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Fusion of Liposome Membranes by the *n*-Alkyl Bromides

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Summary. It has been found that the *n*-alkyl bromides are capable of inducing the fusion of unilamellar liposomes. These compounds can bring about fusion of liposomes composed of either pure phosphatidylcholine or phosphatidylcholine + phosphatidic acid. Fusion of unilamellar liposomes gives rise to multilamellar structures, the morphology of which has been examined by negative staining and freeze-fracture techniques. It has been shown by microelectrophoresis that the *n*-alkyl bromides have no effect on the surface charge of liposomes, and fusion has been further characterized by use of light scattering and differential scanning calorimetry, the latter indicating that true mixing of the fatty acyl chains occurs upon fusion. Finally, fusion occurs at *n*-alkyl bromide levels below that required to saturate the aqueous phase of the system.

The integrity of biological organisms is ensured by the stability of their membranes; i.e., in general the membranes of which organisms are comprised show little or no spontaneous tendency to fuse or coalesce except when a stimulus of some sort is applied. Nevertheless, a wide variety of phenomena involving membrane fusion occur within the organism, and these phenomena are the basis for a variety of related cellular events such as endocytosis, exocytosis, secretion, vertilization, and other processes.

A valuable tool in the study of biological membrane system is the ability to fuse artificial phospholipid vesicles with living cells, and thus to introduce exogenous substances into the cytoplasm (Grant & McConnell, 1973; Papahadjopoulos, Poste & Schaeffer, 1973; Magee et al., 1974; Pagano, Huang & Wey, 1974; Papahadjopoulos et al., 1974*a*; Papahadjopoulos, Poste & Mayhew, 1974*b*; Batzri & Korn, 1975; Huang & Pagano, 1975; Martin & MacDonald, 1976*a–c*). Furthermore, the value of artificial vesicle systems as a model for fusion events *in vivo* has long been recognized, and a variety of fusogens reported. Among these are calcium ions (Okada & Murayama, 1966; Shainberg, Yagit & Yaffe, 1971; Toister & Loyter, 1971; Bosch, van der Schudt & Pette, 1972, 1973; Schudt, Bosch & van der Pette, 1973; Miller & Racker, 1976), phospholipases (der Boer & Loyter, 1971; Sabban & Loyter, 1974), lysolecithin (Howell & Lucy, 1969; Papahadjopoulos et al., 1976*a*), polyethyleneglycol (PEG) (Ahkong, Howell & Lucy, 1975; Maggio, Ahkong & Lucy, 1976), and inactivated Sendai virus (Okada, 1962; Kohn, 1965; Harris et al., 1966; Poste, 1970; Yanovsky & Loyter, 1972; Bachi, Aguet & Howe, 1973; Peretz et al., 1974; van der Bosch & McConnell, 1975).

Recently the existence of a new class of fusion-inducing agents has been reported. These compounds are the *n*-alkyl bromides, such as hexyl bromide, octyl bromide and decyl bromide (Mason, Hladky & Haydon, 1979). Their properties are of interest because not only do they induce fusion among cells and subcellular vesicles, but they do so at low aqueous concentrations – perhaps submicromolar – although their local concentration in a lipid-containing membrane may be higher. Their further advantage lies in the fact that at appropriate concentrations they cause fusion among membranes without affecting cell viability, membrane constitution, or ion transport activities (Mason, 1978; Mason et al.[1]).

Although the use of *n*-alkyl bromides as fusion

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[1] Mason, W.T., Binns, R., Licence, S., Taussig, M. Fusion of lymphocytes. (*in preparation*)

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agents was initially reported for retinal rod disc membranes isolated from frog photoreceptors, a recent report by Mason and Miller (1979) suggested that they also cause the fusion of phospholipid vesicles, or liposomes. In these systems, where membrane composition, surface charge, and other properties can be precisely defined, the possibilities for understanding fusion mechanisms are greatly increased. Furthermore, the possibility that the *n*-alkyl bromides may fuse liposomes with natural membranes compels further study to understand how fusion between the liposome vesicles themselves occurs. It is the purpose of this paper to explore some characteristics of fusion within this model system.

Materials and Methods

Preparation of Liposomes

For all fusion experiments, except those involving differential scanning calorimetry, liposomes were prepared from pure egg phosphatidylcholine (PC) and occassionally included phosphatidic acid (PA) when negatively charged liposomes were required. A quantity of PC (stored under N_2 at -20 °C in chloroform) was evaporated under vacuum in a Buchi rotary evaporator. The dried residue was taken up in a quantity of 100 mM KCl and the lipid allowed to swell in this mixture. This treatment formed multilamellar liposomes. To prepare unilamellar liposomes, the suspension was subsequently sonicated in a Kerry's bath sonicator (160 W) for up to 30 min. During sonication, nitrogen was maintained above the lipid suspension and the temperature monitored to prevent oxidation of lipid. When negatively charged liposomes were required, PA was included in the lipid in chloroform mixture (prior to drying) to give a molar ratio of 9 moles PC to 1 mole PA. The final lipid concentration in fusion experiments was 100 μmol per ml. The purity of all lipids was checked routinely by conventional two dimensional thin-layer chromatography, both for purity of the lipid species used and for oxidation products.

Fusion Techniques

Two methods were used to introduce the *n*-alkyl bromides to the liposomes. In preliminary experiments, bulk *n*-alkyl bromide was added directly to the suspension to give concentrations ranging from 0.1 to 10 μl of *n*-alkyl bromide per ml of lipid suspension, a maximum of about 0.5 moles *n*-alkyl bromide per mole lipid. The liposome mixture was subsequently agitated by vortex mixing for 5–10 sec. This technique thus employed high ratios of *n*-alkyl bromide to lipid, an ultimately undesirable procedure, so that in the second technique the effect of solutions containing saturating or subsaturating amounts of *n*-alkyl bromide was examined. The liposome suspension (100 μmol per ml KCl) was placed in a small-volume dialysis sac which had been boiled in 100 mM KCl containing 10 mM EDTA for 30 min., rinsed well, and soaked in only 100 mM KCl until required. The dialysis sac was tied off and placed into a small volume sealed flask containing 125 ml of 100 mM KCl. Into the flask was placed a quantity of hexyl bromide, usually 100 to 500 μl per 100 ml KCl. The container was stirred continuously with a glass enclosed magnetic stirrer, care being taken to avoid heating of the suspension over the course of the experiment.

The *n*-alkyl bromides were obtained from Koch Light.

Microscopy

Several microscopic techniques were used. The first was to prepare the liposome suspension in a small volume of the negative stain, at the required concentration of stain. This was placed in an inhalation atomizer and sprayed on to Formvar coated grids. The grid was allowed to dry in air for 10 min and examined in the electron microscope. In the second technique, a drop of lipid suspension was placed on a coated grid, allowed to settle for 1 min, and excess liquid removed from the grid with a filter paper. A small drop of negative stain was then added directly on to the grid. After a further minute, the excess moisture was removed with filter paper and the grid allowed to dry in air. Both techniques gave similar results, except that in the latter case of staining a more uniform grid surface was observed.

Two negative stains were employed: 1% sodium phosphotungstate at pH 5.5, and 0.5% ammonium molybdate including 0.02% bovine plasma albumin (BPA).

To investigate the morphology of areas where stain had not penetrated, the technique of freeze-fracture electron microscopy was used. The liposomes were suspended in a solution of 0.5% ammonium molybdate with 0.02% BPA. Both control, unfused samples of vesicles, and samples of vesicles which had been fused by the addition of the alkyl bromides were studied. As a cryoprotectant, glycerol was added to the suspension to a final concentration of 25% (vol/vol), and the preparations left for 10–20 min before freezing. The material was then rapidly frozen in Freon 22 cooled by liquid nitrogen. The samples were stored in liquid nitrogen until use at which time they were placed in a Balzers BA 360 M freeze etching device at -150 °C, and the vacuum reduced to 1×10^{-6} Torr. The replicas were shadowed with tungsten-tantallum, backed with carbon, removed from the chamber, and cleaned in 1% hypochlorite solution. Replicas were washed with double distilled water, mounted on copper grids, and studied in a Phillips EM300. The micrographs of the freeze-cleaved vesicles are mounted with the direction of shadow coming from the bottom or the side.

Microelectrophoresis Studies

To investigate whether the surface charge density of liposomes was affected by the presence of either hexyl bromide or hexane, the microelectrophoresis technique used by Bangham and Dawson (1958) was used. Mobilities of multilamellar liposomes were measured in solutions either saturated with hexane or hexyl bromide (a_i (C/C_o) = 1.0), 10% saturated with these compounds ($a_i = 0.1$), or a control solution of 100 mM KCl. Mobilities were measured by direct observations of the liposomes in the stationary layer of a cylindrical cell mounted in a 25 °C water bath.

Differential scanning calorimetry

As a further criterion of fusion, differential scanning calorimetry (DSC) was carried out on populations of dipalmitoyl lecithin (DPL) and dimyristroyl lecithin (DML) vesicles (Papahadjopoulos et al., 1974*c*, 1976*a–b*, 1977). Experiments used 30 mg of each of these lipids measured out in separate small volume stoppered glass vials. To this was added 100 μl of 100 mM KCl, and both lipid suspensions were allowed to equilibrate in a heated water bath for 1 hr at 45 °C. Following this, samples were mixed together in equal quantities and sonicated for 30 min to 1 hr as described above.

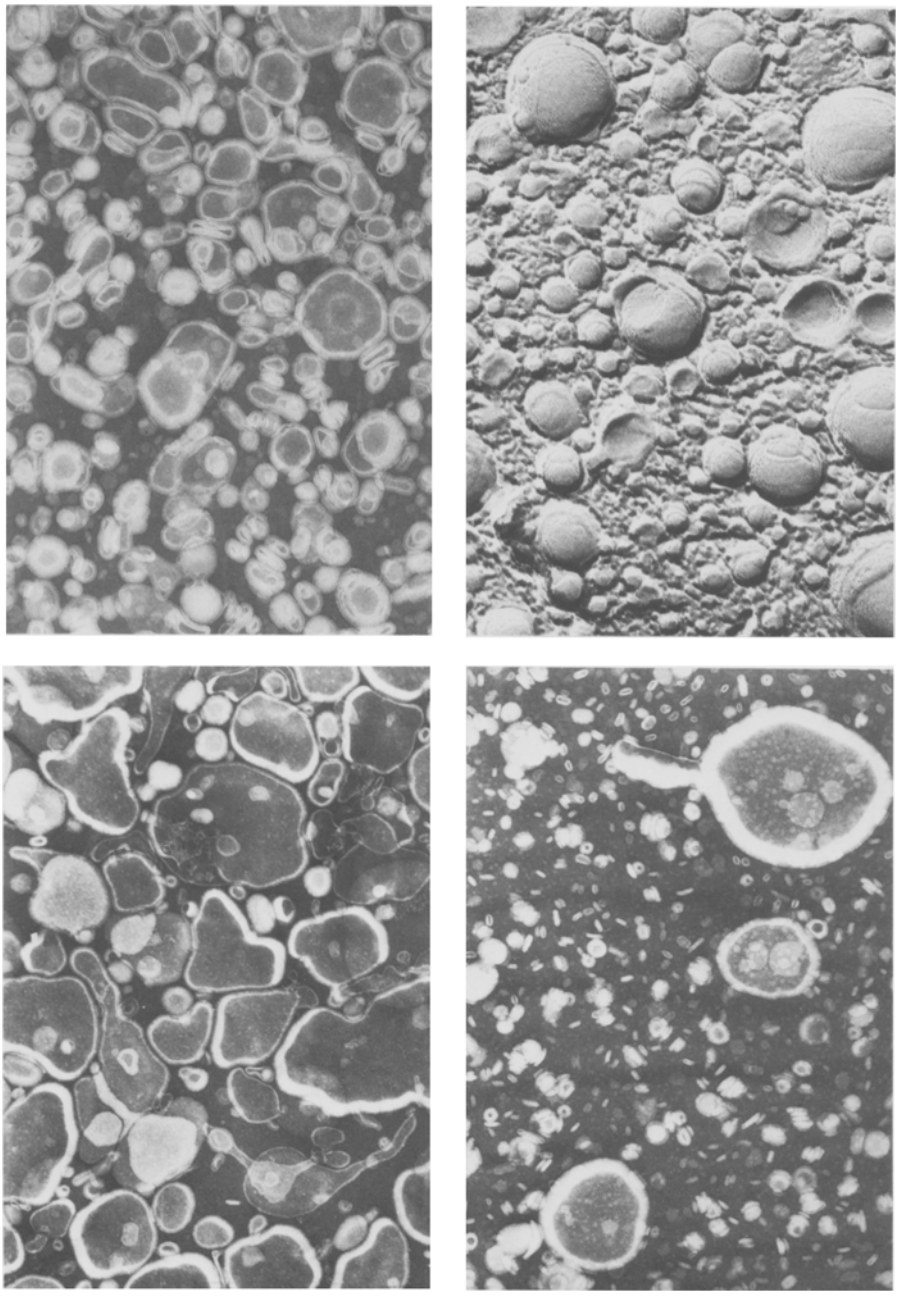

Fig. 1. (*A*): Control PC/PA unilamellar liposome preparations in 100 mM KCl, stained with 0.5% ammonium molybdate containing 0.02% bovine plasma albumin; magnification, ×73,300. (*B*): Freeze-fracture replica of identical PC/PA preparation as in *A*, magnification, ×78,000. (*C*): PC/PA liposomes (100 μmol/ml) fused with excess quantities of the *n*-alkyl bromides and stained with ammonium molybdate as in *A* and *B*. Liposomes fused with 5 μl *n*-hexyl bromide per ml lipid suspension, examined at 200 min after fusion began; magnification, ×55,200. (*D*): Liposomes as in *C* fused with 5 μl of *n*-octyl bromide per ml lipid suspension, examined at 45 min; magnification, ×72,600.

Although this treatment caused substantial reduction in the particle size of the liposome suspensions, it did not in all cases produce true unilamellar liposomal suspensions.

For DSC studies, 14-μl quantities of the lipid suspension with or without hexyl or octyl bromide were placed in sealed aluminium sample pans. A reference sample was prepared using a similar quantity of 100 mM KCl and this was identically sealed. Measurements were carried out on a Perkin Elmer DSC 1B differential scanning calorimeter with the output displayed on a pen recorder.

To examine the effects of temperature and phase transition state, the fusion process was examined by maintaining the lipid samples at room temperature or on ice, although fusion studies were generally carried out in excess of 45 °C to ensure that both DPL and DML were above their transition temperatures.

As controls, samples were taken from a lipid mixture where lipid had initially been dissolved in chloroform (30 mg. lipid per 100 μl chloroform). The dry lipid samples were either combined before dissolving in chloroform, or kept separate before dissolving in chloroform, followed by mixing, and then chloroform removed by flushing with nitrogen. The lipid mixture was then taken up in the usual quantity of the 100 mM KCl and calorimetry samples taken as before.

Gas Liquid Chromatographic (GLC) Analysis of n-Alkyl Bromides

n-Alkyl bromides were analyzed at 80 °C on a Pye series 104 gas liquid chromatograph fitted with dual columns and a heated flame

ionization detector assembly. The column packing was 10% wt/wt silicone gum E30 (SE 30) on acid washed dichloromethyl silane treated 100–120 mesh Diatomite C.

Calibration was routinely made against weighed standards of the *n*-alkyl bromides in pentane.

Light Scattering

Measurements of light scattering indicative of fusion and/or aggregation of the liposomes was monitored in a Perkin Elmer absorption spectrophotometer at 450 nm. Unilamellar liposome preparations were placed into 3 ml cuvettes stoppered with a Teflon plug. The bulk fusogen was introduced into the glass cuvette and the cuvette vortexed as for the usual fusion experiments.

Results

Initially, the effect of the *n*-alkyl bromides on fusion was studied using addition of bulk quantities, i.e., concentrations in excess of the amount required to saturate a saline solution. Figure 1 *a* shows electron micrographs of control liposome preparations, stained with 0.5% molybdate containing 0.02% BPA, and Fig. 1 *b* by freeze fracture. Negatively stained liposomes were bounded by a single membrane, although occasional, slightly larger vesicles appeared by freeze fracture to have multiple fracture planes, indicating the presence of more than several boundary membranes (Fig. 1 *b*). However, this was in contrast to preparations of unsonicated multilamellar liposomes where the number of lamellae was manyfold (>10).

The *n*-alkyl bromides in excess of saturation induced rapid changes in the macroscopic appearance of liposome suspensions, from near transparent to opacity with a time course substantially greater than that required for mixing. Figure 2 shows the typical change in optical density observed for suspensions of unilamellar liposomes exposed to *n*-hexyl bromide, with the optical density increasing over a period up to at least 3 hr. In other experiments liposome suspensions of PC alone or PC/PA were compared. Both lipid suspensions were of identical concentration and were exposed to identical amounts of *n*-hexyl bromide. The increase in optical density of the suspension was 3–5 times greater for pure PC vesicles than for PC/PA vesicles. A suspension of PC/PA vesicles also showed a small absorbance increase when calcium (0.5–2 mM) was added to the suspension, although pure PC vesicles were unaffected by calcium addition in agreement with earlier results (Papahadjopoulos et al., 1974*c*, 1977; Poste & Allison, 1973).

Although light scattering does not necessarily distinguish between membrane fusion and aggregation, it is reported because a strong correlation was found

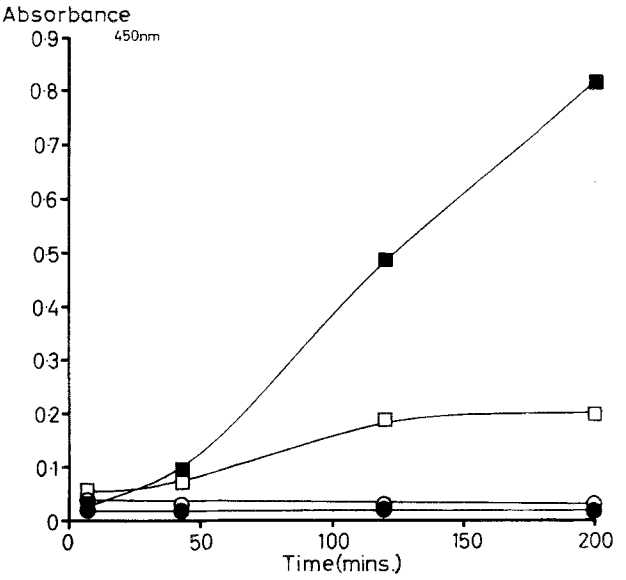

Fig. 2. Change in absorbance of liposome suspensions exposed to excess quantities of *n*-hexyl bromide. PC/PA (empty figures) and PC liposomes (solid figures) at 100 μmol lipid per ml were exposed, at time 0, to 3 μl *n*-hexyl bromide and placed in a sealed 3-ml cuvette. The absorbance at 450 nm was measured at the indicated times. Circles indicate control preparations not exposed to fusogen, and squares indicate those liposomes exposed.

between the amount of fusion observed microscopically and the time course of scattering changes. Preparations used in Fig. 2 were examined in the electron microscope and compared to control samples incubated over a similar period of time, without fusogen, as those shown in Fig. 1. From Fig. 1 *c* and *d* it is seen that the liposomes underwent a marked change in the presence of the *n*-alkyl bromides. Figure 1 *c* illustrates a typical field of liposomes stained with ammonium molybdate, showing the large increase of vesicle diameter which occurred as well as obvious changes in the liposome morphology. Whereas liposomes in Fig. 1 a and *b* are mostly spherical, those in Fig. 1 *c* and *d* have a more asymmetric appearance. Figure 1 d shows more clearly the dramatic change in liposome diameter typical of these preparations.

A further characteristic of fused molybdate-stained liposomes, both charged and uncharged, was a broad outer region which excluded negative stain (Fig. 1 *c* and *d*). The appearance is made further unusual by a single membrane surrounding the entire liposome, distinct from a broader region which excludes negative stain. To investigate this structure, preparations were examined by freeze fracture, and Fig. 3 shows the results of some of these experiments.

Freeze fractured fused liposomes with fracture planes into the liposome interior were distinctly multilamellar in appearance (Fig. 3 *a*), a characteristic also suggested in replicas where complete fracture into

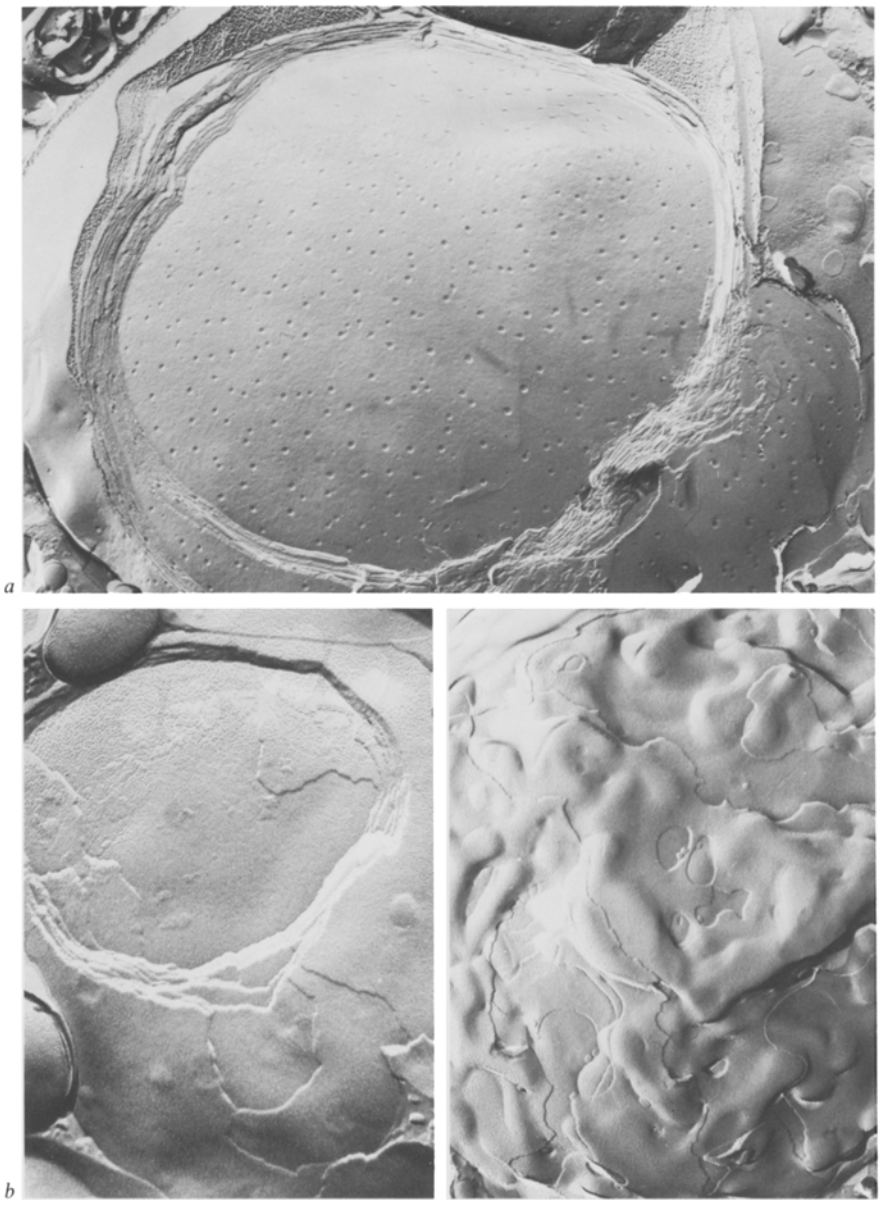

Fig. 3. Freeze fractured preparations of PC/PA liposomes in 100 mM KCl containing 0.5% molybdate and fused with excess *n*-hexyl bromide (3 µl per ml of lipid suspension at 100 µmoles per ml). In *a* is seen a large fused liposome showing numerous lamellae where fracturing into the liposome plane has occurred. Numerous depressions are seen on the inner but not outer faces, while in the same preparation (*b*) a similar fracture into the liposome interior can be seen, but without the depressions. *c* shows the typical appearance of many liposomes, with numerous superficial fractures into the interior revealing a number of lamellae. Liposomes were frozen 160 min after fusion began. Magnifications are (*a*) ×17,800, (*b*) ×39,00, and (*c*) ×58,100

the interior had not occurred (Fig. 3*c*). In some replicas were noted both particles and depressions on the liposome fracture planes. In Fig. 3*a*, numerous depressions on the fracture plane were observed, whereas in Fig. 3*c* particles which may correspond to these depressions were seen. A percentage (~20%) of the liposomes had no discernible particles on their inner fracture planes (Fig. 3*b*). These particles were not contaminants due to shadowing or to the fracture process, as they never occurred on the external surface (Fig. 3*a*).

In further experiments using liposomes fused with bulk *n*-alkyl bromide, the multilamellar appearance observed in freeze-fracture replicas was confirmed when liposomes were stained with phosphotungstic acid (such as in Figs. 4 and 5). Phosphotungstic acid gave a more uniform penetration into the multilamellar regions surrounding the fused liposome than did the ammonium molybdate (as in Fig. 1*e* and *d*).

Fusion with Subsaturated Quantities of n-Alkyl Bromides

The use of bulk quantities of the *n*-alkyl bromides in saline suspensions of liposomes presents several problems in interpretation, i.e., the presence of microdroplets of alkyl bromides. An obvious experimental

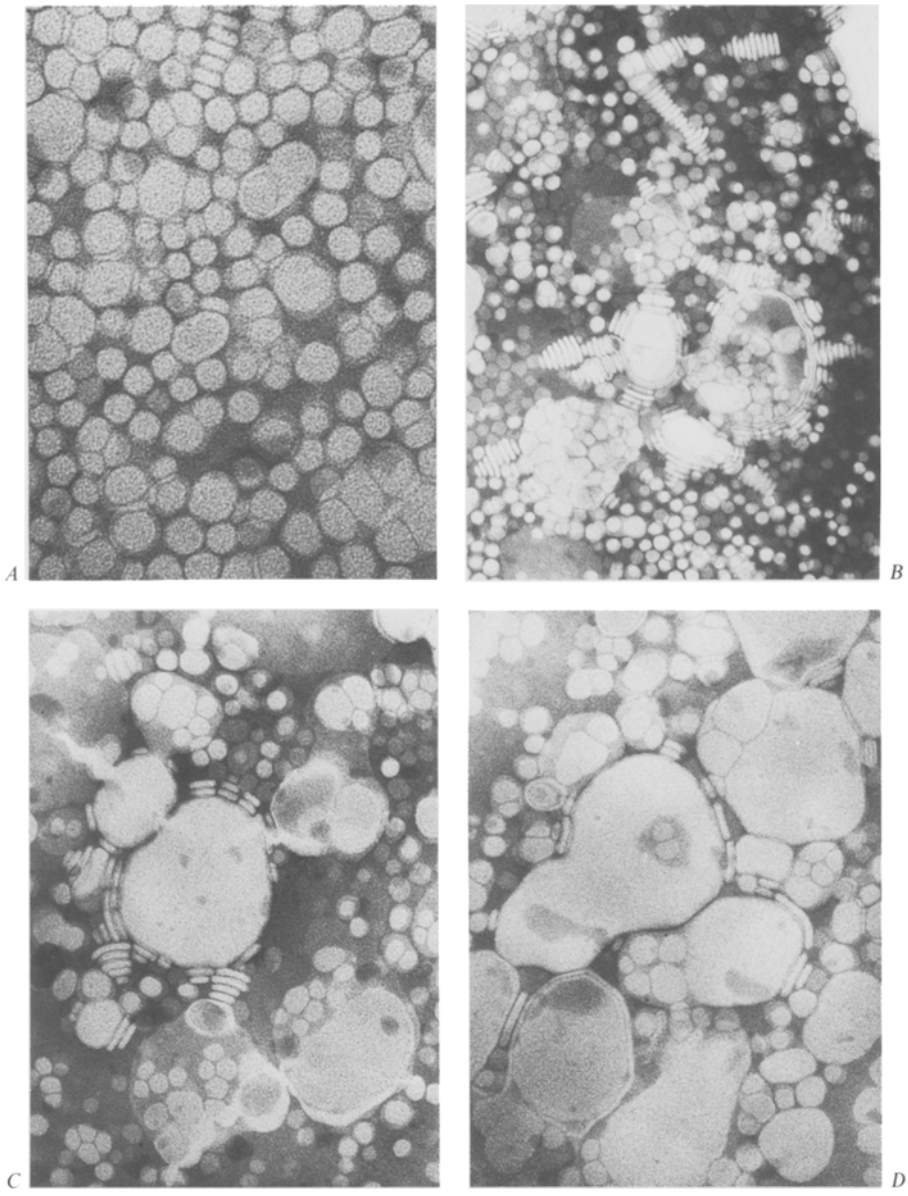

Fig. 4. Liposomes fused with subsaturating quantities of *n*-alkyl bromides by the dialysis fusion technique and stained with phosphotungstate 6 hr after dialysis began. (*A*): Control sample of PC liposomes dialyzed against 100 mM KCl in the absence of fusogen; ×19,500. (*B*): PC liposomes dialyzed against *n*-hexyl bromide in 100 mM KCl; ×77,800. (*C*): PC/PA liposomes dialyzed against *n*-octyl bromide in 100 mM KCl; ×114,800. (*D*): PC liposomes dialyzed against *n*-octyl bromide in 100 mM KCl; ×129,600.

approach was thus to limit the access of bulk hydrocarbon to the liposome suspension, and this was accomplished by enclosing the liposome suspension in a semi-permeable dialysis sac such that only molecular forms of the *n*-alkyl bromides gained access to the lipid, an assumption confirmed by GLC analysis of the aqueous sac contents. Entry of molecular hexyl bromide or octyl bromide into the dialysis sac in the absence of liposomes was slow, with a time course of 3–4 hr to reach 95% of aqueous saturation, the saturating concentration of hexyl bromide in KCl being about 1 mM. However, hexyl bromide entry when liposomes were present in the sac followed a rate over several hours which approximated the initial rapid rate of entry of hexyl bromide into a sac with only aqueous contents. On this basis the hexyl bromide content was determined when samples were examined microcopically from 6 hr to ascertain a molar ratio of fusogen/lipid critical for fusion.

As seen in Figs. 4 and 5, over periods up to 3 days no appreciable aggregation or fusion was observed in the control samples dialysed against 100 mM KCl. From periods of 6 hr to 3 days, liposome fusion was observed in samples in which hexyl bromide or octyl bromide was present in the external KCl. As in liposomes fused with bulk *n*-alkyl bromide, these suspensions also showed a multilamellar appearance, and particle diameters considerably in excess of 1 μm were frequently observed.

Figure 6 shows a histogram of measured liposome

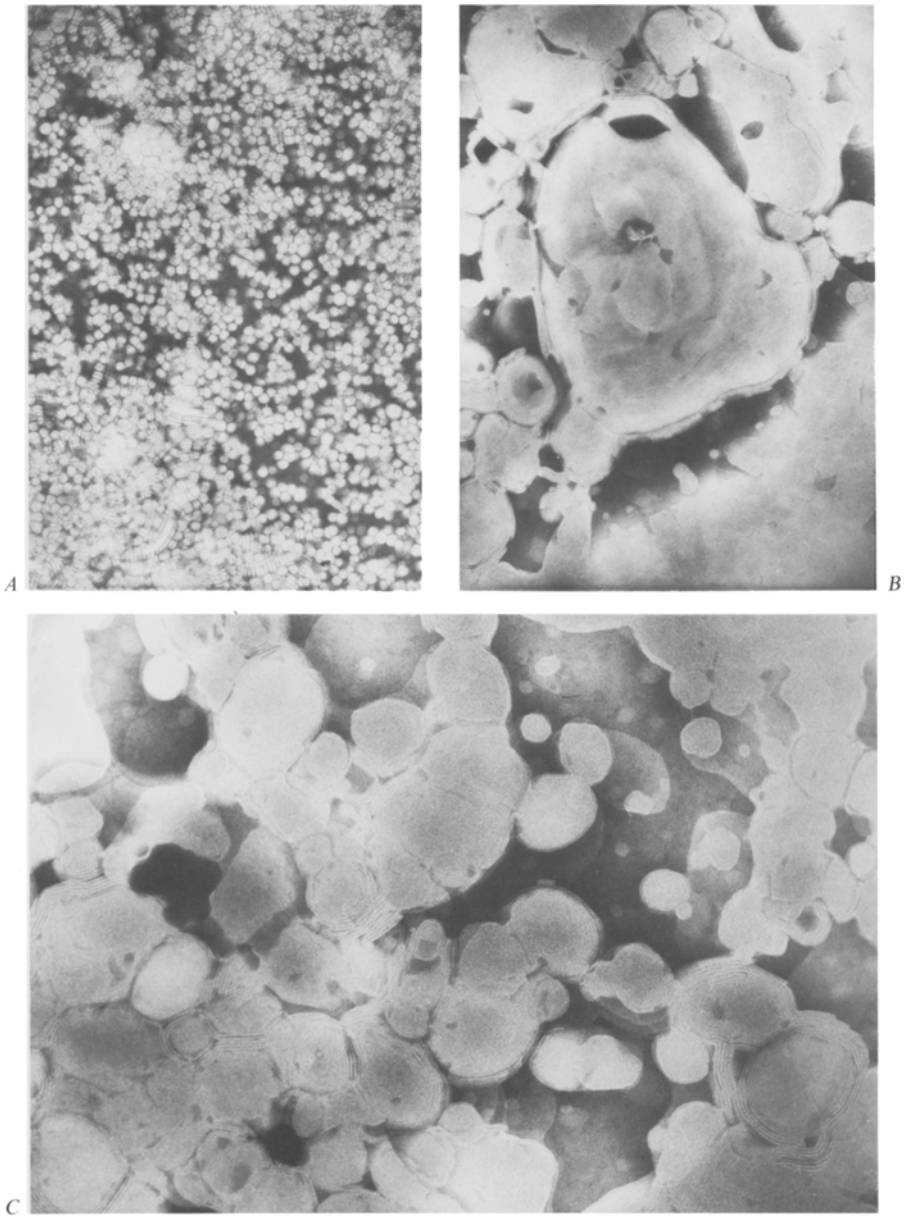

Fig. 5. PC liposomes dialyzed for various times against the *n*-alkyl bromides. (*A*): Control preparations of liposomes dialyzed for 72 hr against 100 mM KCl only; × 43,000. (*B*): Liposomes dialyzed against *n*-octyl bromide for 18 hr; × 56,100. (*C*): Liposomes dialyzed against *n*-hexyl bromide for 72 hr; × 67,800. In all experiments, the lipid contents of the dialysis sacs were analyzed after the experiment, and no oxidation of lipid was detected by thin-layer chromatographic techniques.

diameters before and after fusion and compares the average vesicle diameters of liposomes fused by the two techniques. These measurements reflect the substantial increase in vesicle size observed, although the histograms do not reflect the occasional observation of fused liposomes above 1 μm diameter; but notably, although invisible in the light microscope prior to fusion, examination of preparations after *n*-alkyl bromide fusion clearly revealed fused structures of greater than 1 μm in diameter, with various appearances, sizes, and shapes. Unlike native multilamellar liposomes allowed to swell in 100 mM KCl, liposomes fused by this technique were not birefringent, an observation which may reflect substantial membrane rearrangement. As an estimate of the consequence of fusion noted in Fig. 6, if it is assumed that surface area is conserved during fusion and that unilamellar vesicles fuse to form multilamellar structures with, say, 10 lamellae on average, it would be required for 500–600 vesicles as in Fig. 6*A* to fuse to form *one* vesicle as in Fig. 6*B*.

Microelectrophoretic Mobilities

The effect of *n*-hexane and *n*-hexyl bromide on the electrophoretic mobility of PC/PA liposomes was also studied. Neither compound was found to significantly

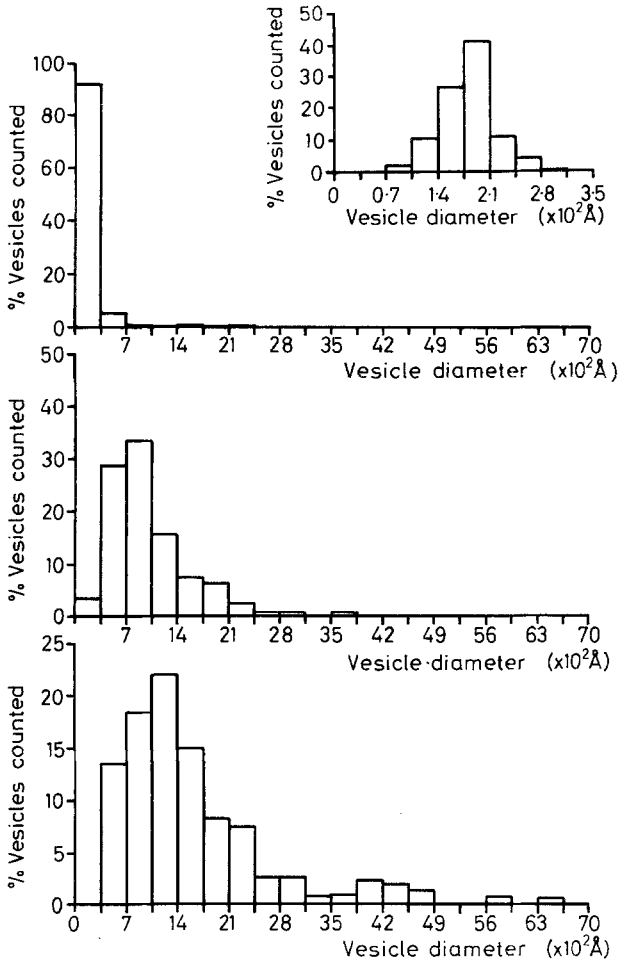

Fig. 6. Histograms of PC/PA liposome size distributions in control and preparations fused with subsaturated and excess quantities of *n*-hexyl bromide. Staining was with phosphotungstate. *Upper:* Control preparation of liposomes not exposed to *n*-hexyl bromide; *Inset:* an expanded version of the same measurements. *Middle:* Liposomes fused with subsaturated fusogen by the dialysis fusion technique, sampled and observed at 18 hr. *Lower:* Liposomes fused with excess fusogen (5 µl *n*-hexyl bromide per 100 µmol lipid per ml) and examined at 180 min. In the middle and lower parts of the figure, liposome diameters were evaluated as the average of the short and long axis due to their assymetry, and liposomes of diameter less than 350 Å diameter excluded. This is justified by the fact that (as per text) ~500–600 liposomes must, on average, fuse to form one fused vesicle.

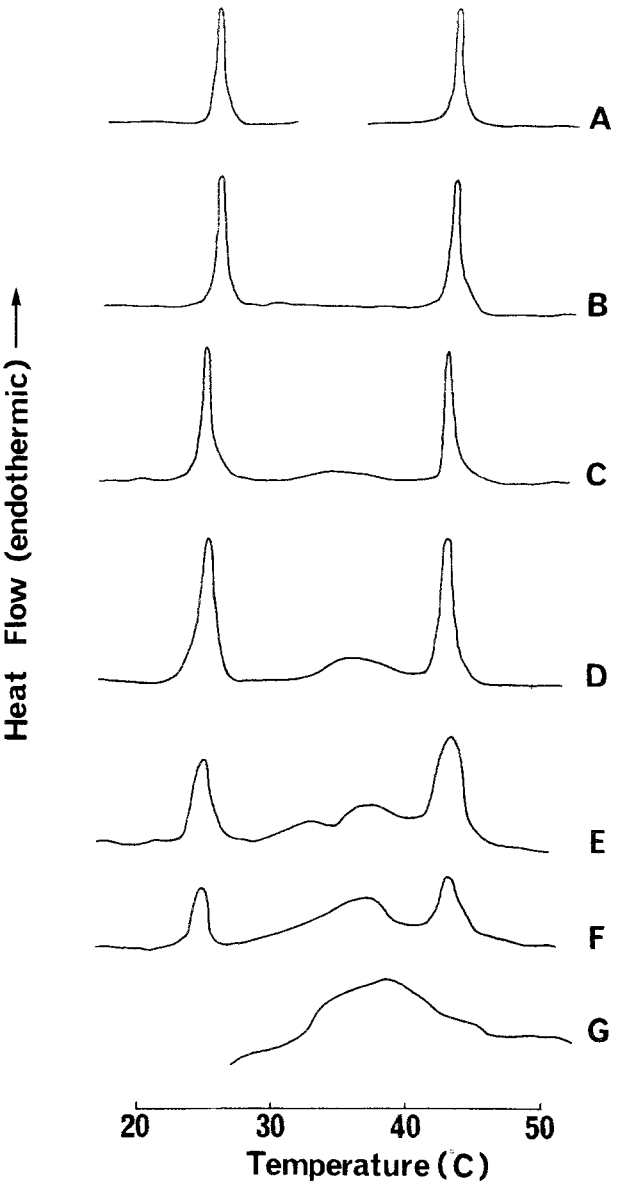

Fig. 7. Tracings of differential scanning calorimetry records of liposome suspension exposed to *n*-hexyl bromide in excess quantities. (*A*): DPL and DML scanned seperately in 100 mM KCl in the absence of fusogen, and prior to mixing the two components. (*B*): DPL and DML mixed in approximately equimolar concentrations and incubated above their transition temperatures (48° C) for 260 min, followed by the scan. (*C–F*): 100 µl DPL/DML suspension exposed to 0.2 µl *n*-hexyl bromide, incubated in sealed sample pans for the indicated time at 48 °C and scanned at (*C*) 25 min, (*D*) 55 min, (*E*) 120 min, and (*F*) 250 min. (*G*): DPL and DML were mixed together in equimolar quantities in chloroform. The chloroform was blown off under N_2 and the dried lipid mixture taken up in 100 mM KCl and scanned.

modify the surface charge density/mobility ($\sim 1.17\,\mu \cdot \sec^{-1} \cdot V^{-1} \cdot cm^{-1}$) of liposome from that of liposomes not exposed to fusogen. This observation ruled out the possibility that a modification of liposome surface charge played a role in induction of fusion.

Differential Scanning Calorimetry Experiments

One criterion available in artificial membrane studies which establishes that membrane fusion has occurred (as opposed to rupture and resealing of the membranes, or some other mechanism) is the evidence by DSC of molecular mixing amongst pure lipid species prepared as liposomes (Papahadjopoulos et al., 1974*c*, 1976*a* and *b*, 1977). Figure 7 shows scans

of vesicles of DPL and DML prepared in 100 mM KCl and sonicated as described in the Methods section. When the lipid suspensions were assayed either separately or mixed after swelling the lipid and subsequent sonication together, distinct endothermic peaks corresponding to the lipid phase transitions were detected, indicating the absence of any molecular mixing of the two species of hydrocarbon chains. As a corollary to this experiment, when the lipids were dissolved together in chloroform followed by evaporation and subsequent swelling of the liposomes from the lipid mixture, a complex and broad transition was observed, obscuring the identification of pure molecular species from the thermal profile.

Addition of *n*-hexyl bromide to lipid suspensions of DPL or DML on their own, had only a small effect on the transition temperature when used at the concentrations recorded for fusion with bulk hydrocarbon, the transition temperature being lowered by 1–2 °C, but the transition shape being unaltered. On the other hand, if DPL and DML liposomes were mixed together and exposed to *n*-hexyl bromide a similar lowering in temperature and broadening of both transition peaks was noted, with the appearance of a new broad transition peak at a temperature intermediate to the two lipid transitions. The size of this broad intermediate transition varied with time, increasing in area under the peak with increased length of exposure of the lipid preparation to hexyl bromide and observed only when the DPL/DML suspension was incubated at 45 °C in the presence of *n*-hexyl bromide. Incubation below 45 °C with or without *n*-hexyl bromide resulted in the retention of discrete lipid peaks, indicating that fusion had not occurred. Incubations of the lipid suspension for a similar period of time in the absence of hexyl bromide indicated no detectable mixing of the lipid peaks, and it may thus be concluded that fusion resulting in molecular mixing of the lipid phases was induced by hexyl bromide and confirms the work of Papahadjopoulos and co-workers, which suggested that a successful fusion of DPL and DML required the lipid to be in a liquid, semi-crystalline state above their mutual transition temperature.

Discussion

The experiments presented here indicate that the *n*-alkyl bromides induce fusion of pure phospholipid vesicles by a mechanism at least superficially different from that noted for other fusogens. Significantly, this work is consistent with fusion using these compounds being independent of surface charge, a property thought to be the basis of other fusion systems such as those utilizing calcium ions (Breisblatt & Ohki, 1976; Papahadjopoulos et al., 1976*b*, 1977), and compared to fusion observed with calcium ions and other agents, several points are clear. The first is the observation that alkyl bromide-induced fusion of both PC and PC/PA vesicles shows no dependence on calcium concentration, over a range of 0.5 to 10 mM, assayed either by light scattering or by direct microscopic observation. Further, uncharged PC vesicles do not fuse spontaneously nor, more importantly, have they been found by other workers to fuse upon addition of known fusogens. Finally, the microelectrophoresis studies carried out indicate that neither hexane nor the *n*-hexyl bromides have any demonstrable effect on the surface charge of PC/PA or PC vesicles. Thus the role of surface charge in alkyl bromide fusion does not appear to be a major factor. However, one condition for fusion with the *n*-alkyl bromides in common with other fusion processes does appear to be that the lipid species involved are in a "fluid" state above their transition temperature, as the fusion behavior (appearance of a third melting peak) of DPL and DML was not observed below the mutual lipid transition temperatures. Thus, perhaps the most convincing evidence that fusion of liposome membranes has occurred comes from experiments with DPL and DML liposomes which show that vesicle interaction in the presence of the *n*-alkyl bromides results in molecular mixing of the lipid fatty acyl chains, leading to disappearance of the two pure lipid phase transition peaks, and to the appearance of a third broad transition, a result in contrast to the calorimetric profile obtained for vesicles mixed together in the absence of the *n*-alkyl bromides.

One point which distinguishes the fusion process in liposomes from fusion of natural membranes is the generation of multilamellae. Natural membranes from retinal photoreceptors (Mason et al., 1979), brain synaptosomes (W.T. Mason, *unpublished observations*), red cells (D. Brooks & D.A. Haydon, *personal communication*), and lymphocyte preparations (Mason et al.)[2], yield structures with a single limiting membrane. One reason for this difference might be the presence of protein in or near the lipid bilayer in natural membranes but not in liposomes. The appearance of the *n*-alkyl bromide fused liposomes resembles the fused proteoliposome system reported by Miller et al. (1976). Consistent with their observations, fusion giving rise to large vesicle structures results in a broad outer membrane region attributable to multilamellae. Miller et al. (1976) suggested that the driving force for fusion was a transmembrane osmotic gradient leading to a rise in membrane surface free energy,

[2] *See* footnote 1, p. 69.

and in the present case a similar explanation may be advanced on the basis that alkyl bromide uptake into the liposome bilayer would also lead to an increase in surface free energy. These liposomes might then be expected to achieve a more stable, lower state of surface free energy via fusion leading to a lowering of their surface/volume ratio.

Although the dialysis experiments described here have not sought to establish the quantitative level at which fusion occurs, and thus the absolute concentration of hexyl bromide necessary, the time course of hexyl bromide equilibration across the dialysis sac indicates that as little as 1 mole of hexyl bromide per 20–30 moles of lipid is required to induce fusion, assuming a high partition coefficient of hexyl bromide into the lipid membrane, i.e., that the concentration of hexyl bromide in the lipid bilayer is substantially greater than that in the aqueous phase and thus the bilayer is a "sink" for the fusogen.

Considerable scope exists for applying the alkyl bromides to fusion between liposomes and natural membranes in order to introduce extrinsic substances into cell interiors, although it remains to compare the enhanced rate of fusion by the alkyl bromides with that occurring spontaneously between charged liposomes and natural membranes (Batzri & Korn, 1975).

Finally, the utility of the alkyl bromides as a fusogen of wide application is here extended (Mason et al., 1979; Mason & Miller, 1979), and the use of liposomes as a model system to study this fusion may in the future provide a more complete answer to how these compounds act on membranes.

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