Physical Studies on Phosphonium Phosphatidylcholine

A UNIQUE [^{31}P]PHOSPHORUS NUCLEAR-MAGNETIC-RESONANCE PROBE FOR MODEL AND BIOLOGICAL MEMBRANES

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1. Distearoyl phosphatidylcholine and the phosphonium analogue, in which the nitrogen atom is replaced by phosphorus, show similar gel-liquid crystalline transition temperatures as detected by differential scanning calorimetry. 2. The temperature-dependence of the ^{31}P n.m.r. (nuclear-magnetic-resonance) linewidths of the phosphate resonances of sonicated vesicles of distearoyl phosphatidylcholine and the phosphonium analogue are similar. Below the phase-transition temperature the linewidths decrease as the temperature is raised. Above the phase-transition temperature the phosphate resonances are relatively temperature-independent. The phosphonium ^{31}P n.m.r. signal exhibits the same pattern of temperature-dependence. 3. The ^{31}P n.m.r. phosphonium resonance is sensitive to the paramagnetic shift reagent, $\text{K}_3\text{Fe}(\text{CN})_6$. Use of $\text{K}_3\text{Fe}(\text{CN})_6$, together with $\text{Nd}(\text{NO}_3)_3$, enabled the determination of the trans-bilayer distribution of egg-yolk phosphatidylcholine and its phosphonium analogue in co-sonicated vesicles. Both are distributed comparably across the bilayer of the vesicles. 4. The phosphonium ^{31}P n.m.r. signal is much sharper than the corresponding phosphate resonance in both sonicated and unsonicated dispersions of the phosphatidylcholine analogue. 5. The properties of the phosphonium analogue of phosphatidylcholine are discussed in terms of its suitability as a probe of membrane structure.

[^{31}P]Phosphorus n.m.r. has been shown to be a powerful technique in the study of model membrane systems (Michaelson *et al.*, 1973). In particular, for sonicated vesicles of two or more phospholipids, well-resolved ^{31}P n.m.r. signals have been observed and the distribution of each species on the inside and outside of the bilayer vesicles may be determined (Berden *et al.*, 1974). For biological membranes such resolution is not possible because much broader ^{31}P n.m.r. signals are obtained (Davis & Inesi, 1972).

Edwards (1973) demonstrated that the phosphonium analogue of 1,2-dioctadecanoyl-*sn*-glycero-3-phosphorylcholine (distearoyl phosphatidylcholine), in which the nitrogen atom is replaced by phosphorus, gives two distinct ^{31}P n.m.r. absorption peaks. The phosphonium resonance is 28 p.p.m. downfield from the phosphate resonance of phosphatidylcholine (Edwards, 1973) and is thus distant from other biological phosphate ^{31}P n.m.r. signals (Henderson *et al.*, 1974; Hoult *et al.*, 1974). Further, the phosphonium analogue of choline is incorporated into phosphatidylcholine by rats (Edwards, 1973), *Neurospora crassa* (J. Grayheb, unpublished work) and a variety of cultured cell lines (Pasternak *et al.*, 1975) without causing any apparent adverse effects. The phosphonium

analogue of phosphatidylcholine therefore seems to cause minimal perturbation of biological membranes. This is in contrast with electron-spin-resonance labels (Kornberg & McConnell, 1971) and fluorescent probes (Radda & Vanderkooi, 1972), which have been used in membrane studies and may excessively perturb the system.

As a necessary preliminary to investigations on natural membranes labelled *in vivo* with the phosphonium analogue of choline, we describe here some physical studies of phosphonium phosphatidylcholine in model membrane systems, by using ^{31}P n.m.r. and differential scanning calorimetry.

Experimental

Lipids

The phosphonium analogue of distearoyl phosphatidylcholine was synthesized by Edwards (1973) by the method of Aneja & Chadha (1971). Distearoyl phosphatidylcholine was obtained from Calbiochem Ltd., London W1, U.K.

Egg-yolk lecithin (Fisons, Loughborough, Leics., U.K.) was purified initially by the method of Hanahan *et al.* (1951) and further purified by t.l.c. on 1 mm-thick activated silica gel H (E. Merck, Darmstadt,

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Germany) in solvent system I (chloroform-methanol-7M-NH₃, 46:18:3, by vol.) (Quinn, 1973). After exposure to iodine vapour, the phosphatidylcholine band was removed, eluted with ethanol-chloroform-water (10:3:2, by vol.) and dried under reduced pressure.

The phosphonium analogue of egg-yolk phosphatidylcholine was prepared from the purified egg-yolk phosphatidylcholine by using the alcohol transferase activity of cabbage phospholipase D (EC 3.1.4.4), purchased from Sigma, Kingston-upon-Thames, Surrey, U.K., in the presence of excess of phosphonium choline chloride (synthesized as described by Edwards, 1973). A modification of the procedure of Yang *et al.* (1967) was used. Purified egg-yolk phosphatidylcholine (1 g) was dispersed in 60 ml of 20 mM-sodium acetate buffer (pH 5.5) containing 4 M-phosphonium choline chloride and 25 mM-CaCl₂. Phospholipase D (1700 units, where 1 unit releases 1 μ mol of choline from phosphatidylcholine/h) was then added with diethyl ether (15 ml). The resulting mixture was shaken vigorously at room temperature (23°C) for 4 h. The ether was then removed and the residue extracted (Lands & Hart, 1965), dried under reduced pressure and resuspended in chloroform-methanol (2:1, v/v).

The phosphonium egg-yolk phosphatidylcholine was separated from egg-yolk phosphatidylcholine and 1,2-diacyl-*sn*-glycerol 3-phosphate by two-dimensional t.l.c. on silica gel H by using solvent system I in the first dimension, and in the second dimension system II, chloroform-methanol-water-acetic acid (50:50:4:1, by vol.) (Table 1). The spots were detected by exposure to iodine vapour and identified by comparison with chromatograms of 1,2-diacyl-*sn*-glycerol 3-phosphate (Lipid Products, Redhill, Surrey, U.K.), distearoyl phosphatidylcholine and phosphonium distearoyl phosphatidylcholine. Appropriate regions of the silica gel were removed, eluted and dried as above and then resuspended in chloroform-methanol (2:1, v/v).

Preparation of samples

Vesicles of distearoyl phosphatidylcholine or the phosphonium analogue were prepared by sonicating

approx. 20 mg of either phospholipid in 1.5 ml of ²H₂O made 0.2 mM with respect to EDTA and 25 mM with respect to Tris-HCl (pH 7.4) under nitrogen by using a Dawes Soniprobe, power setting 2. The sonication vial was immersed in a water bath (60°C), above the expected phase-transition temperature, and the dispersion was sonicated for 4 min until it became clear.

Vesicles were also prepared from a mixture of egg-yolk phosphatidylcholine and the phosphonium egg-yolk phosphatidylcholine. Solutions of egg-yolk phosphatidylcholine and its phosphonium analogue (dissolved in chloroform-methanol, 2:1, v/v) were combined to give a mixture containing egg-yolk phosphatidylcholine and the phosphonium analogue (6:4, mol/mol). The final composition of the mixture was determined from the phospholipid content (Bartlett, 1959) of the constituent phosphatidylcholines after separation, as described above, on 0.25 mm-thick silica gel H. Corrections were made for the contribution of the phosphonium group to the total phosphorus content, since the phosphonium group was oxidized to phosphate under the conditions used for phosphate ester hydrolysis [70% (w/v) HClO₄ at 210°C for 1 h]. The mixture was dried under a stream of N₂ and sonicated in 2 ml of 50 mM-Tris-HCl, pH 7.4, in H₂O/²H₂O (1:1, v/v). The sample was sonicated as described above except that the sonication vessel was immersed in an ice-water bath. Additions of paramagnetic shift reagents to the co-sonicated vesicles were made to give final concentrations of 0.1 M-K₃Fe(CN)₆ (Fisons) or 3 mM-Nd(NO₃)₃ (Koch-Light Laboratories, Colnbrook, Bucks., U.K.).

Unsonicated lipid dispersions (liposomes) of distearoyl phosphatidylcholine and the phosphonium analogue were prepared for differential scanning calorimetry by mixing approx. 5 mg of either phosphatidylcholine with an excess of water (50 μ l) above the expected phase-transition temperature on a vortex mixer. Liposomes of phosphonium distearoyl phosphatidylcholine were prepared for ³¹P n.m.r. experiments in a similar manner, 17 mg of lipid being dispersed in ²H₂O (1.5 ml) made 0.2 mM with respect to EDTA and 25 mM with respect to Tris-HCl (pH 7.4).

Nuclear magnetic resonance

³¹P n.m.r. spectra were recorded on two spectrometers, both of which were interfaced with Nicolet B-NC-12 computers and operated in the Fourier transform mode. The lower-frequency (36.4 MHz) machine was a Bruker WH-90 spectrometer equipped with temperature control, broad-band proton decoupling and field stabilization via a deuterium lock. The higher-frequency (129 MHz) machine was built in this laboratory (Hoult, 1973) and was similarly equipped except for proton decoupling. Accumulated free induction decays were obtained from up to 2000

Table 1. Separation of egg-yolk phosphatidylcholine and the phosphonium analogue by two-dimensional t.l.c.

Conditions for separation are described in the text.

	<i>R_F</i>	
	System I	System II
Egg-yolk phosphatidylcholine	0.19	0.18
Phosphonium analogue	0.31	0.21
1,2-diacyl- <i>sn</i> -glycerol 3-phosphate	0.08	0.68

transients by using a 2 s interpulse time, except for experiments on co-sonicated egg-yolk phosphatidylcholine vesicles where a 4 s interpulse time was used. Spectra of egg-yolk phosphatidylcholine were recorded at 30°C, and those of distearoyl phosphatidylcholine were recorded at a variety of temperatures.

Differential scanning calorimetry

Heating curves of unsonicated dispersions of distearoyl phosphatidylcholine or the phosphonium analogue were recorded at a heating rate of 5°C/min on a Perkin-Elmer DSC-2 differential scanning calorimeter. The phase-transition temperature was measured at the point of departure from the baseline, as defined by Ladbroke & Chapman (1969).

Electron microscopy

Small samples of each sonicated vesicle preparation were negatively stained with 1% (w/v) phosphotungstic acid at room temperature. Examination in an

AE1 801 electron microscope revealed the presence of small closed bilamellar vesicles for all samples, with some aggregates for the distearoyl phosphatidylcholines.

Solvents

Ethanol was obtained from Burroughs, London S.E.11, U.K. All other solvents were A.R. grade (Fisons). $^2\text{H}_2\text{O}$ was obtained from Ryvan Chemical Co., Southampton, Hants., U.K.

Results

^{31}P n.m.r. spectra of sonicated vesicles of distearoyl phosphatidylcholines

The 36.4 MHz ^{31}P n.m.r. spectra of sonicated vesicles of distearoyl phosphatidylcholine and the phosphonium analogue obtained above the phase-transition temperature are shown in Fig. 1. The phosphonium resonance is 28 p.p.m. downfield from the phosphate signal, in agreement with previous results (Edwards, 1973) for solutions of phosphonium distearoyl phosphatidylcholine in deuterated chloroform. A splitting of the phosphate signal (Fig. 1) is observed for both vesicle preparations, which is due to the chemical-shift differences between the resonances arising from phospholipid molecules on the inside and outside of the vesicle bilayer (Berden *et al.*, 1975). A similar splitting of the phosphonium resonance is not observed.

Phase transitions

Distearoyl phosphatidylcholine and the phosphonium analogue in the presence of excess of water, exhibit very similar phase-transition temperatures as detected by differential scanning calorimetry (Table 2). The temperature-dependences of the phosphate ^{31}P n.m.r. spectral linewidths ($\Delta\nu_{1/2}$) for both lipid species are also similar. As illustrated in Fig. 2 the linewidths of both phosphate signals decrease

Fig. 1. 36.4 MHz ^{31}P n.m.r. spectra of sonicated vesicles of distearoyl phosphatidylcholine and the phosphonium analogue

Vesicles were prepared and 36.4 MHz ^{31}P n.m.r. spectra were recorded as described in the text. (a) Phosphonium distearoyl phosphatidylcholine recorded at 60°C. (b) Distearoyl phosphatidylcholine recorded at 65°C. The ordinate scales are shown in arbitrary units. The inserts show horizontal expansions of the phosphate region of both spectra and correspond to 3 p.p.m. H→ indicates the direction of increasing field.

Table 2. Thermal transition temperatures of distearoyl phosphatidylcholine and the phosphonium analogue

Gel-liquid crystalline transition temperatures were obtained as described in the Experimental section.

Sample	Transition temperature (°C)	Reference
Distearoyl phosphatidylcholine	58.0	Ladbroke & Chapman (1969)
Distearoyl phosphatidylcholine	56.3	This study
Phosphonium analogue	55.5	This study

Fig. 2. Temperature-dependence of 36.4 MHz ^{31}P n.m.r. linewidths of sonicated vesicles of phosphatidylcholine and the phosphonium analogue

Vesicles of distearoyl phosphatidylcholine and the phosphonium analogue were prepared by sonication as described in the text; 36.4 MHz ^{31}P n.m.r. spectra were recorded at a variety of temperatures. Linewidths ($\Delta\nu_{1/2}$) were measured at half height. For partially resolved phosphate resonances (above 60°C) the combined peak widths of both components at half the height of the downfield outside component (Fig. 1) was determined. Peak widths of the phosphate resonance of distearoyl phosphatidylcholine vesicles (○), of the phosphate resonance (△) and of the phosphonium resonance (■) of vesicles of the phosphonium analogue of distearoyl phosphatidylcholine are shown as a function of temperature. The scale on the left axis refers to linewidth ($\Delta\nu_{1/2}$) of both phosphatidylcholine phosphate resonances (Hz). The scale on the right axis refers to linewidth of the phosphonium resonance.

markedly as the temperature is raised to the region of the thermal phase transition. At higher temperatures the linewidths remain at 10 Hz for both phosphate resonances. The linewidths of the phosphonium distearoyl phosphatidylcholine phosphate resonances are slightly broader than those of distearoyl phosphatidylcholine below the phase-transition temperature. This difference may be attributed to a greater tendency of the phosphonium vesicles to aggregate below the phase-transition temperature; a greater degree of aggregation has been observed in electron micrographs of vesicles compared with micrographs of vesicles of distearoyl phosphatidylcholine.

The narrower phosphonium resonance also exhibits its temperature-dependent linewidths, which decrease up to the phase-transition temperature and then remain relatively constant with further increase in temperature (Fig. 2). This result indicates that the onset of motion, which produces a narrowing of the phosphate resonance as the temperature is raised, affects the entire polar headgroup region, in agreement with ^1H n.m.r. results (Lee *et al.*, 1972). The positions of the phosphonium and phosphate ^{31}P n.m.r. signals do not change with temperature.

Trans-bilayer distribution

Addition of 0.1 M- $\text{K}_3\text{Fe}(\text{CN})_6$ to co-sonicated vesicles of egg-yolk phosphatidylcholine and the phosphonium analogue results in a splitting of the phosphonium resonance (Figs. 3a and 3c). The shifted upfield component is derived from phosphonium phosphatidylcholine molecules on the outside of the vesicle bilayer. Related effects have been reported by Kostelnik & Castellano (1972) in ^1H n.m.r. studies on phosphatidylcholine vesicles in the presence of $\text{K}_3\text{Fe}(\text{CN})_6$. The ratio of the number of phosphonium phosphatidylcholine molecules on the outside to the number on the inside of the vesicles can be determined from the ratio of the area of the shifted to unshifted component of the phosphonium resonance (Table 3). $\text{K}_3\text{Fe}(\text{CN})_6$ does not affect the phosphate resonance (Fig. 3c). Addition of the cationic paramagnetic-shift reagent $\text{Nd}(\text{NO}_3)_3$ causes a marked downfield shift and broadening of the phosphate resonance arising from egg-yolk phosphatidylcholine and phosphonium egg-yolk phosphatidylcholine molecules on the outside of the vesicle bilayer (Fig. 3b) (Levine *et al.*, 1973). Measurement of the ratio of the areas of the shifted

Fig. 3. Effects of paramagnetic shift reagents on ^{31}P n.m.r. spectrum of co-sonicated vesicles of egg-yolk phosphatidylcholine and the phosphonium analogue

Co-sonicated vesicles of egg-yolk phosphatidylcholine and the phosphonium analogue were prepared as described in the text; 36.4 MHz ^{31}P n.m.r. spectra of the vesicles were recorded at 30°C (a) before and after addition of either (b) $\text{Nd}(\text{NO}_3)_3$ to a final concentration of 3 mM or (c) $\text{K}_3\text{Fe}(\text{CN})_6$ to a final concentration of 0.1 M. All spectra are shown with the same ordinate expansion. H → denotes the direction of increasing field.

Table 3. Comparison of the distribution of egg-yolk phosphatidylcholine and the phosphonium analogue across the bilayer of co-sonicated vesicles

Sonicated vesicles of egg-yolk phosphatidylcholine and the phosphonium analogue were prepared and ^{31}P n.m.r. spectra recorded as described in the legend to Fig. 3. R_0/i is the ratio of the areas of the shifted to unshifted components of the phosphonium or the phosphate resonances in the presence of $\text{K}_3\text{Fe}(\text{CN})_6$ (0.1M) or $\text{Nd}(\text{NO}_3)_3$ (3mM) respectively. R_0/i for the phosphonium resonance was computed graphically from an expanded spectrum (500Hz plot width) of the phosphonium region. R_0/i for the phosphate resonance was determined from a computer integration of the peaks.

Addition	R_0/i
$\text{K}_3\text{Fe}(\text{CN})_6$	1.4
$\text{Nd}(\text{NO}_3)_3$	1.3

Fig. 4. ^{31}P n.m.r. spectrum of unsonicated liposomes of the phosphonium analogue of distearoyl phosphatidylcholine

An unsonicated dispersion of the phosphonium analogue of distearoyl phosphatidylcholine was prepared as described in the text. The 129 MHz ^{31}P n.m.r. spectrum was recorded at 65°C. H→ denotes direction of increasing field.

to unshifted phosphate resonances gives the distribution of all phosphatidylcholine molecules across the bilayer (Table 3).

Although the shift induced by addition of $\text{K}_3\text{Fe}(\text{CN})_6$ is small, which makes accurate measurement of the trans-bilayer distribution of phosphonium egg-yolk phosphatidylcholine difficult, the ratios obtained for shifted to unshifted components for phosphonium and phosphate are not significantly different (Table 3). This implies that phosphonium egg-yolk phosphatidylcholine is distributed similarly to egg-yolk phosphatidylcholine across the vesicle bilayer.

^{31}P n.m.r. of unsonicated lipid dispersions

The ^{31}P n.m.r. (129 MHz) spectrum of unsonicated liposomes of the phosphonium analogue of distearoyl phosphatidylcholine above the thermal phase transition is shown in Fig. 4. The broad phosphate signal has the characteristic 'solid state' shape obtained at high magnetic field strengths which arise from the effects of chemical-shift anisotropy of the phosphate

group in slowly tumbling liposomes (A. C. McLaughlin & P. R. Cullis, unpublished work). That such effects are not observed for the much narrower phosphonium resonance may be attributed to the more symmetrical electronic environment of the phosphonium phosphorus together with a greater motional freedom of the quaternary phosphonium group.

Discussion

Replacement of the nitrogen atom of distearoyl phosphatidylcholine by phosphorus does not cause any marked alteration of the gel-liquid crystalline phase-transition temperature of the hydrocarbon chains as measured by differential scanning calorimetry. Further, the temperature-dependence of the ^{31}P n.m.r. phosphate resonance linewidth in sonicated dispersions of distearoyl phosphatidylcholine or the phosphonium analogue shows that phase-transition effects in the polar headgroup region are not significantly affected by the substitution. The packing properties of phosphonium egg-yolk phosphatidylcholine and normal phosphatidylcholine are also similar, since both species show comparable trans-bilayer distributions in co-sonicated vesicles. Phosphonium phosphatidylcholine is a biologically active analogue (Pasternak *et al.*, 1975), implying that replacement of nitrogen by phosphorus introduces negligible structural and functional differences. This conclusion is fully supported by our results.

It has recently been observed that phospholipids in many biological membranes (*Acholeplasma laidlawii*, erythrocytes, rat muscle and myelin) have broad 'solid state' ^{31}P n.m.r. phosphate resonances at high magnetic field strengths (P. R. Cullis, B. De Kruffy & A. C. McLaughlin, unpublished work). These phosphate resonances resemble the phosphate signal for unsonicated liposomes of phosphatidylcholine above the phase-transition temperature (Fig. 4). In conditions where the phosphate ^{31}P n.m.r. signal is broad the phosphonium resonance is relatively narrow and easily resolved.

Our results show that the phosphonium ^{31}P n.m.r. signal is sensitive to the hydrocarbon phase transition (Fig. 2). The incorporation of the phosphonium analogue of choline into biological membranes which exhibit phase transitions (De Kruffy *et al.*, 1972) offers the possibility of investigating whether the motion of the phosphatidylcholine polar headgroup is also sensitive to the hydrocarbon phase transition.

The phosphonium ^{31}P n.m.r. signal is altered in the presence of paramagnetic shift reagents (Fig. 3). The availability of the polar region of phosphatidylcholine in cell membranes for interaction with paramagnetic ions may be studied by using natural membranes labelled with phosphonium phosphatidylcholine.

The hydrophobic region of phospholipid bilayers (Seelig & Seelig, 1974) and the headgroup region of phospholipids in cell membranes (Arvidson *et al.*, 1975) have been studied by ^2H n.m.r. by using selectively deuterated analogues of phosphatidylcholine in both cases. ^2H n.m.r. has the advantage that the spectra are more easily interpretable in terms of molecular interactions than are ^{31}P n.m.r. spectra (Seelig & Niederberg, 1974). On the other hand, ^{31}P is six times more sensitive than ^2H for n.m.r. studies (Emsley *et al.*, 1965). The phosphonium resonance in a ^{31}P n.m.r. spectrum of an unsonicated dispersion of the phosphonium analogue is well resolved (Fig. 4). This is also true for deuterium signals in ^2H n.m.r. spectra obtained from unsonicated dispersions of selectively deuterated phosphatidylcholines (Seelig & Seelig, 1974), whereas proton n.m.r. spectra of unsonicated lipid dispersions are ill-defined (Sheetz & Chan, 1972).

^{31}P n.m.r. using the phosphonium analogue of phosphatidylcholine shares the advantage of n.m.r. studies on other selectively introduced magnetic nuclei, e.g. ^2H (Seelig & Niederberg, 1974), ^{19}F (Birdsall *et al.*, 1971) and ^{13}C (Metcalf *et al.*, 1972) in that spectra are simple and the signals can be identified unambiguously. This is not always true for proton n.m.r. and natural-abundance ^{13}C n.m.r. (Robinson *et al.*, 1972) where assignment of resonances may be uncertain. Most n.m.r. studies on phospholipids using selectively introduced magnetic nuclei have been restricted to the fatty acyl chains, although the technique is adaptable to the headgroup region (Arvidson *et al.*, 1975). The phosphonium analogue of choline provides a means of labelling, biosynthetically, the headgroup of phosphatidylcholine in natural membranes for subsequent ^{31}P n.m.r. studies.

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References

- Aneja, R. & Chadha, J. S. (1971) *Biochim. Biophys. Acta* **248**, 455–457
- Arvidson, G., Lindblom, G. & Drakenberg, T. (1975) *FEBS Lett.* **54**, 249–252
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Berden, J. A., Cullis, P. R., Hoult, D. I., McLaughlin, A. C., Radda, G. K. & Richards, R. E. (1974) *FEBS Lett.* **46**, 55–58
- Berden, J. A., Barker, R. W. & Radda, G. K. (1975) *Biochim. Biophys. Acta* **375**, 186–208
- Birdsall, N. J. M., Lee, A. G., Levine, Y. K. & Metcalfe, J. C. (1971) *Biochim. Biophys. Acta* **241**, 693–696
- Davis, D. G. & Inesi, G. (1972) *Biochim. Biophys. Acta* **282**, 180–186
- De Kruffy, B., Demel, R. A. & van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* **255**, 331–347
- Edwards, R. G. (1973) D.Phil. Thesis, University of Oxford
- Emsley, J. W., Feeney, J. & Sutcliffe, L. H. (1965) *High-Resolution Nuclear Magnetic Resonance Spectroscopy*, vol. 1, Appendix A, Pergamon Press, Oxford
- Hanahan, D. J., Turner, M. B. & Jayko, M. E. (1951) *J. Biol. Chem.* **192**, 623–628
- Henderson, T. O., Costello, A. J. R. & Omachi, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2487–2490
- Hoult, D. I. (1973) D. Phil. Thesis, University of Oxford
- Hoult, D. I., Busby, S. J. W., Gadian, D. G., Radda, G. K., Richards, R. E. & Seeley, P. J. (1974) *Nature (London)* **252**, 285–287
- Kornberg, R. D. & McConnell, H. M. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2564–2568
- Kostelnik, R. J. & Castellano, S. M. (1972) *J. Magn. Reson.* **7**, 219–223
- Ladbrooke, B. D. & Chapman, D. (1969) *Chem. Phys. Lipids* **3**, 304–367
- Lands, E. M. L. & Hart, P. (1965) *Biochim. Biophys. Acta* **98**, 532–538
- Lee, A. G., Birdsall, N. J. M., Levine, Y. K. & Metcalfe, J. C. (1972) *Biochim. Biophys. Acta* **255**, 43–56
- Levine, Y. K., Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C. & Robinson, J. D. (1973) *Biochim. Biophys. Acta* **291**, 592–607
- Metcalf, J. C., Birdsall, N. J. M. & Lee, A. G. (1972) *FEBS Lett.* **21**, 335–340
- Michaelson, D. M., Horwitz, A. F. & Klein, M. P. (1973) *Biochemistry* **12**, 2637–2645
- Pasternak, C. A., Strachan, E., Micklem, K. J. & Duncan, J. (1975) in *Cell Surfaces and Malignancy* (Mora, P. T., ed.), Fogarty International Center Proceedings no. 28, U.S. Government Printing Office, Washington, D.C., in the press
- Quinn, P. J. (1973) *Biochem. J.* **133**, 273–281
- Radda, G. K. & Vanderkooi, J. (1972) *Biochim. Biophys. Acta* **265**, 509–549
- Robinson, J. D., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1972) *Biochemistry* **11**, 2903–2909
- Seelig, A. & Seelig, J. (1974) *Biochemistry* **13**, 4839–4845
- Seelig, J. & Niederberg, W. (1974) *J. Am. Chem. Soc.* **96**, 2069–2072
- Sheetz, P. & Chan, S. I. (1972) *Biochemistry* **11**, 4573–4581
- Yang, S. F., Freer, S. & Benson, A. A. (1967) *J. Biol. Chem.* **242**, 477–484