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PHYSICAL STUDIES OF PHOSPHOLIPIDS

X. THE EFFECT OF SONICATION ON AQUEOUS DISPERSIONS OF EGG YOLK LECITHIN

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SUMMARY

Increasing use is being made of sonication of aqueous phospholipid and membrane systems prior to physical and biochemical studies. We have examined the effect of sonication on the structure of aqueous dispersions of egg yolk lecithin. Using electron microscopy (both freeze-etching and negative-staining methods), low-angle X-ray diffraction and high resolution NMR spectroscopy, we confirm the breakdown in size from large particles (0.5 μ–20 μ) containing extensive lamellar order to smaller particles less than 1000 Å diameter which still retain the same 66-Å lamellar order originally present in the coarse dispersion.

INTRODUCTION

The technique of using ultrasonication as a method for producing homogeneous aqueous dispersions of phospholipids has been described by many authors[1–4]. Experiments have shown that sonication of the lipid dispersion is essential for many biochemical studies of, for example, the interaction of phospholipids with proteins[5,6], enzymatic studies[7], *etc.* SAUNDERS and co-workers[1,4] have used both ultracentrifugation and light scattering to study changes in size and shape of sonicated aqueous lipid dispersions. FINEAN *et al.*[8] have also reported briefly the effect of sonication on dispersions of the lamellar, membrane system myelin.

Aqueous lipid and membrane systems are currently being studied in our laboratory by magnetic resonance spectroscopy[9–11] where the effect of sonication appears to be of paramount importance. In particular we have found that a high resolution spectrum is obtained from egg yolk lecithin only after sonication.

Here we report the conclusions of a series of experiments, involving electron microscopy, X-ray diffraction, and NMR spectroscopy, on the effect of sonication on aqueous dispersions of egg yolk lecithin. The experiments were organised such that information from the three techniques were obtained from the sample. In this paper we pose the question — does sonication merely cause a change in size of the liquid crystalline aggregates, or does some phase change occur from, say, the usual lamellar phase to some other micellar phase?

MATERIALS AND METHODS

Egg yolk lecithin (Koch Light grade I-α-lecithin) was used without further purification. Egg yolk lecithin prepared according to the procedure described by

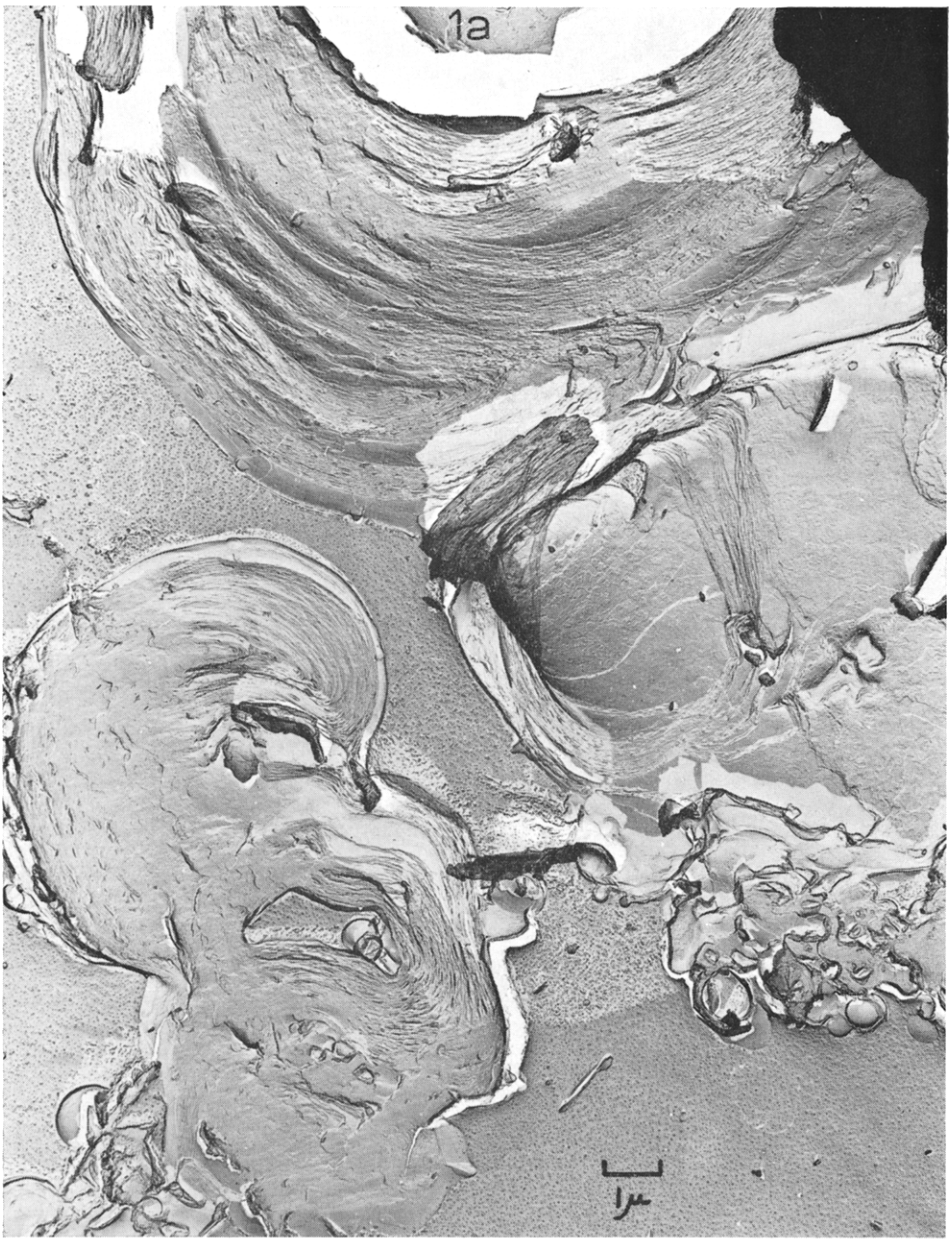

Fig. 1. Electron micrographs of aqueous dispersions of egg yolk lecithin. (a) unsonicated, freeze-etched; (b) sonicated, freeze-etched; (c) as (a) at higher magnification; (d) as (b) at higher magnification; (e) sonicated, negatively stained.

SINGLETON *et al.*[12] was also used. Both samples were shown to be pure by thin-layer chromatography.

The coarse dispersions were made by dissolving the lecithin in ether, adding water which caused the material to swell, and then evaporating off all the ether. The fine dispersions were made by sonicating the coarse dispersions at 20 kcycles/sec with a Dawe Instruments soniprobe, type 1130, fitted with a microtip. The sonication procedure was as follows: 1 ml of the dispersion was placed in a strong plastic tube. The microtip was immersed in the liquid and the tube was surrounded by an ice-water bath; the liquid was sonicated at power level 3 for various time intervals.

Electron microscopy

Electron microscopy was carried out using a JEM-7 microscope operating at 80 kV. Freeze-etched samples were prepared using a Balzer's freeze-etching unit[13], and were etched for 60 sec at −100°. For negative staining a 2% solution of sodium phosphotungstate was used at pH 7. Equal volumes of stain and 5% lipid suspension were mixed and a drop applied to a carbon-coated grid. Excess liquid was removed with filter paper, and the samples allowed to dry.

X-ray diffraction

Low-angle X-ray diffraction patterns were recorded on a Kratky camera using Cu K_α radiation from a Philips PW 1010 generator. Samples were mounted in thin-walled glass capillaries (internal diameter 1 mm) and the diffracted radiation detected by a proportional counter, the angular 2θ position being controlled by a step scanning mechanism. All experiments were performed at room temperature.

High resolution NMR spectroscopy

NMR spectra were obtained using a Perkin Elmer R.10 NMR spectrometer operating at 60 Mcycles/sec. The sample in this instrument is thermostatically maintained at 33.4°. Integrations of signal areas were also obtained using this instrument.

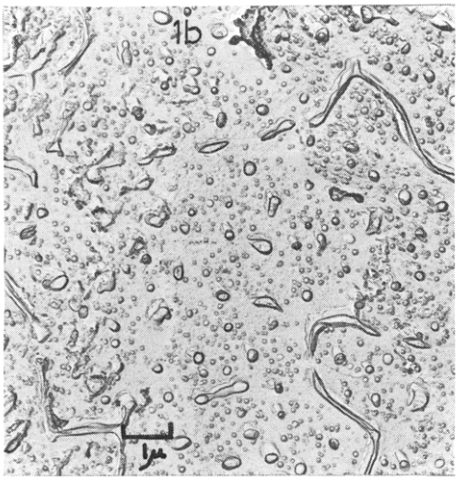

Fig. 1b.

RESULTS AND DISCUSSION

Electron micrographs of freeze-etched samples of unsonicated 5% dispersions show that practically all the lipid is included in large globules, from 0.5 μ up to more than 20 μ in diameter. Both Figs. 1(a) and 1(c) illustrate well the lamellar nature of the system. Figs. 1(b) and 1(d) are freeze-etched specimens of similar material which has been sonicated for 15 min, and they confirm the breakdown of the material into much smaller particles. The minimum size is about 200 Å diameter, and from this size up to about 800 Å they appear to be roughly spherical. Above this, they become elongated, extending in size up to about 5000 Å × 1000 Å. The smaller particles give

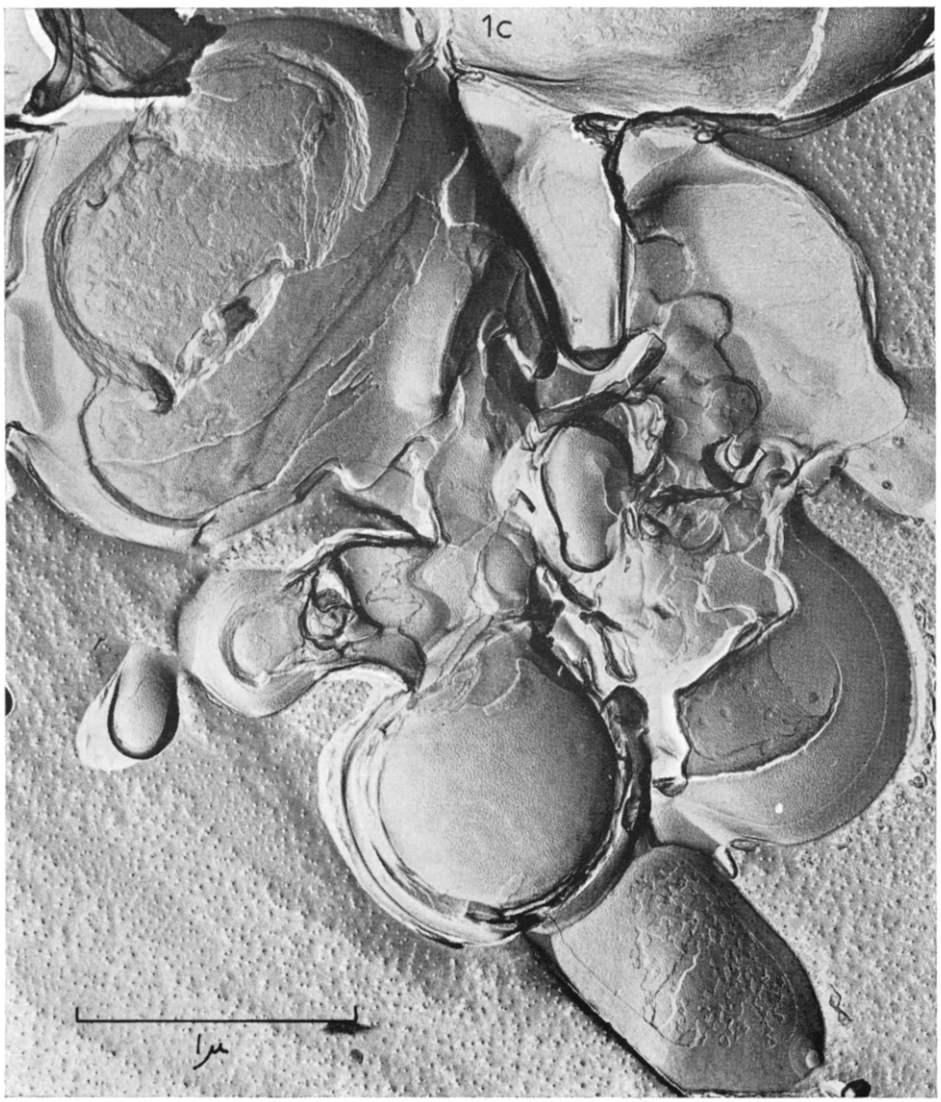

Fig. 1c.

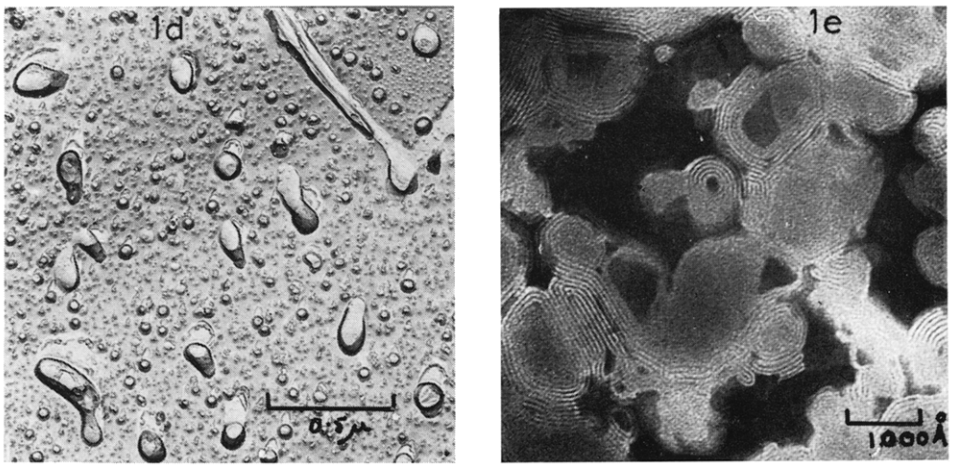

Fig. 1d and e.

no indication of lamellar structure, for the line of fracture, during the preparation of the sample, always appears to pass over rather than through them, but the lamellar nature is evident in some of the larger ones. This is confirmed in the negatively stained specimen, Fig. 1(e), which exhibits a range of particle sizes from about 300 Å upwards, with extensive evidence of lamellar structure.

In Figs. 1(b) and 1(d) of the freeze-etched specimens after 15 min sonication, a small proportion of the material is present as long strings or sheets up to about 1000 Å thick. Prolonged sonication (for 1 or 2 h) results in a much higher proportion of this material, as though aggregation has occurred and there are indications that these aggregates are at least partly composed of, or coated with, globules about 200 Å diameter. This phenomenon has not yet been examined in detail.

Fig. 2(a) shows the low-angle diffraction pattern of a 5% egg yolk lecithin

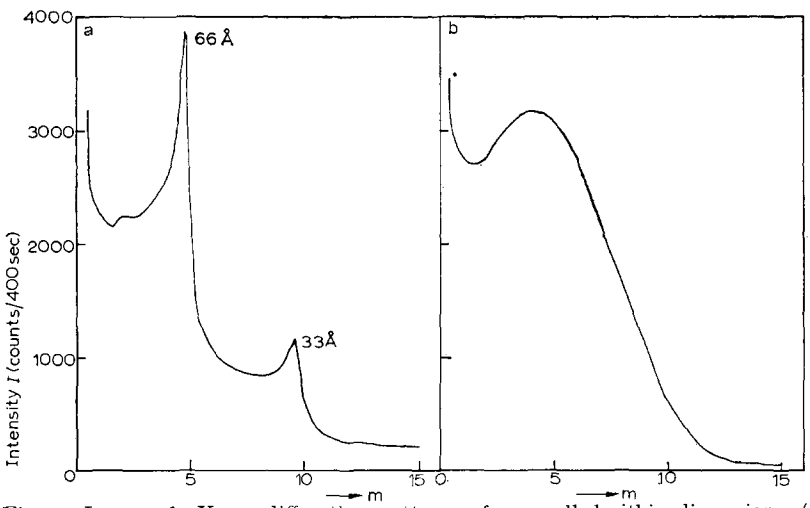

Fig. 2. Low-angle X-ray diffraction patterns of egg yolk lecithin dispersions. (a) unsonicated; (b) sonicated for 10 min. $m = a \tan 2\theta$; $a = 20.5$ cm.

dispersion before sonication. The sharp diffraction peaks at 66 Å and 33 Å are the first and second order diffractions from the lamellar two phase structure described by SMALL[14] and by REISS-HUSSON[15]. Fig. 2(b) shows the effect of 10 min sonication on the diffraction pattern. Clearly the diffraction peak at 66 Å has become very much broader but without a marked loss in peak intensity. The weaker intensity peak at 33 Å is much less obvious, presumably due to (a) a broadening and (b) a masking by the overlapping very broad 66-Å peak.

These results are consistent with the picture of a breakdown of the large lamellar particles of the coarse dispersion containing extensive 66-Å order. The line broadening of the 66-Å diffraction peak without accompanying marked loss of intensity indicates that the lamellar order remains in the smaller particles produced by sonication. Prolonged sonication ($< 1\frac{1}{2}$ h) appears to have little effect on the diffraction pattern, perhaps suggesting that a limiting size of particle has been reached.

The NMR spectrum of egg yolk lecithin dispersed in ^2H_2O changes on sonication from a broad line (approx. 500 cycles wide) to a narrow line spectrum which consists of a series of narrow, chemically shifted signals, see Fig. 3. The intensity of the narrow signals increases exponentially with sonication time[9] until, after about 20 min, constant intensities are observed. The integrated area of the choline, $N^+(CH_3)_3$,

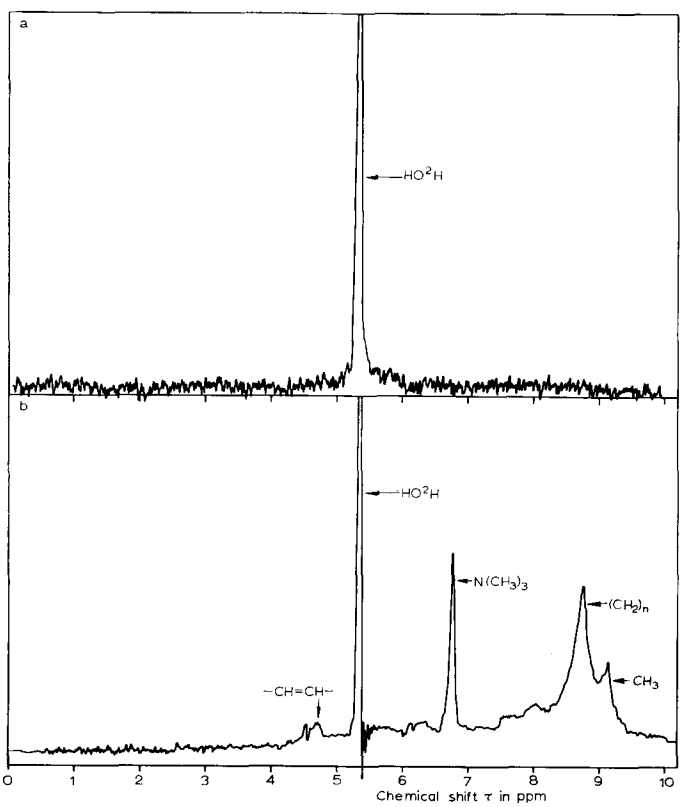

Fig. 3. High resolution NMR spectra of aqueous dispersions of (a) unsonicated egg yolk lecithin; (b) sonicated egg yolk lecithin.

signal of the sonicated lecithin dispersion was compared with that of an equimolar solution of lysolecithin. The areas were the same, within experimental error, showing that most, if not all, of the sonicated lecithin molecules were giving rise to the high resolution spectrum.

The combination of the results from this series of experiments confirms that the coarse dispersions, before sonication, are large particles (0.5 μ–20 μ diameter) with extensive lamellar ordering. The effect of sonication is to reduce the overall size of the particles to particles with diameters less than 1000 Å (in reasonable agreement with ATTWOOD AND SAUNDERS, who found a mean diameter of the order of 250 Å (micellar weight $2 \cdot 10^6$) after *prolonged* sonication[1]) and that during the breakdown of the particles lamellar order is retained (*i.e.* no phase change occurs). The phospholipids therefore give high resolution NMR spectra whilst retaining a lamellar structure, and suggestions have been made by PENKETT, FLOOK AND CHAPMAN[9] that this is associated with the removal of magnetic effects present in the large particles.

Our results suggest that a major reason why phospholipids need to be sonicated before interaction with proteins can occur is concerned with the increased surface area of the lipid rather than the production of a different phase. There may, however, be other more subtle effects or changes not revealed by our present techniques.

REFERENCES

1 D. ATTWOOD AND L. SAUNDERS, *Biochim. Biophys. Acta*, 98 (1965) 344.
2 A. D. BANGHAM AND R. W. HORNE, *J. Mol. Biol.*, 8 (1964) 660.
3 J. A. LUCY AND A. M. GLAUERT, *J. Mol. Biol.*, 8 (1964) 727.
4 L. SAUNDERS, J. PERRIN AND D. GAMMACK, *J. Pharm. Pharmacol.*, 14 (1962) 567.
5 I. SEKUZU, P. JURTSHUK AND D. E. GREEN, *J. Biol. Chem.*, 238 (1963) 975.
6 D. E. GREEN AND S. FLEISCHER, *Biochim. Biophys. Acta*, 70 (1963) 554.
7 M. KATES, J. R. MADELEY AND J. L. BEARE, *Biochim. Biophys. Acta*, 106 (1965) 630.
8 J. B. FINEAN, R. COLEMAN, W. G. GREEN AND A. R. LIMBRICK, *J. Cell. Sci.*, 1 (1966) 287.
9 S. A. PENKETT, A. G. FLOOK AND D. CHAPMAN, *Chem. Phys. Lipids*, in the press.
10 D. CHAPMAN, *Advances in Chemistry*, in the press.
11 D. CHAPMAN, V. B. KAMAT, J. DE GIER AND S. A. PENKETT, *J. Mol. Biol.*, 31 (1968) 101.
12 W. S. SINGLETON, M. S. GRAY, M. L. BROWN AND J. WHITE, *J. Am. Oil Chemist's Soc.*, 42 (1965) 53.
13 H. MOOR, K. MUHLETHALER, H. WALDMER AND A. FREY-WYSSLING, *J. Biophys. Biochem. Cytol.*, 10 (1961) 1.
14 D. M. SMALL, *J. Lipid Res.*, 8 (1967) 551.
15 F. REISS-HUSSON, *J. Mol. Biol.*, 25 (1967) 363.