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FUSION OF CHARGED AND UNCHARGED LIPOSOMES BY N-ALKYL BROMIDES

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SUMMARY: Uncharged and charged liposomes, consisting of pure phosphatidyl choline (PC) or PC with phosphatidic acid (PA) are shown by electron microscopy to form structures consistent with the occurrence of membrane fusion upon exposure to small amounts of the n-alkyl bromides. Control vesicles similarly treated with n-hexane or calcium ions showed no evidence of fusion.

Membrane fusion is a fundamental process in biological organisms, manifest in a variety of cellular events[1]. The development of artificial lipid membranes has made it possible to introduce exogenous substances into living cells by membrane fusion[2,3], and these membranes have as a model system proved a useful paradigm for the occurrence of fusion in biological systems. This paper presents electron microscopic evidence which suggests that the n-alkyl bromides of greater than 5-carbon chain length bring about a structural reorganisation of liposomes, consistent with the occurrence of membrane fusion of these structures. This interaction occurs independently of membrane surface charge, and at a low molar ratio of fusogen to phospholipid.

While fusion of natural membranes *in vivo* occurs spontaneously in cells and organisms, albeit at different levels or organisation, fusion *in vitro* requires the introduction of some stimulus. Studies into mechanisms of membrane fusion have revealed a variety of agents capable of triggering a model fusion event, e.g. polyethyleneglycol (PEG)[4,5], lysolecithin[6,7], some phospholipases[8,9], inactivated Sendai virus[10-16], concanavalin A[17], and calcium ions[18-25].

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More recently, it was reported that the n-alkyl bromides, e.g. the homologous series from hexyl to decyl bromide, bring about membrane reorganisation consistent with fusion in a natural membrane system, the photoreceptor disc membranes of retinal rod outer segments[26]. However, the mechanism of fusion by these compounds is unclear.

It is, however, implicit in studies of fusion occurring in natural systems that the influence of membrane composition is unknown. Therefore the effect of the n-alkyl bromide series was examined in artificial lipid membranes of known composition and size, i.e. the unilamellar liposome, either without net surface charge, as in the case of pure phosphatidyl choline (PC) liposomes or membranes with finite charge such as PC membranes with small quantities of phosphatidic acid (PA) included.

MATERIALS AND METHODS

Liposomes were prepared by evaporating from chloroform either pure PC (from egg lecithin, greater than 99% pure), or mixtures of PC and PA (9 : 1 mole/mole) respectively. Multilamellar liposomes were made by swelling the dried lipid mixtures usually in 100 mM KCl. Unilamellar vesicles were produced by sonication under nitrogen in a controlled temperature bath. In this preparation greater than 99% of the vesicles are single - walled when observed by negative staining and electron microscopy (figure 1a) and the resulting suspension of unilamellar vesicles, unlike the multilamellar liposomes, is optically transparent.

An aliquot of the sonicated vesicle suspension was retained as a control and the remainder of the suspension used for fusion studies. In initial experiments 1 - 10µl of alkyl bromide (Koch Light or Eastman Kodak) was added to 1 ml of the lipid suspension (50 – 100µmoles lipid/ml). This quantity of fusogen exceeded the aqueous solubility, in 100 mM KCl (about 1 mM for hexyl bromide). The mixture was vortexed for 5-10 sec to promote dispersion of the alkyl bromide, resulting in a time dependent increase in the optical density of the lipid suspension (at 450 nm), from the optically transparent solution of unilamellar liposomes to a cloudy suspension typical of multilamellar liposomes.

RESULTS AND DISCUSSION

When examined by negative staining with either 1% sodium phosphotungstate or 1% ammonium molybdate containing .05% bovine serum albumin, a dramatic size increase in the average membrane vesicle

diameter had occurred (fig. 1b-1d) relative to that of the control (fig. 1a). The untreated suspension showed no increased particle size or aggregation, nor did a suspension of liposomes of similar lipid concentration when treated identically with the same quantity of n-hexane. A further characteristic of the fused liposomes was the appearance of a broad membrane region which excluded the molybdate stain, but when stained with phosphotungstate appeared to consist of numerous lamellar membranes, an appearance distinct from native multilamellar liposomes, in that the membrane region did not invade the liposome interior but was confined instead to the outer region of the vesicle (fig. 1b-1d).

Fusion was observed microscopically in lipid mixtures containing a surface charge approximating that of natural membranes, i.e. a mixture of PC : PA (9 : 1, mole/mole (fig. 1b, 1c)). To investigate the role surface charge plays in n-alkyl bromide induced membrane reorganisation, and the possibility that hexyl bromide induces this reorganisation by modifying the membrane charge density, liposomes with zero net surface charge, i.e., pure PC vesicles, were prepared. Their behaviour (fig. 1d) was indistinguishable from liposomes with net charge. Addition of 0.5 - 5.0 mM Ca^{++} to PC vesicles had no effect on alkyl bromide fusion, nor did Ca^{++} induce fusion of PC vesicles in its own right; however, subsequent addition of the alkyl bromides to liposome suspensions containing Ca^{++} brought about the previously described behaviour, i.e. a large increase in particle size of the liposomes in suspension.

In this work there was concern as to the role which a bulk hydrocarbon phase might play in the fusion process. To examine this, access of hexyl bromide to the vesicle suspension was limited by placing a sonicated unilamellar vesicle suspension inside a small volume (1.0ml) dialysis sac and placing this in a sealed container with a large

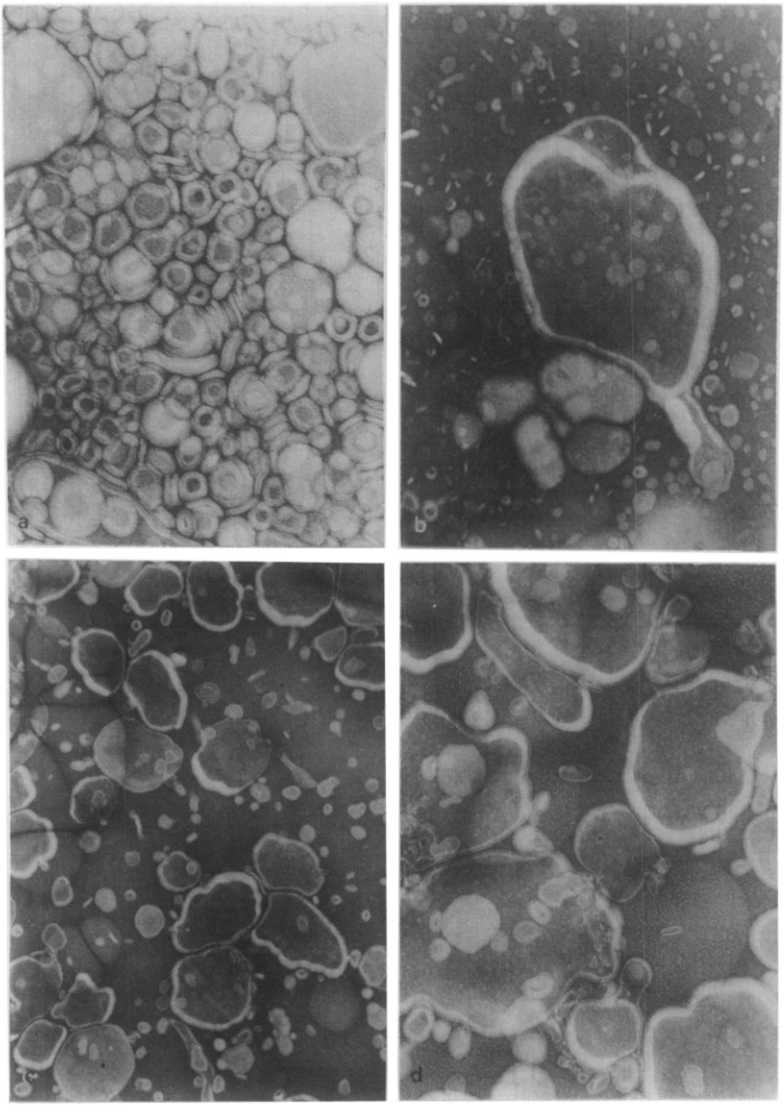

Figure 1 (a) Electron micrograph of sonicated unilamellar liposomes (PC/PA). Control unexposed to n-alkyl bromides. Mag. X 238,000. Molybdate stained. (b) Sonicated unilamellar liposomes (PC/PA) exposed for 1 hour to 0.5µl hexyl bromide per ml of lipid suspension at a concentration of 50 µmoles lipid/ml. Note the large central fused liposome surrounded by a background of unfused material of small dimensions. Mag. X 86,400. Molybdate stained. (c) Sonicated unilamellar liposomes (PC/PA) exposed to 0.75µl octyl bromide for 1.5 hours in a 1 ml suspension containing 100 µmoles lipid/ml. The field shows a number of fused liposomes characterised by a broad membrane region which excludes negative stain. Mag.X34,600. Molybdate stained. (d) Sonicated unilamellar liposomes composed of PC only. Liposomes were exposed to 0.3µl hexyl bromide per ml of lipid suspension (100 µmoles/ml) for 1 hour, at a lipid concentration of 93 µmoles per ml. Mag. X 79,600. Molybdate stained.

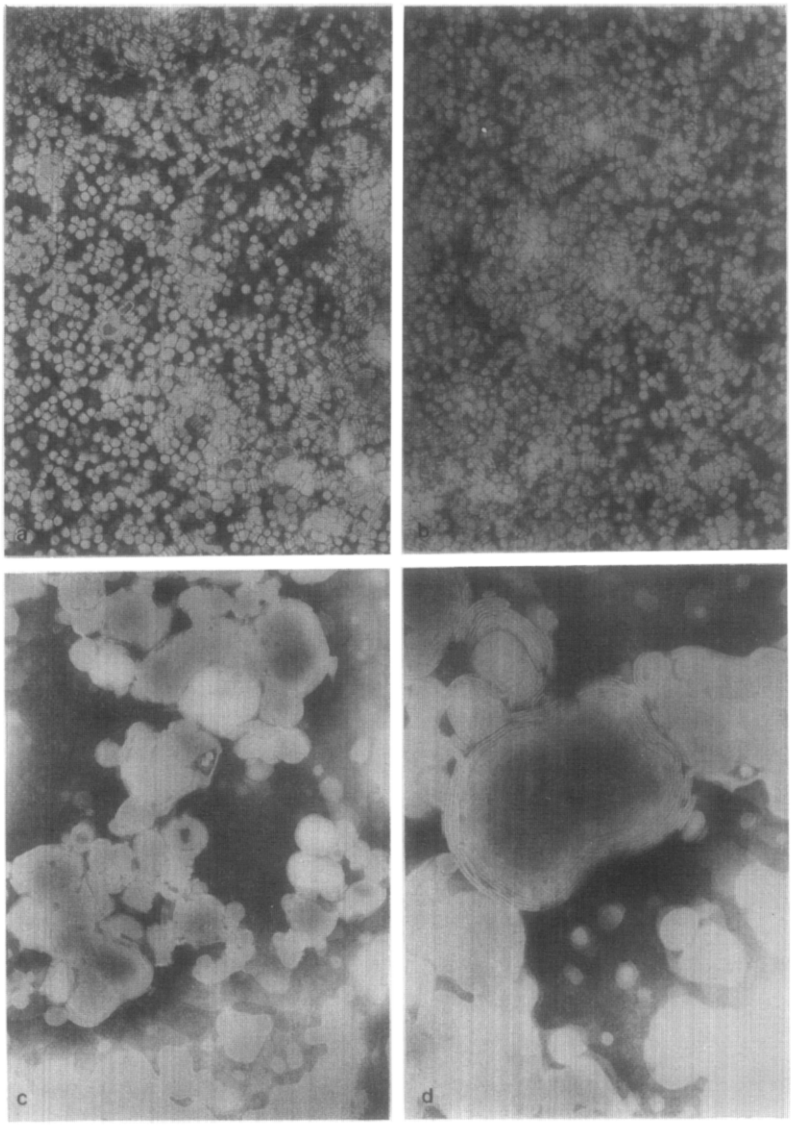

Figure 2. Sonicated uncharged unilamellar liposomes (PC) exposed to n-alkyl bromides by the dialysis fusion technique described in the text. Lipid concentrations in the dialysis sac were 100 µmoles/ml, with a typical sac volume of 1 ml. (a) Control sample in 100 mM KCl, dialysed against 100 mM KCl for 3 days. No evidence of aggregation or fusion was seen in these samples. Mag. X 54,000. Phosphotungstate stained. (b) Sample dialysed against 100 mM KCl containing 0.5 ml hexane per 100 ml volume. No evidence was seen which might suggest that hexane aggregates or fuses sonicated unilamellar liposomes after the three day exposure used in this instance. Mag. X 51,500. Phosphotungstate stained. (c) A sample dialysed for 3 days under identical conditions to 2(b), except the external medium of 100 mM KCl contained 0.5µl hexyl bromide. Substantial fusion producing large structures was seen, and earlier samples showed signs of fusion at as little as 6 hours. The large fused lipo-

volume of electrolyte (100 mls) to which 0.5 mls of hexyl bromide was added. This was stirred at room temperature and the contents of the dialysis sac removed, negatively stained and examined in the electron microscope at various times. Under these conditions bulk hydrocarbon could not cross the dialysis sac, an assumption confirmed by direct sampling of the sac contents and analysis of the hexyl bromide content of the saline by gas liquid chromatography (GLC). Liposome suspensions sampled from 3 to 72 hours following exposure to hexyl bromide in this way showed an increase in diameter (fig.2c-2d), although liposome samples identically dialysed against either saline, or saline containing the identical volume of n-hexane, showed no increase in vesicle size (fig. 2a-2b). Considerable fusion, compared to the control, could be observed at as little as three hours. This time course was of interest because GLC analysis showed that diffusion of hexyl bromide across a dialysis sac into the identical volume used in the fusion experiments, came to $\pm$ 5% of saturation in 100 mM KCl without lipid present in 120 - 180 mins. However, with a depot of lipid as used in the fusion experiments, the time required for saturation of the lipid membranes and the medium would be considerably longer, perhaps days. These experiments suggest, therefore, that liposome fusion by the alkyl bromides is not dependent on bulk hydrocarbon nor on lipid membranes saturated with bulk hydrocarbon, and that fusion may occur at alkyl bromide concentrations substantially below that of saturation of the lipid membrane.

At this time it is not clear what may be the mechanism of n-alkyl bromide induced membrane reorganisation/fusion. However,

somes showed evidence of membrane rearrangement consistent with fusion, and a conjoint process aside from simple fusion is indicated by the multilamellar appearance of the large liposomes. Mag. X 61,000. Phosphotungstate stained. (d) A sample dialysed for 6 hours against 100 mM KCl containing 0.5µl octyl bromide per 100 mls. The multilamellar appearance, as in figure 2(c), was typical of the appearance of the preparation. Mag. X 101,000. Phosphotungstate stained.

preliminary studies using a microelectrophoresis chamber indicate that membrane-saturating quantities of the n-alkyl bromides (from 6 to 10 carbon chain length) do not alter the surface charge density of charged liposome systems, such as the PC/PA mixture.

The studies of liposome fusion presented here have employed conditions which must favour frequent contact of liposomes in suspension. At low lipid concentrations, membrane fusion is observed microscopically down to a concentration of 15-20 µmoles of lipid/ml. Below this concentration the use of electron microscopic criterion for fusion is limited by the innate heterogeneity of size within the liposome vesicle population. That is to say, although fusion probably occurs, it cannot be resolved as a single fusion event between two vesicles. However, introduction of exogenous substances into natural cells, and the subsequent modification of cellular properties, due to the intracellular presence of these substances, may be more sensitive and would hopefully provide a good criterion for the utility of this technique.

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