C the shape of the NMR spectrum changes to that observed for phospholipids organised in extended bilayers. This reversible temperature-dependent 'bilayer' to 'isotropic' transition was also observed in rabbit liver microsomes [4]. Possible sources of the isotropic motion at 37°C include particle tumbling, lateral diffusion around the microsomal vesicle and the presence of non-bilayer lipid structures [1,3,4] in the membrane. The first two mechanisms are dependent on the size of the membrane system. The correlation time for isotropic motion of a lipid molecule in a spherical vesicle is related to the radius of the particle (a) and the rotational diffusion, D, according to [17]

$$\frac{1}{\tau_c} = \frac{6}{a^2} D \tag{1}$$

where D is composed of a tumbling dependent part $D_t = kT/8\pi a\eta$, in which η is the solution viscosity, and a lateral diffusion dependent part D_d. Freeze-fracturing of the microsome used in this study shows that the predominant size of the spherical particles is about 2000 Å in diameter, as was also found for rabbit liver microsomes [4]. The lateral diffusion rate of a lipid in microsomal membranes was estimated to be 10^{-7} cm²/s [18]. From these data and the water viscosity, it can be calculated that for the microsomes at 37°C vesicle tumbling contributes only 13% to D. The main source for isotropic motional averaging is lateral diffusion of the lipid molecules around the vesicle. That vesicle tumbling does not significantly contribute to the isotropic motion of the phospholipid

molecules is also experimentally well established [3,4]. On considering the possibility that lateral diffusion around the vesicle could cause the observed spectral changes, it is relevant to estimate τ_c for the microsomes at 37°C and 4°C. Using the published value for D_d of microsomes for all the microsomal lipids, and assuming that the temperature dependence of D_d in microsomes is similar to that reported in egg lecithin bilayers [18], it can be estimated that at 37°C $\tau_c = 10^{-4}$ s and at 4°C $\tau_c = 2.5 \cdot 10^{-4}$ s.

Recently the ^{31}P-NMR lineshapes for phospholipids in bilayers were calculated for various values of τ_c [19]. Application of these results to our situation shows that a change in lineshape from that of a bilayer to a more symmetrical spectrum occurs over a narrow range of τ_c between $0.7 \cdot 10^{-4}$ and $3 \cdot 10^{-4}$ (Burnell, E., unpublished observations). It is therefore possible that changes in the rate of lateral diffusion of the phospholipids in the microsomal membrane could produce spectral changes similar to those observed in Fig. 1. However, in view of the assumptions made, it is at the moment not possible to say whether lateral diffusion of the lipid molecules alone could produce the observed spectral effects. Therefore, the third possibility that non-bilayer phases in the microsomal membrane at 37°C give rise to the observed spectral changes cannot be excluded; in particular since the microsomal phosphatidylethanolamine which amounts to 20—25% of the total lipids prefers the hexagonal H_{II} phase above 5°C (Fig. 4). Some of the functional properties of the microsomal membrane certainly do suggest that this membrane is unique and possesses more dynamic properties than are normally encountered in plasma membranes. Firstly, phospholipid flip-flop is very fast at 37°C [20,21] and appears to be much slower at lower temperatures [21]. Secondly, as shown in Fig. 5, the Mn^{2+} permeability is very fast at 37°C and much slower at 4°C. Whether these features are related to possible non-bilayer phospholipid phases or whether they are associated with the slight changes occurring in the ^{31}P-NMR spectrum of the microsomes during incubation at 37°C for times on the order of minutes is not known.

There is a remarkable analogy between these observations and the behaviour of phosphatidylcholine/cardiolipin (1 : 1) bilayers in the absence and presence of Ca^{2+}. An aqueous dispersion of phosphatidylcholine/cardiolipin (1 : 1) is organized in extended bilayers [22] which are impermeable to Mn^{2+} and in which no phosphatidylcholine flip-flop can be demonstrated [23]. Addition of Ca^{2+}, which can induce the hexagonal H_{II} phase with pure cardiolipin [24,25], does induce nonbilayer structures in mixtures with phosphatidylcholine which are possibly of an inverted micellar nature [22,26]. In this situation, Mn^{2+} permeability and phosphatidylcholine flip-flop are markedly increased [22,23]. In passing, another aspect of the Mn^{2+} results obtained on the microsomal membrane at 4°C is that a new mild method for the measurement of the transmembrane distribution of the total phospholipids across a biomembrane is demonstrated. The data of Fig. 5 show that at 4°C 43% of the membrane phospholipids are located in the outer half of the microsomal membrane.

The major goal of the present study was the determination of membrane structure in functionally fully active liver cells, which is a more biologically relevant system than the various isolated membranes. It should be emphasized that although our ^{31}P-NMR approach to the study of macroscopic lipid orga-

nization has some definite disadvantages as compared to other techniques such as X-ray [1], in the case of metabolically active biological membranes and even more so in the case of cells in intact tissue, at present only NMR techniques can give quantitative insight concerning the structural organization of the membrane phospholipids.

The ^{31}P-NMR data on the liver slices in oxygenated buffer show the high metabolic activity of the system as judged from the very rapid decrease in ATP levels. Whether this has any consequence for the structure of endogenous membranes is unknown. The ^{31}P-NMR data on these 'ATP-depleted' liver slices show that at $4°C$ virtually all the phospholipids have motional characteristics typical for lipids organized in extended bilayers. Therefore, in the great majority of the membranes in this tissue, e.g., rough and smooth endoplasmic reticulum membranes, mitochondrial inner and outer membranes, plasma and nuclear membranes, the phospholipids are organized in a lipid bilayer. However, with increasing temperature the amount of 'bilayer' signal decreases in conjunction with the appearance of a broad isotropic peak such that at $37°C$ $10 \pm 3\%$ of the ^{31}P-NMR signal may be attributed to phospholipids undergoing isotropic motion. Since $70 \pm 4\%$ of the phosphorus in rat liver slices is present in phospholipids, this means that 14% of the phospholipids are involved in this transition. ^{13}C-NMR data on ^{13}C-enriched phosphatidylcholine incorporated into the endogenous membranes of liver slices also demonstrated the existence of isotropic motion of part of the lipids at $37°C$ [27]. At present it is not known in which membrane(s) these phospholipids are located. In view of temperature-dependent 'bilayer' $\rightleftharpoons$ 'isotropic' transition in microsomes, it is tempting to speculate that they originate in the endoplasmic reticulum, which is the most abundant membrane in the liver cell [28]. However, the possibility that they are localized (in part) in the mitochondrion cannot be excluded, since evidence of isotropic motion of part of the phospholipids in the isolated inner mitochondrial membrane of rat liver has been obtained under certain conditions [29].

Our preliminary data on the perfused liver, under conditions of full metabolic activity, also suggest the occurrence of predominantly bilayer structure in the various membranes but also point to the occurrence of a small fraction of phospholipids undergoing isotropic motion at $37°C$. As in the case of the isolated microsomes, there is no information available about the precise nature of the isotropic motion of the phospholipids. In view of the preceding arguments, tumbling of small membrane fragments or vesicles does not seem to play an important role. Whether it arises from the diffusion of phospholipids along curved surfaces or from phospholipids in non-bilayer phases is unknown. Saturation transfer ^{31}P-NMR techniques are presently being applied to these systems in order to get a better understanding of the possible occurrence of non-bilayer phases in biological membranes.

Acknowledgements

References

1 Cullis, P.R. and de Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399—420
2 Cullis, P.R. and Grathwohl, C. (1977) Biochim. Biophys. Acta 471, 213—226
3 De Kruijff, B., van den Besselaar, A.M.H.P., Cullis, P.R., von den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 514, 1—8
4 Stier, A., Finch, S.A.E. and Bösterling, B. (1978) FEBS Lett. 91, 109—112
5 Krebs, H.A. (1950) Biochim. Biophys. Acta 4, 249—256
6 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
7 Folch, L., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
8 Cullis, P.R. and de Kruijff, B. (1976) Biochim. Biophys. Acta 436, 523—540
9 Moore, N.F., Patzer, E.J., Wagner, R.R., Yeagle, P.L., Hutton, W.C. and Martin, R.B. (1977) Biochim. Biophys. Acta 464, 234—244
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Fiske, C.H. and Subbarrow, Y. (1925) J. Biol. Chem. 66, 375—379
12 Broeckhuyse, R.M. (1969) Clin. Chim. Acta 23, 457—461
13 Ansell, G.B. and Hawthorne, J.N. (eds.) (1964) Biochim. Biophys. Acta Library III; Phospholipids, chemistry, metabolism and function. Elsevier, Amsterdam
14 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 513, 31—42
15 Cohen, S.M., Ogawa, S., Rottenberg, H., Glynn, P., Yamare, T., Brown, T.R. and Schulman, R.G. (1978) Nature 273, 554—556
16 Salhany, J.M., Stohs, S.J., Reinke, L.A., Pieper, G.M. and Hansing, J.M. (1979) Biochem. Biophys. Res. Commun. 86, 1077—1083
17 Cullis, P.R. (1976) FEBS Lett. 70, 223—228
18 Galla, H.J., Hartmann, W., Theilen, U. and Sachmann, E. (1979) J. Membrane Biol. 48, 215—234
19 Campbell, R.F., Melrovitch, E. and Freed, J.H. (1979) J. Phys. Chem. 83, 525—533
20 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110
21 Van den Besselaar, A.M.H.P., de Kruijff, B., van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242—255
22 de Kruijff, B., Verkleij, A.J., van Echteld, C.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and de Gier, J. (1979) Biochim. Biophys. Acta 555, 200—209
23 Gerritsen, W.J., de Kruijff, B., Verkleij, A.J., de Gier, J. and van Deenen, L.L.M. (1980) Biochim. Biophys. Acta, in the press
24 Rand, R.P. and Sengupta, S. (1972) Biochim. Biophys. Acta 255, 484—492
25 Cullis, P.R., Verkleij, A.J. and Ververgaert, P.H.J.T. (1978) Biochim. Biophys. Acta 513, 11—20
26 Verkleij, A.J., Mombers, C., Leunissen-Bijvelt, J. and Ververgaert, P.H.J.T. (1979) Nature 279, 162—163
27 De Kruijff, B., Rietveld, A. and van Echteld, C.J.A. (1980) Biochim. Biophys. Acta, in the press
28 Jelsema, C.L. and Morre, D.J. (1978) J. Biol. Chem. 253, 7960—7971
29 Cullis, P.R., de Kruijff, B., Hope, M.J., Nayar, R., Rietveld, A. and Verkleij, A.J. (1979) Biochim. Biophys. Acta, in the press