THE USE OF NMR SPECTRA OF SONICATED PHOSPHOLIPID DISPERSIONS IN STUDIES OF INTERACTIONS WITH THE BILAYER

E.G. FINER, A.G. FLOOK and H. HAUSER
Biophysics Division, Unilever Research Laboratory, Colworth/Welwyn, The Frythe, Welwyn, Herts, England

Received 23 August 1971

1. Introduction

Recent investigations of dispersions of phospholipids and phospholipid-containing systems have tended to avoid high resolution proton NMR studies of sonicated dispersions, a technique which was increasingly used a few years ago [1]. More difficult and expensive methods (e.g. Fourier transformed ^{13}C time domain spectra [2], selective deuterium substitution and deuteron resonance [3]) have become the vogue, but little extra information has become available from these techniques. The reasons why the earlier technique has lost favour are twofold: firstly, it has not been clear whether sonication introduces some unknown change in the molecular packing of the system in addition to reducing the particle size, and secondly, there has been doubt as to the extent to which NMR line broadening in the high resolution spectra of sonicated systems can be interpreted in terms of a reduction in segmental or molecular motion. In order to resolve these problems, it is necessary to understand the following items: the mechanism of sonication (including a method for following the course of the sonication process), the nature of the resultant vesicles (including details of the molecular packing), the nature and origin of the NMR spectrum obtained before sonication, the reason why sonicated systems give a high resolution spectrum, and the nature and origin of the details of that spectrum. In this letter we present results obtained with egg yolk lecithin dispersions which help the understanding of these points.

Our results show that breakdown of lipid particles by sonication of aqueous egg yolk lecithin dispersions occurs by collisions between particles given high kinetic energy by the sonicator. These collisions produce complete disruption of the multilamellar particles, with the formation of short-lived bilayer fragments or other forms of small lecithin aggregates. These fragments then reaggregate to form single-shelled vesicles. The process follows second-order kinetics and its efficiency is defined by a particle half-life (0.9 min for the sonication conditions used in this study). The single-shelled vesicles are closed spheres with a lipid bilayer shell, the molecular organization and packing of which is the same as in the unsonicated state. Equal areas per molecule are occupied by molecules in the inside and outside surfaces of the vesicle bilayers. Packing of the molecules in the bilayer is tightest in the region of the glycerol residues, and so the glycerol protons and the first two or three chain methylene groups near them are relatively immobile. The other methylene groups and the polar head groups are mobile. These variations in segmental mobility produce different linewidths in the high resolution spectrum observed after sonication, because the rapid tumbling of the small vesicles which averages out the dipolar interactions (responsible for the broad lines observed before sonication) will give only completely narrow lines which are already well narrowed by segmental motion. Since the bilayer is retained after sonication, and since the spectral linewidths are completely dipolar in origin (although showing some unusual field/frequency dependent properties), experiments based on line broadening in the high reso-

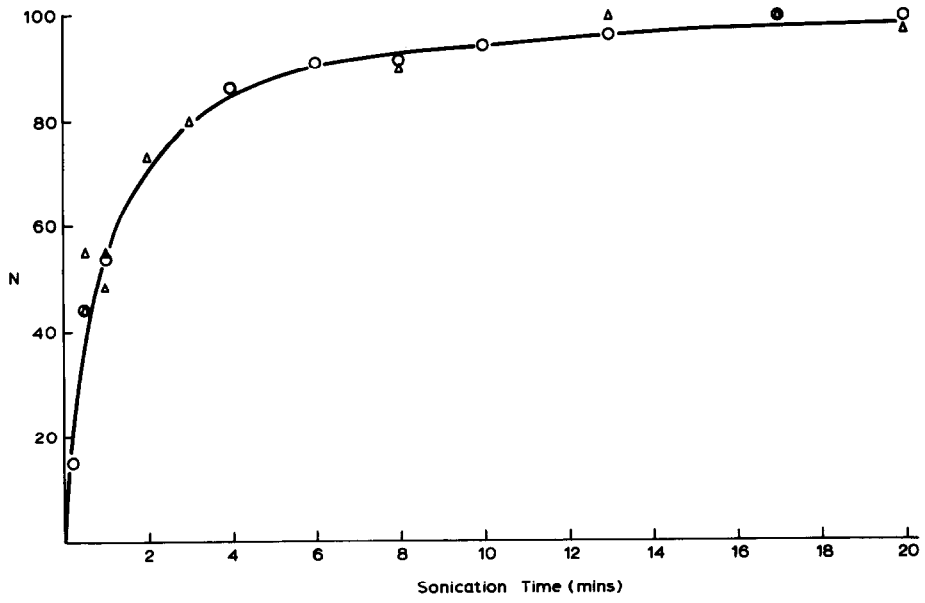

Fig. 1. Fraction of molecules N (%) in single-shelled vesicles as a function of sonication time, as measured by gel filtration (○—○) and NMR (△—△). The solid curve is calculated for a particle half-life of 0.88 min for the rate process described in the text.

lution spectra of sonicated phospholipid dispersions can be interpreted in terms of a reduction in segmental molecular motion if such effects as viscosity and particle size and structure changes are allowed for.

2. Materials and methods

Methods of preparation and sonication of egg yolk lecithin dispersions, and NMR techniques, have been described elsewhere [4, 5]. Analytical gel filtration was carried out on Sepharose 4B [4], and phospholipid concentrations were estimated by phosphorus [6] or dry weight determination.

3. Results and interpretation

Analytical gel filtration of sonicated egg yolk lecithin dispersions on Sepharose 4B gives two fractions (I and II) [4], of particle diameter 0.2–4μ and 190–300 Å, respectively. The proportion of molecules in fraction II, $N(t)$, which was estimated by phosphorus determination, grows rapidly as the time of sonication (t) is lengthened; its time dependence follows exactly the growth of high resolution NMR signal (fig. 1), as measured by integration with respect to a standarised external reference [4]. After any given sonication time $N(t)$ is equal (± 4%) to the proportion of molecules giving a high resolution spectrum, and so it is clear that all molecules in fraction II particles, and no others, give such a spectrum. Since the nature (e.g. linewidths) of the spectrum is constant, and only its intensity varies with sonication time, it follows that the sonication process which produces fraction II particles from fraction I particles is an all-or-nothing process. The nature of the process is revealed by the kinetics of production of fraction II particles, which is second order. A second order process follows the equation

$$\frac{1}{N} = 1 + \frac{t}{t_{1/2}} \qquad (1)$$

where $t_{1/2}$ is the half-life of the process. Least squares fitting to this equation of the two sets of data for N, i.e. from analytical gel filtration and from the growth of high resolution NMR signal, gave intercepts of

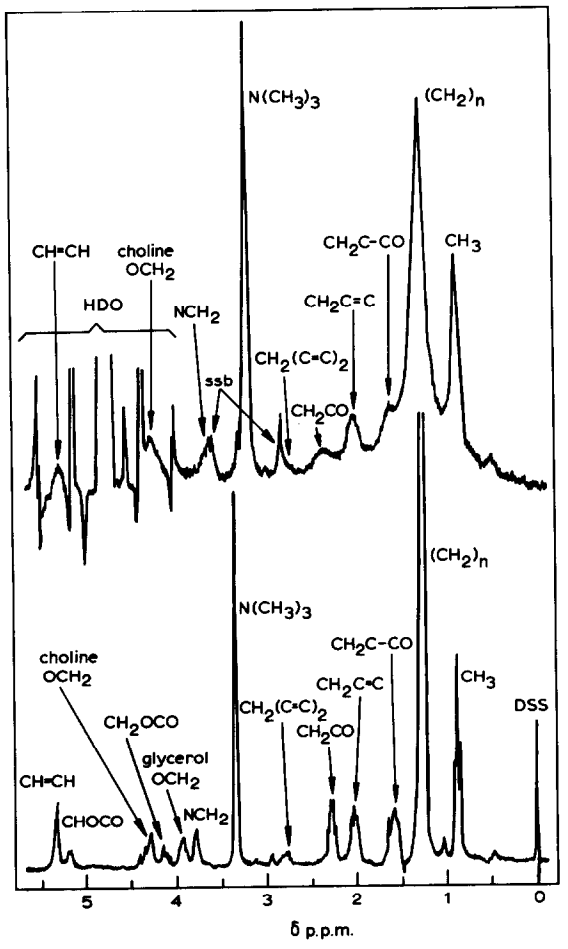

Fig. 2. 220 MHz NMR spectra of 2% (w/v) egg yolk lecithin: upper figure: sonicated dispersion in D_2O, lower figure: $CDCl_3$ solution.

0.97 ± 0.02 and 0.96 ± 0.08, slopes of 0.88 ± 0.06 min and 0.94 ± 0.19 min, and correlation coefficients of 0.997 and 0.985, respectively. The rate process is therefore of the form

$$\frac{dN}{dt} = \frac{(1-N)^2}{t_{1/2}} \quad , \qquad (2)$$

implying a collision mechanism between (fraction I) particles which contain a time-invariant average number of lecithin molecules. Thus the multilamellar particles, after colliding under the influence of the sonicator, must disintegrate completely. The fact that the size of fraction II particles is independent of the sonication conditions used shows that small fragments must be formed as an intermediate step, these fragments then reaggregating to form the fraction II particles. Calculations have shown that this mechanism is consistent with the energy produced by the sonicator. Such a process is also fully consistent with the changes in optical density of the dispersion observed on sonication [5].

Other evidence has suggested that fraction II particles consist of vesicles of average radius 115 Å surrounded by a single lipid bilayer shell of thickness 46 Å [7]. We have confirmed this by studying the effect on the fraction II high resolution spectrum of the dropwise addition of a 0.01 M manganese chloride solution. The paramagnetic ions caused severe broadening of the $N(CH_3)_3$ peak such that eventually only 28% of the original intensity was left in the high resolution spectrum. The residual peak in the presence of excess manganese, together with the hydrocarbon chain peak, showed no broadening. This is the behaviour expected from a bilayer organized into a closed spherical shell, with the inside surface inaccessible to the bulk water, and the hydrocarbon chains in the interior of the bilayer and also inaccessible to the water. If molecules in the interior and exterior surfaces of a bilayer of thickness 46 Å, closed into a sphere of radius 115 Å, occupy equal areas/molecule, 26.5% of the head groups are expected to be in the interior. Clearly agreement with this model is excellent. A comparison of these results with other qualitative [8] and quantitative [9] results on the same topic is given elsewhere [5].

The chemical shift differences observed between spectra of egg yolk lecithin in $CDCl_3$ solution and in sonicated D_2O dispersion (fig. 2) confirm that the hydrocarbon chains remain in an apolar environment in both solvents, while the polar groups undergo a change in environment when the bilayer structure is assumed. Differential line broadening in the spectrum of the sonicated dispersion shows that the glyceride backbone protons are least mobile, followed by the first two methylenes nearest the polar ends of the chains, thus packing is tightest in the glycerol backbone regions of the bilayer, as has been suggested elsewhere [10]. The production of such a differentially broadened spectrum by averaging of broad lines of different linewidths, due to rapid tumbling

of the single-shelled vesicles, can be predicted from calculations based on the work of Kubo and Tomita [11] linking residual dipolar-induced linewidth to tumbling rate. The large multilamellar particles, which are the only other particles present in an appreciable concentration, tumble much too slowly to produce any narrowing of the broad line spectrum observed with unsonicated systems. The widths in this broad line spectrum are simply controlled by dipole-dipole interactions which are not completely averaged out by the anisotropic segmental molecular motions. The postulated inhomogeneity broadening [12, 13] has not been shown to exist [14–16], and in any case calculations show that it would be small.

The dipolar origin of the broad line obtained from unsonicated lecithin dispersions is shown by the angular dependence of the linewidth when a macroscopically ordered sample is rotated with respect to the applied magnetic field [5]. A minimum is observed when the angle θ between the field and the hydrocarbon chain long axes is such that $3\cos^2\theta - 1 = 0$. Further evidence is provided by the fact that some peaks in the ^{13}C spectrum with (incomplete) proton noise decoupling are sharp [2]. However, although the proton linewidths are field/frequency independent below about 60 MHz, for a reason not yet understood they show a strong dependence between 60 MHz and 300 MHz. This does not indicate a non-dipolar broadening mechanism, because the same phenomenon has been discovered with some rubbers [5] which also give an NMR linewidth completely originating in dipolar interactions.

Finally, one further advantage of studying sonicated dispersions should be mentioned. This is that the phenomenon of spin diffusion, which may lead to considerable difficulty in the interpretation of spin-lattice relaxation time data, is unlikely to take place in lipid systems giving high resolution spectra, unless there is gross line broadening.

References

[1] B. Sheard and E.M. Bradbury, Prog. Biophys. Mol. Biol. 20 (1970) 187.
[2] E. Oldfield and D. Chapman, Biochem. Biophys. Res. Commun. 43 (1971) 949.
[3] E. Oldfield, D. Chapman and W. Derbyshire, FEBS Letters 16 (1971) 102.
[4] H. Hauser, E.G. Finer and D. Chapman, J. Mol. Biol. 53 (1970) 419.
[5] E.G. Finer, A.G. Flook and H. Hauser, submitted to Biochim. Biophys. Acta.
[6] P.S. Chen, T.Y. Tribara and H. Warner, Anal. Chem. 28 (1956) 1756.
[7] C. Huang, Biochemistry 8 (1969) 344.
[8] L.D. Bergel'son, L.I. Barsukov, N.I. Dubrovnia and V.F. Bystrov, Dokl. Akad. Nauk. SSSR Biofizika 194 (1970) 703.
[9] R.D. Kornberg and H.M. McConnell, Biochemistry 10 (1971) 1111.
[10] W.L. Hubbell and H.M. McConnell, J. Am. Chem. Soc. 93 (1971) 314.
[11] R. Kubo and K. Tomita, J. Phys. Soc. Japan 9 (1954) 888.
[12] J.R. Hansen and K.D. Lawson, Nature 225 (1970) 542.
[13] S. Kaufman, J.M. Steim and J.H. Gibbs, Nature 225 (1970) 743.
[14] G.J.T. Tiddy, Nature Physical Sci. 230 (1971) 136.
[15] J. Charvolin and P. Rigny, J. Mag. Res. 4 (1971) 40.
[16] S.I. Chan, G.W. Feigenson and C.H.A. Seiter, Nature 231 (1971) 110.