*Biochimica et Biophysica Acta*, 598 (1980) 206—211
© Elsevier/North-Holland Biomedical Press

## BBA Report

BBA 71458

# APPLICATION OF $^{31}$P-NMR SATURATION TRANSFER TECHNIQUES TO INVESTIGATE PHOSPHOLIPID MOTION AND ORGANIZATION IN MODEL AND BIOLOGICAL MEMBRANES

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

[a] *Department of Biochemistry and* [b] *Department of Chemistry, University of British Columbia, Vancouver, B.C. (Canada)*

(Received January 24th, 1980)

*Key words: $^{31}$P-NMR; Saturation transfer; Phospholipid organization; Motional characteristics; Lateral diffusion*

## Summary

The potential of $^{31}$P-NMR saturation transfer experiments for determining motional characteristics (in the millisecond to second time scale) of phospholipids in model and biological membranes is demonstrated. A technique to separate membrane phospholipid $^{31}$P-NMR signals from those of small water-soluble phosphates in intact cells in liver tissue is also illustrated.

Saturation transfer $^{31}$P-NMR techniques have recently been applied to studies of ATP kinetics in cells [1]. The principle of the technique involves 'saturating' the NMR response of a given nucleus in a particular chemical environment and detecting the transfer of this saturation to nuclei in other chemical environments. This allows the elucidation of exchange processes with rate constants of the order of seconds or less [1]. We show in this work that saturation transfer methods are also useful to study phospholipid dynamics in model and biological membranes. In particular, exchange processes between different phospholipid structures and environments can be monitored. Furthermore, this technique can be employed to resolve the phospholipid $^{31}$P-NMR response from that of other phosphorous-containing compounds in intact tissue.

The methods for obtaining egg lecithin, dipalmitoyl phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), rat liver microsomes and intact perfused rat liver are indicated elsewhere [2,3]. Small unilamellar vesicles were

* Present address: Physical Chemistry Laboratory, South Parks Road, Oxford, OX1 3QZ, U.K.

prepared by sonication [4], large unilamellar vesicles by the ether-evaporation method [5] and partly-hydrated phospholipid dispersions as described before [6]. A standard Bruker WP 200 wide-bore NMR spectrometer equipped with proton decoupling was used for all NMR measurements using a sweep width of 20 kHz and 4K data points. Saturation of a particular resonance was achieved employing the DANTE pulse train of Morris and Freeman [7] using a slightly modified version of the standard Bruker FTQNMR program. The pulse sequence used was $[D_0 - (P_1 - D_1)_{N_p} - P_1 - D_2 - P_w - D_e - \text{Acquisition}]_{N_s}$. $D_0$ and $D_2$ are variable delays. $P_1$ and $D_1$ are the pulse width and delay between the saturation pulses. This sequence is repeated $N_p$ times. $P_w$ is the 90° radio-frequency pulse used for data acquisition. $D_e$ is a delay (10 $\mu$s) between $P_w$ and the subsequent data acquisition and $N_s$ is the number of times the whole pulse sequence was repeated. Using this pulse sequence the saturation irradiation lasts approx. $N_p \times D_1$ s with a power level proportional to $\nu_1$ $(P_1/D_1)$, where $\nu_1$ is the pulse power $(1/4P_{w,90°})$ which in general was 25 kHz. The saturation is found at frequencies $O_1 \pm n/D_1$, where $O_1$ is the carrier frequency for $^{31}P$ and $n = 0, 1, 2, 3$ .... By setting $O_1$ and selecting appropriate values for $D_1$, saturation (resulting in decreasing signal intensity or even total elimination of the signal) will be observed only in discrete regions of the spectrum. The actual values of $D_0$, $P_1$, $D_1$, $D_2$, $N_p$, $P_w$ and $N_s$ are given in the legends of the figures.

The proton-decoupled $^{31}P$-NMR spectrum of lamellar phospholipids in large (greater than 4000 Å diameter) model and biological membranes has a characteristic line shape [8]. This line shape results from the large chemical shift anisotropy experienced by phospholipid phosphorus, which is only partially averaged by the restricted modes of motional averaging available. Under these circumstances, the chemical shift of each phospholipid $^{31}P$-NMR resonance is dependent on the orientation of the phospholipid with respect to the applied magnetic field, and the observed line shape results from a summation over all available orientations [9]. In contrast, in small (less than 500 Å diameter) phospholipid structures, tumbling and lateral diffusion of the lipids are rapid on the NMR time scale. This results in isotropic motional averaging and a sharp symmetrical resonance at a characteristic chemical shift position [8,9]. This is illustrated in Fig. 1A for a mixture of large unilamellar vesicles (average size ± 4000 Å diameter [5]) and small unilamellar vesicles (average size 250 Å in diameter [4]) of egg lecithin. The narrow resonance of the small unilamellar vesicles is superimposed on the broad asymmetrical resonance arising from the large unilamellar vesicles (in the absence of small unilamellar vesicles no narrow resonance is present).

Applying a train of saturation pulses at the high-field side of the large unilamellar vesicle spectrum (see arrow in Fig. 1A) results in virtually complete elimination (saturation) of the entire large unilamellar vesicle spectrum (Fig. 1B). The signal from the small unilamellar vesicles is not significantly affected (see the difference spectrum, Fig. 1C). A similar result is obtained when the saturation is applied to the low-field side of the large unilamellar vesicle spectrum (Fig. 1D—F). This behaviour can be rationalized as follows. At any given moment, only a small proportion of the phospholipid $^{31}P$ nuclei are saturated. These nuclei correspond to those phospholipids which have an orientation with

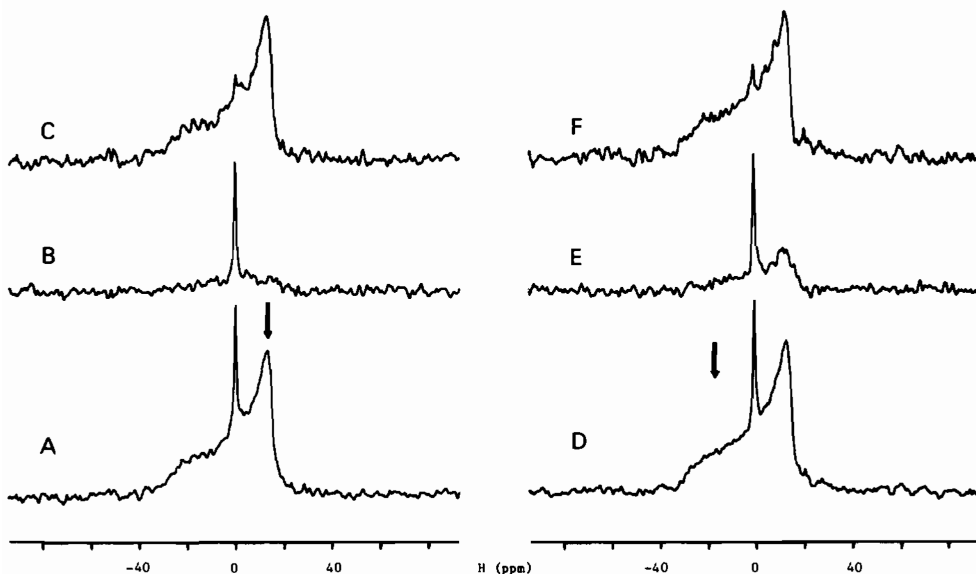


Fig. 1. Saturation transfer $^{31}$P-NMR of a mixture of large unilamellar vesicles and small unilamellar vesicles of egg lecithin at 30°C. Large unilamellar vesicles were prepared by injection at a rate of 0.25 ml/min of 4 ml diethyl ether (2.5 mM in egg lecithin) in 8 ml 100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7, buffer at 55°C with a motor-driven syringe. This was repeated eight times and the combined aqueous fractions containing 80 μmol of phosphatidylcholine were centrifuged for 10 min at 3000 × g. The pellet was dissolved in 2.5 ml of the buffer and dialyzed at 4°C overnight against 1 l of the above buffer to remove traces of ether. 0.5 ml small unilamellar vesicles (containing 7 μmol of phosphatidylcholine) prepared in the $^2H_2O$ analogue of the buffer was added and the mixture was transferred to a 10 mm diameter NMR tube. Spectra were recorded without (A and D) and with (B and E) saturation. Saturation conditions employed: $D_0$ =0, $D_1$ = 100 μs, $D_2$ = 10 μs, $P_1$ = 0.4 μs and $N_p$ = 10 000. The arrows in spectra A and D (and also in Figs. 2—4) indicate the carrier frequency at which the saturation was applied. C and F are difference spectra of A — B and D — E, respectively. For all spectra $P_w$ = 15 μs and $N_s$ = 500. In spectra A and D a delay of 1 s was used in between pulses.

respect to the magnetic field such that their chemical shift coincides with the frequency of the selective irradiation. The duration of the saturating pulse train (1 s in this experiment) is long compared to the time for a lipid molecule to experience reorientation via large unilamellar vesicle tumbling or lateral diffusion around the large unilamellar vesicle. The correlation time for this isotropic motion ($\tau_c$) can be estimated [10] to be approx. 0.1 s. Thus, during the saturation period, virtually all the phospholipid phosphorus nuclei will experience the irradiation with concomitant loss of signal intensity. The relatively large residual (non-saturated) signal from the large unilamellar vesicles when saturating at the low-field side of the spectrum can be attributed to the smaller density of spins in this spectral region. As no exchange of lipid molecules occurs between the large unilamellar vesicles and small unilamellar vesicles, the signal of the small unilamellar vesicles will not be affected by exchange processes, and the slight decrease in signal intensity upon saturation arises from the limited selectivity of the saturating pulse train employed.

These experiments were also conducted on partially-hydrated liquid crystalline dipalmitoyl phosphatidylcholine bilayers (Fig. 2). In this system, application of a short (0.15 s) pulse train resulted in 'hole-burning' (see Fig. 2B) at the

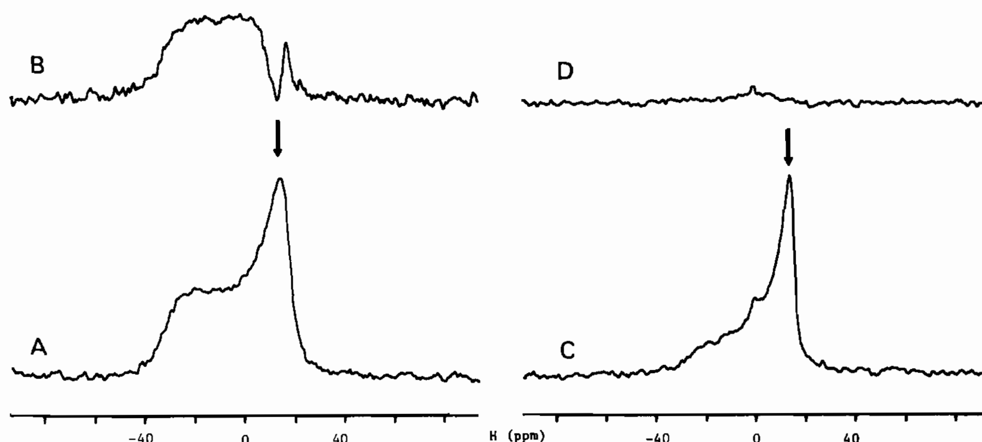


Fig. 2. Saturation transfer $^{31}$P-NMR of dipalmitoyl phosphatidylcholine bilayers at 60°C. $^{31}$P-NMR spectra of partly-hydrated dipalmitoyl phosphatidylcholine without (A) and with (B) saturation. $^{31}$P-NMR spectra of the same sample after the addition of 1.0 ml of 100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0, without (C) and with (D) saturation. Saturation conditions: $D_0$ = 0.85 s, $D_1$ = 200 $\mu$s, $D_2$ = 2 $\mu$s, $P_1$ = 1 $\mu$s, $N_p$ = 250. In all experiments $P_w$ = 11 $\mu$s and $N_s$ = 750. In spectra A and C a delay of 1 s was used between pulses. The partly-hydrated sample was obtained by incubating 100 $\mu$mol of dry lipid in a closed 10 mm NMR tube containing an open reservoir of a saturated NaCl solution for 12 h at 60°C. In this way the lipid takes up approx. 16% by weight of water [6].

irradiation frequency. It appears that in this system bilayer tumbling and/or lateral diffusion of the lipids around curved bilayer surfaces is slow ($\tau_c >>$ 0.15 s) reducing the rate at which the saturation is transferred over the entire spectrum. In contrast, repetition of the same experiment on this sample after the addition of excess buffer (resulting in the formation of liposomes) results in the virtually total saturation of the signal (Fig. 2C, D). This agrees with the data obtained for the large unilamellar vesicles composed of egg lecithin. Very similar results were obtained on biological membranes such as that of the rat liver microsome at 4°C (Fig. 3). The spectrum is composed of a broad asymmetrical component typical of extended bilayers and a narrow resonance from water-soluble phosphates [3]. Saturation at the position indicated by the arrow eliminates most of the phospholipid signal without significantly affecting the narrow component.

These experiments clearly show the sensitivity of the saturation transfer technique to phospholipid dynamics in the second to millisecond time scale. Measurement of exchange rates of lipid molecules between different phospholipid phases, such as the hexagonal $H_{II}$ and, in particular, 'isotropic' phases often encountered in aqueous dispersions of membrane phospholipids [8], is one of many potential applications. These studies are presently being undertaken. Similarly, exchange processes of lipids between biological membranes or large unilamellar vesicles and small unilamellar vesicles or other small lipid aggregates such as micelles could be conveniently monitored. Furthermore, selective hole-burning experiments, as depicted in Fig. 2 on large unilamellar vesicles of defined sizes could be used to obtain measures of lateral diffusion rates without the use of probe molecules.

An alternative and potentially most useful application of the saturation

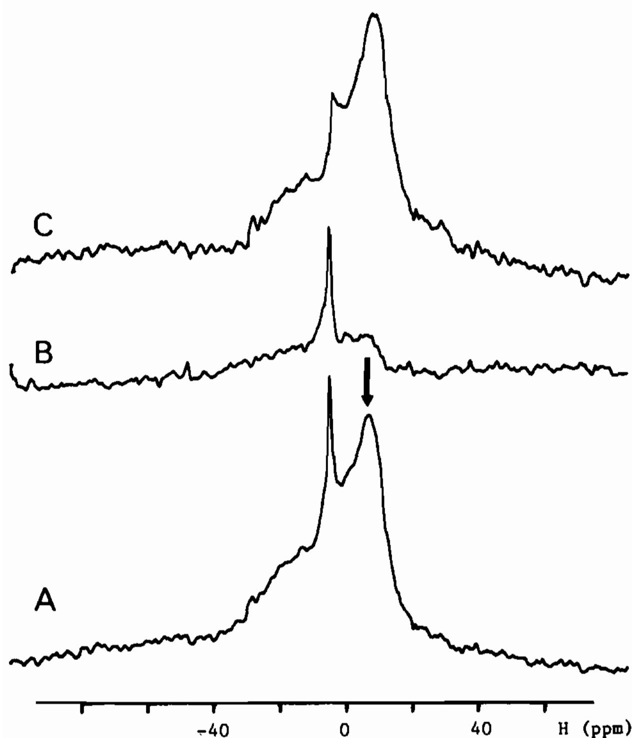


Fig. 3. Saturation transfer $^{31}$P-NMR of rat liver microsomes at 4°C. The sample consists of 4 ml of 100 mM NaCl, 10 mM Tris-HCl, pH 7.0, 0.5 mM EDTA containing 10% $^2H_2O$ and approx. 200 mg membrane protein in a 15 mm diameter NMR tube. (A) Spectrum without and (B) with saturation. (C) Difference spectrum of A and B. Saturation conditions: $D_0 = 0$, $D_1 = 100\ \mu s$, $D_2 = 1\ \mu s$, $P_1 = 0.5\ \mu s$ and $N_p = 3000$. In all experiments $P_w = 17\ \mu s$ and $N_s = 500$. In spectrum A a delay of 0.3 s was used between pulses.

transfer technique involves the elucidation of macroscopic phospholipid organization in intact cells and tissues. In such systems the presence of non-phospholipid phosphates (e.g., ATP, ADP, $P_i$, etc.) often masks the underlying phospholipid resonances. This is illustrated in Fig. 4a for perfused rat liver (for details on the spectrum see Ref. 3). It is clearly difficult to extract the phospholipid component of this spectrum unambiguously. However, the application of selective saturation at a frequency between the $\alpha$ and $\beta$ ATP resonances reduces the amplitude of the broad phospholipid resonance without markedly affecting the metabolite signals (Fig. 4B). Thus, subtraction of the spectrum of Fig. 4B from that of Fig. 4A gives rise to the spectrum of Fig. 4C where the contribution from non-phospholipid phosphates is much reduced. This allows the phospholipid resonance to be more clearly resolved as an asymmetrical spectrum characteristic of phospholipids in extended bilayers. Improvements in the selectivity of the saturation irradiation can be expected to result in the quantitative elimination of small molecule resonances, providing a new and direct means of determining phospholipid organization in intact living systems.

This work was supported by the Canadian Heart Foundation and the Medical Research Council of Canada. The support of the Killam Foundation (Postdoctoral Fellowship to G.A.M.) is gratefully acknowledged. P.R.C. is a research

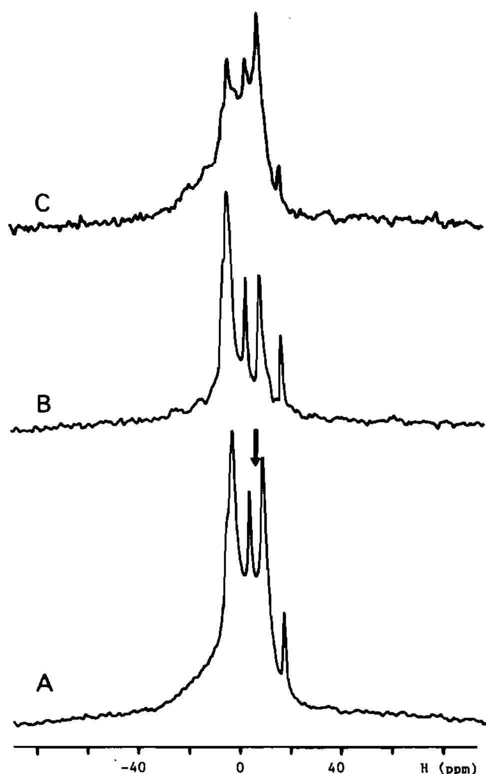


Fig. 4. Saturation transfer $^{31}$P-NMR of intact perfused rat liver at 37°C. The rat liver was perfused in a 20 mm diameter NMR tube with oxygenated Krebs-Ringer buffer [3]. Spectrum without (A) and with (B) saturation. (C) Difference spectrum of A and B. Saturation conditions: $D_0 = 0$, $D_1 = 100$ $\mu$s, $D_2 = 1$ $\mu$s, $P_1 = 0.6$ $\mu$s and $N_p = 3000$. For all measurements $P_w = 30$ $\mu$s and $N_s = 2000$. In spectrum A a delay of 0.3 s was used between pulses.

scholar of the M.R.C. and B. de K. is a Visiting Scientist of the M.R.C. and Canadian Heart Foundation (1979—1980).

## References

1 Brown, T.R., Ugurbil, D. and Schulman, R.G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5551—5553
2 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207—218
3 De Kruijff, B., Rietveld, A. and Cullis, P.R. (1980) Biochim. Biophys. Acta 599, in the press
4 De Kruijff, B., Cullis, P.R. and Radda, G.K. (1976) Biochim. Biophys. Acta 436, 729—740
5 Deamer, D.W. and Bangham, A.D. (1976) Biochim. Biophys. Acta 443, 629—634
6 Hemminga, M.A. and Berendsen, H.J.C. (1972) J. Magn. Resonance 8, 133—143
7 Morris, G.A. and Freeman, R. (1978) J. Magn. Resonance 29, 433—453
8 Cullis, P.R. and de Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399—420
9 Seelig, J. (1978) Biochim. Biophys. Acta 575, 105—140
10 Cullis, P.R. (1976) FEBS Lett. 70, 223—228