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Hybridization by Cosonication of Pigeon Erythrocyte Membrane with Exogenous Lipid Vesicles

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Summary. Concentrated mixtures of lipid vesicles and pigeon erythrocyte membrane were cosonicated in order to produce functional hybrid vesicles. From the properties of the resulting material, we conclude that hybrids were very probably formed. These properties were as follows: (i) The presence of membrane increased the sonic fragmentability of lipid vesicles. Sonic fragmentability was assessed by measuring sonication-induced release of previously trapped [^{14}C]-choline and trapping of external [^{3}H]-choline. (ii) Space enclosed by lipid was served by the membrane-like properties of $^{36}Cl^-$ permeability and ATP-dependent $^{45}Ca^{++}$ uptake activity. (iii) ^{36}Cl-permeability was more readily and fully induced into the more easily fragmented lipid vesicles. Further sonication caused loss of the induced $^{36}Cl^-$-permeability. This loss was less rapid with the less easily fragmented lipid vesicles; i.e., less easily fragmented lipids protected $^{36}Cl^-$-permeability better. (iv) Glycine uptake activity was partially protected from sonic damage by the presence of lipid vesicles. (v) On centrifugation in bovine serum albumin density gradients, cosonicated material showed lipid properties (enclosed choline and $^{32}P_i$ space and [^{3}H]-cholesterol) and membrane properties ($^{36}Cl^-$-permeability and ATP-dependent $^{45}Ca^{2+}$ uptake) coinciding at a density intermediate between those reached by separately sonicated membrane and lipid vesicles. (vi) Electron micrographs showed the disappearance of pure membrane-like structures and the appearance of large amounts of new vesicles whose appearance is consistent with a hybrid structure.

Membranes and lipids can be caused to fuse by many different treatments. These include treatment with lysolecithin [1, 2, 14], polyethylene glycol [3, 5, 29] and a variety of other fusogens [4, 9, 10, 13, 15]. Fusion can also be induced by heating [2, 12], coincubation [7, 21, 22], treatment with Sendai virus [6, 20], and, for phosphatidylserine-rich lipids, incubation with Ca^{2+} [19, 23, 24]. Since so many treatments can cause fusion, it seemed possible that cosonication of membrane and lipid vesicles might

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cause them to fuse. Racker [25, 26] had already shown that functional membrane transport proteins could be incorporated into lipid vesicles by cosonication.

Our ultimate goal is to fractionate membrane-lipid hybrids which are sufficiently small and dilute that individual vesicles contain either one or no protein molecule or molecular assemblages. Cofractionation of different proteins would suggest their association in the original membrane. The first step toward this goal is to prepare membrane-lipid hybrids retaining membrane activities by a procedure that never removes membrane proteins from a lipid environment. We describe a cosonication procedure and a number of properties of cosonicated lipid vesicles and pigeon erythrocyte membranes indicating that cosonication forms hybrids which possess several transport activities of the native membranes.

Materials and Methods

Materials

Asolectin (soybean lecithin[1]) was obtained from Associated Concentrates, 32–34 61st Street, Woodside, L.I., N.Y.; Blue Dextran 2000 was obtained from Pharmacia, Uppsala, Sweden; Scintisol, the solubilizer used for serum albumin gradient fractions, was obtained from Isolab, Akron, Ohio, the scintillation cocktail 3a70B, the scintillator, 2a70 and scintillation grade toluene were obtained from Research Products International Corp., Elk Grove Village, Ill.; [G-^{3}H] cholesterol, [Me-^{14}C] choline, [Me-^{3}H] choline, and [l-^{14}C] glycine were obtained from Amersham/Searle, Arlington Heights, Ill.; $H^{36}Cl$, $^{45}CaCl_2$ and [^{32}P] H_3PO_4 were obtained from ICN Life Sciences Group, 2727 Campus Drive, Irvine, Calif.; bovine serum albumin[2] (Cohn fraction V), heparin (sodium salt, grade 1, approx. 160 USP U/mg), L-α-phosphatidylcholine (egg yolk lecithin, type III-E) and the other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo.; inorganic chemicals were reagent grade or better.

Solutions

Stock solutions of asolectin, 10% wt/vol in $CHCl_3$, neutralized with 0.054 ml 4.45 N KOH/g lipid, were stored in brown bottles at −20°. Lipid vesicle suspensions were prepared from solutions of lipids in $CHCl_3$ by drying overnight with a stream of dry N_2, suspending in the appropriate media (figure legends) and sonicating. Sonication was typically at 35–40° at 510 cal/ml [31] (e.g., 5 min, setting 3, 1 ml vol) under wet N_2. "Wet N_2" is N_2 bubbled through water at room temperature.

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1 Asolectin phospholipids are comprised of 40% phosphatidylcholine, 33% phosphatidylethanolamine, 14% phosphatidylinositol, 5% lysophosphatidylcholine and 4% cardiolipin [19].

2 *Abbreviations:* EGTA = ethyleneglycol-bis-(β-aminoethyl ether) N, N′-tetraacetic acid; BSA = bovine serum albumin.

EGTA-Ca and EGTA-Mg stock solutions were prepared by adding $CaCl_2$ or $MgCl_2$ to EGTA and titrating to pH 7.1 with KOH. EGTA solutions with various amounts of Ca and/or Mg were prepared by mixing neutralized stock solutions to avoid pH changes on divalent ion ligation.

BSA solutions for gradient preparation were made as described [17].

Sonication buffer (pH 7.1) contained (in mM): 3.0 KH_2PO_4, 6.0 K_2HPO_4, 1.0 EGTA-Ca, 1.0 EGTA-Mg, 3.0 $MgCl_2$, 1.0 NaCl, and 129.6 KCl. The EGTA-Ca and EGTA from the EGTA-Mg constitute a Ca^{++} buffer. The Na^+ and K^+ forms of glycine uptake media contained (in mM): 3.0 Na_ or KH_2PO_4, 6.0, Na_2_ or K_2HPO_4; 3.67 $CaCl_2$; 0.30, $MgCl_2$; 10.7, D-glucose; 0.27, labeled glycine; and 130.6 Na_ or KCl.

The ATP-containing and ATP-free forms of the Ca^{++}-uptake incubation medium contained (in mM): 7.0 KH_2PO_4, 2.0 K_2HPO_4, 3.0 $MgCl_2$, $^{45}CaCl_2$ (sp act approx. 16 mCi/mg), 6.7 NaCl, and either 3.3 K_2MgATP plus 128 KCl (+ATP medium) or 132.8 (−ATP medium).

Column wash buffer, used to equilibrate and elute the analytical sephadex columns, was described previously [31].

Scintillation cocktails were, for the approx. 0.5 ml BSA gradient fractions, 2.4 g 2a70, 100 ml scintisol, and 500 ml toluene [17] and for the 5-ml sephadex column fractions, the 3a70B.

Methods

Pigeon red cell membrane was prepared as described [16, 17] up to the second sonication step. The protein content was 46.2 ± 3.5 mg/g wet wt of membrane ($n=5$).

Protein was determined by a modified Lowry procedure [18]. Protein-lipid mixtures were made 0.48 M in NaCl and 1.25% in sodium dodecylsulfate to dissolve the lipid without precipitating potassium dodecyl-sulfate. One vol of alkaline copper reagent and 4 vol of Folin-Ciocalteu reagent were added for color development [28].

To hybridize membrane and lipid vesicles, a concentrated mixture was sonicated in a cut-off conical centrifuge tube under a stream of wet N_2 by a Branson sonifier, model W 185 D equipped with a microtip probe. Energy delivered to the sample (cal/ml) was estimated as described [31]. Samples were briefly centrifuged ($480 \times g$, 30 sec) to remove any Ti particles, and then "annealed" by incubating 15 min at 40° in sonication buffer. Typical conditions were: concentrations, 18.6 mg (dry wt) presonicated lipid/ml, 200 mg (wet wt; approx. 6.2 mg lipid) membrane/ml; volume, 1.0 ml; bath temperature 18–23 °C (calculated maximum sample temperature, 35–40 °C); sonicator setting, 3 (1.7 cal/sec). The sonic dose delivered and (maximum) sample temperature during sonication were calculated as described previously [31].

Properties of cosonicated samples were compared with the properties of mixtures of membrane and lipid vesicles which had been separately sonicated and separately annealed at the same concentrations and conditions as the cosonicated samples.

Sodium ion-dependent glycine uptake by vesicles was measured by mixing aliquots 1:3 (vol/vol) with Na^+ and K^+ glycine uptake medium and incubating for 5 min at 40°. The difference in uptake from Na^+ and K^+ media is Na^+-dependent glycine uptake. ATP-dependent $^{45}Ca^{++}$ uptake was measured analogously but with a 10-min incubation time.

For measurements of the amount of isotope taken up or trapped at any stage, a sephadex filtration procedure was used [31].

Density gradients were linear 2–15% wt/vol gradients of BSA in column wash buffer in 13-ml tubes ($^9/_{16}$ inch $\times$ $3^1/_2$ inch nitrocellulose). Gradient linearity was checked by density measurements [30]. 9.5 ml of gradient was formed over a 0.5-ml 20% BSA shelf.

The osmotic contribution of K^+ from the KOH used to neutralize the BSA was included in the adjustment of osmotic strength to 308 *i*mosM. Samples of 3 ml were applied on top. Gradient tubes were centrifuged in a Beckman L5-65 ultracentrifuge for 45 min, 40,000 rpm in a SW 41 Ti rotor at 0–5°. In most cases, 0.5-ml fractions were taken from the bottom and collected directly in scintillation vials containing 12 ml 2a70 scintillation cocktail. Samples were counted in a Packard Tricarb scintillation counter. For multiple isotope experiments, spill corrections were made, quench corrections were unnecessary. For quadruple label experiments, the samples were counted twice at different window settings.

Samples were prepared for electron microscopy as follows. One-tenth ml samples were held in an ice bath, and 2% agar in 0.1 M pH 7.4 sodium phosphate buffer was held in a 50–60° bath. Then the sample tubes were placed in a 40° bath for 10 sec, 0.1 ml agar was immediately added and mixed, and 0.1 ml of the mixture was immediately placed on a microscope slide resting on ice. One-mm^3 cubes were cut in the cold room, placed in 3% glutaraldehyde, held overnight in the cold, and then rinsed with phosphate buffer. Specimens were fixed [8, p. 14], dehydrated with ethanol, embedded in Epon 812 [8, p. 56], sectioned (60–70 nm) and stained with uranyl acetate and lead citrate [27]. The grids were examined and recorded photographically with a Philips 201 electron microscope operated at 60 kV.

Results

A. Effects of Sonicating Membrane Alone and Lipid Alone

The membrane vesicles were prepared with a very sonic dose, 5.4 cal/ml. They were large, 200–1400 nm (Fig. 1 *a*), and very sensitive to further sonication. Figure 1 *b* and *c* show electron micrographs of membrane

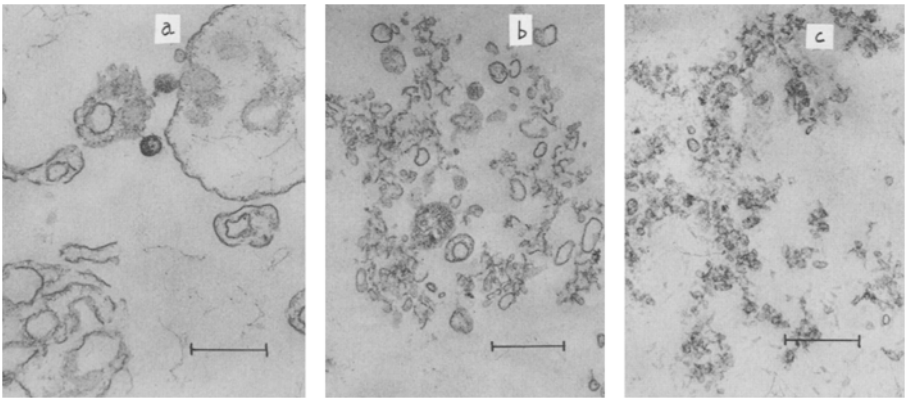

Fig. 1. Effect of sonication on the size of membrane vesicles. Membrane vesicles were prepared as described in *Methods,* resonicated at 1.7 $cal \cdot ml^{-1} \cdot sec^{-1}$ at 35–40° to give the indicated sonic doses, annealed in sonication buffer 15 min at 40°, and electron micrographs were made. The bar represents 500 nm. (*a*): No resonication, total sonic dose = 5.4 cal/ml. (*b*): Resonicated, total sonic dose = 56 cal/ml. (*c*): Resonicated, total sonic dose = 617 cal/ml

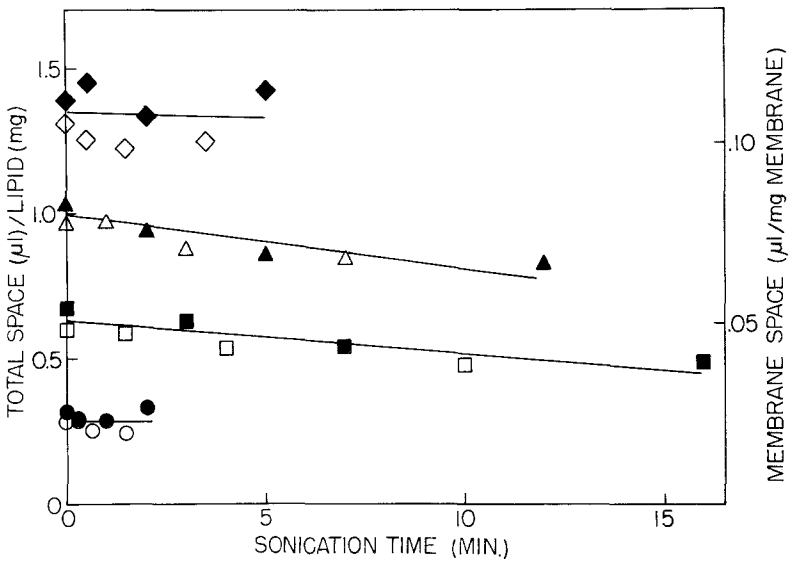

Fig. 2. Effect of sonication on total vesicle-enclosed space of lipid vesicles and membrane vesicles. Lipid, 74 mg (dry wt)/ml, was sonicated in 1.5 ml sonication buffer containing [^{14}C]-choline (0.5 μCi/ml, 8 μM) for a sonic dose of 510 cal/ml. It was diluted fourfold with sonication buffer containing [^{3}H]-choline (1.2 μCi/ml, 12 μM) and resonicated at 35 °C and 1.7 cal · ml^{-1} · sec^{-1} in 1.0 ml portions for the time indicated on the abscissa. Membrane vesicles containing [^{14}C]-choline were prepared similarly and resonicated with [^{3}H]-choline the same way except that 200 mg (wet wt)/ml were sonicated with [^{14}C]-choline for a sonic dose of 68 cal/ml prior to the sonication with [^{3}H]-choline. After the sonication with [^{3}H]-choline, lipid vesicles were diluted fourfold and membrane vesicles diluted twofold with sonication buffer and vesicle-trapped ^{14}C and ^{3}H determined by sephadex filtration (*Methods*). Total vesicle-enclosed space is the sum of [^{3}H]- and [^{14}C]-choline space calculated after correcting [^{14}C]-choline space for recapture of released [^{14}C]-choline. "Space" is given in units of μl/mg (dry wt) of lipid or μl/mg (wet wt) of membrane. Solid and open symbols show data from different experiments. ■, □: egg yolk lecithin/cholesterol (4:1, wt/wt). ▲, △: egg yolk lecithin/asolectin/cholesterol (2:2:1, wt/wt/wt). ◆, ◇: asolectin/cholesterol (4:1, wt/wt). ●, ○: membrane (note the different ordinate scale)

vesicles given progressively larger sonic doses. The size (20–60 nm, Fig. 1 *c*) of vesicles given 617 cal/ml is close to the minimum size (20 nm) for sonicated lipid vesicles [11]. This size was already reached with a sonic dose of 260 cal/ml (not shown).

Sonic fragmentability of membrane vesicles and of lipid vesicles was also measured by determining vesicle-trapped choline space (Figs. 2 and 3). Fifty cal/ml of sonic energy drastically reduced the total trapped space of pure membrane (data not shown). Further sonication of membrane had little effect on total space (Fig. 2, bottom curve). The lipid vesicles had been prepared with a high sonic dose and their total space was also little affected by further sonication (Fig. 2).

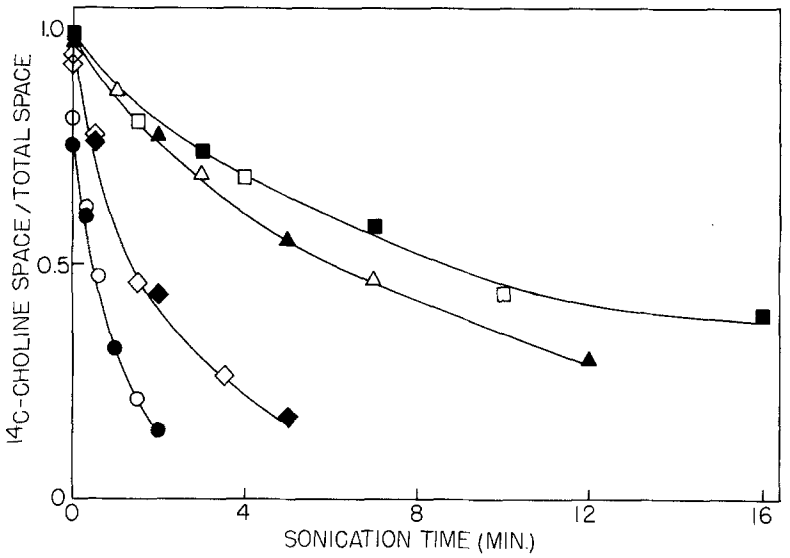

Fig. 3. Sonic fragmentability of lipid vesicles and membrane vesicles. The data was obtained from the experiments of Fig. 2, but [^{14}C]-choline space/total choline space is plotted. The same symbols are used for the membrane and lipids as in Fig. 2

Although size reduction ceases, further sonication is still rupturing the bilayers of both lipid and membrane vesicles (Fig. 3). [^{14}C] choline was introduced into lipid and membrane vesicles by sonication (510 cal/ml and 68 cal/ml, respectively). The samples were diluted with [^{3}H]-choline buffer and resonicated for various times. The total space (^{14}C-plus ^{3}H-space) changes little in this sonic dose range (Fig. 2), but with increasing sonication, vesicles progressively release ^{14}C-choline and trap ^{3}H-choline (Fig. 3). The sonic fragmentability of lipid vesicles increases as the asolectin/egg yolk lecithin ratio is increased. Membrane vesicles are more easily fragmented than the most susceptible of these lipid vesicles (Fig. 3).

B. Effects of Cosonicating Membrane and Lipid

In order to discuss the evidence for formation of membrane-lipid hybrids, a working definition of hybrids is needed. Hybrids are defined as structures in which membrane components and exogenous lipid coexist in a common bilayer. The membrane components are functional and approximately native and are drawn unselectively from the original membrane.

Table 1. The increased sonic-susceptibility of lipid vesicles in the presence of membrane

Lipid (wt/wt/wt)	sonication time (min)	Total space (μl/mg lipid)		[^{14}C]-choline space (μl/mg lipid)		[^{14}C]-choline space ratio $\frac{\text{lipid + membrane}}{\text{lipid}}$
		Lipid alone	Lipid + membrane	Lipid alone	Lipid + membrane	
A/C	0.5	1.25	1.20	0.96	0.29	0.30
(4:1)	0.5	1.45	1.57	1.10	0.51	0.46
E/A/C	1.0	0.96	1.29	0.84	0.69	0.82
(2:2:1)	2.0	0.93	1.29	0.72	0.52	0.72
E/C	1.5	0.59	0.98	0.47	0.42	0.89
(4:1)	3.0	0.63	0.88	0.46	0.42	0.90

The experiments were done as for Fig. 2, but (where indicated) the sonication of [^{14}C]-choline-containing lipid vesicles with [^{3}H]-choline was done in the presence of 200 mg membrane (wet wt)/ml of suspension. The concentration and specific activity of [^{3}H]-choline were the same as for Fig. 2.
A: asolectin; C: cholesterol; E: egg yolk phosphatidylcholine.

If hybrids are formed by cosonication of membrane and lipid vesicles, (i) membrane-like properties should be imparted to the lipid vesicles, (ii) lipid-like properties should be imparted to the membrane, and (iii) there should be features of the process readily explained by hybrid formation but difficult to explain otherwise. The observations corresponding to these expectations are described below.

1) *Sonic fragmentation of lipid vesicles is increased by the presence of membrane.* The data of Table 1 (last column) shows that membrane present during sonication increases the release of [^{14}C]-choline from lipid vesicles. This effect is greatest with the asolectin-cholesterol vesicles. This loss of [^{14}C]-choline is not due to an effect of membrane on total space (column 3 *vs.* column 4) as this is either unchanged (A/C) or increased (E/A/C and E/C). It is not due to loss of membrane-contributed ^{14}C space since with membrane present, ^{14}C space is less than with lipid vesicles alone.

2) *Membrane induces $^{36}Cl^-$ permeability into lipid-enclosed space.* In the experiments of Figure 4 most of the trapped space in the cosonicated preparation is attributable to the lipid. The space trapped by the membrane, if present alone, would be at most 20–25% of the space trapped by the cosonicated material and with the lipid (dry wt)/membrane (wet

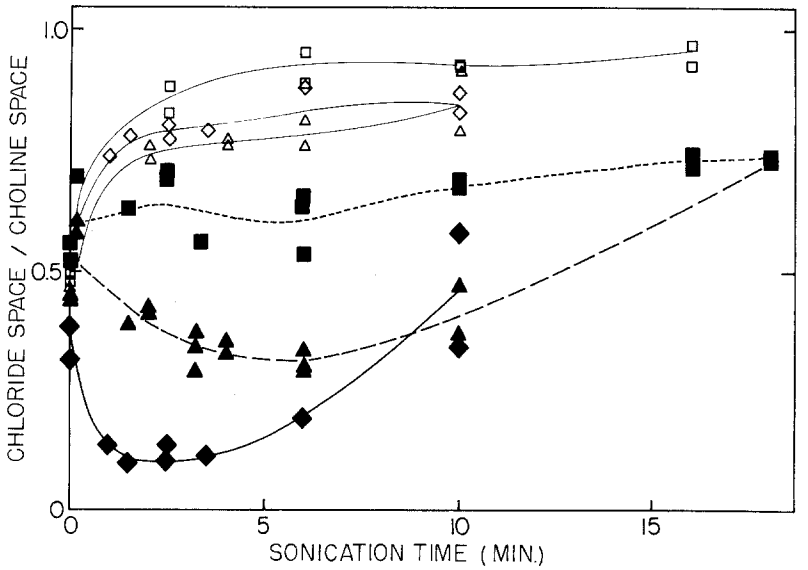

Fig. 4. The effect of sonic dose and lipid composition on $^{36}Cl^-$ permeability of cosonicated lipid and membrane vesicles and mixtures of separately sonicated lipid and membrane vesicles. Suspensions of lipid vesicles, 74 mg (dry wt)/ml were prepared in sonication buffer containing either ^3H- or ^{14}C-labeled choline (^3H: 1 μCi/ml and 0.1 or 10 μM; ^{14}C: 0.25 μCi/ml and 4 μM) and $^{36}Cl^-$ (0.1 or 0.2 μCi/ml). Membrane was prepared in the same doubly labeled buffer by the procedure described in *Methods*. At this point it had received only 5.4 cal/ml sonic energy and consisted of large vesicles. Lipid vesicle suspensions were (i) mixed with membrane to give 18.5 mg (dry wt) lipid/ml and 200 mg (wet wt) membrane/ml and unchanged labeled choline and $^{36}Cl^-$ concentrations and specific activities or (ii) simply diluted with the doubly labeled buffer. Membrane, lipid vesicles, and membrane-lipid vesicle mixtures were sonicated at 35 or 40 °C at 1.7 cal·ml^{-1}·sec^{-1} for the indicated times. Next, all samples were incubated at 40 °C for 15 min ("annealed"), and then membrane and lipid vesicle samples were mixed together, and cosonicated membrane plus lipid vesicle samples diluted with the doubly labeled buffer. At this point, all samples, whether cosonicated or separately sonicated and then mixed, had the same membrane, lipid, labeled choline and $^{36}Cl^-$ concentrations. All samples were then diluted with 3 vol glycine incubation medium (*Methods*) containing enough labeled choline and $^{36}Cl^-$ to keep unchanged the concentrations and specific activities of choline and $^{36}Cl^-$, incubated 5 min at 40 °C, and then 1.0-ml aliquots were analyzed for vesicle-trapped isotopes by passage through sephadex columns. (Glycine uptake was also measured in these experiments; *see* Fig. 6.) The only opportunity for $^{36}Cl^-$ loss was during passage through the sephadex columns (1–2 hr at 5–8°). Large filled symbols are cosonicated membrane plus lipid vesicles; small open symbols are mixtures of separately sonicated membrane plus lipid vesicles. ◆, ◇: Lipid is asolectin/cholesterol, 4:1 (wt/wt). ▲, △: Lipid is asolectin/egg yolk, lecithin/cholesterol 2:2:1 (wt/wt/wt). ■, □: Lipid is egg yolk lecithin/cholesterol, 4:1 (wt/wt)

wt) = 0.093, the added lipid vesicles have 3 times as much lipid as the membrane. In these experiments a membrane-like property, $^{36}Cl^-$ permeability, is seen associated with a lipid property, a large vesicle-enclosed space. This induction of $^{36}Cl^-$ permeability is greatest with asolectin-

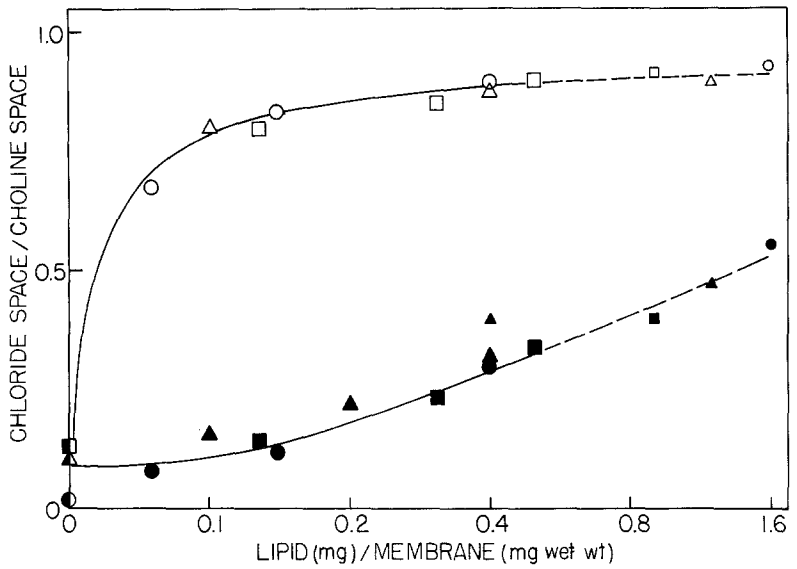

Fig. 5. The effect of the lipid/membrane ratio on $^{36}Cl^-$-permeable space of cosonicated and separately sonicated lipid and membrane vesicles. Lipid vesicles were prepared by sonicating (at 35 °C and 510 cal/ml) 300 mg asolectin/cholesterol, 4:1, wt/wt, in 1.5 ml sonication buffer containing 10 μM [^{3}H]-choline, 1 μCi/ml, and $^{36}Cl^-$, 0.2 μCi/ml. Membrane was prepared in the same doubly labeled medium. For the filled data points (cosonicated samples) through which the solid curve is drawn, membrane was mixed with the lipid vesicle suspension plus the doubly labeled sonication buffer to give 200 mg (wet wt/ml) membrane and lipid (dry wt)/membrane (wet wt) ratios between zero and 0.5, and unchanged labeled choline and $^{36}Cl^-$ concentrations. These samples were sonicated 2.5 min at 35° and 1.7 cal·ml^{-1}·sec^{-1} (255 cal/ml). These cosonicated samples were diluted twofold with the doubly labeled sonication buffer, annealed 15 min at 40° and diluted fourfold with Ca^{++} uptake media with or without ATP, containing $^{45}Ca^{++}$ (0.5 μCi/ml), and the same [^{3}H]-choline and $^{36}Cl^-$ concentrations and specific activities as above, and then incubated 10 min at 40°. The "mixed" samples were not diluted; membrane and lipid vesicle suspensions were incubated in the Ca^{++}-uptake media and then mixed. The final concentrations of membrane, lipid, and labeled solutes were thus the same for the cosonicated and mixed samples with the same lipid/membrane ratio. For data points through which the dashed line is drawn, membrane was mixed with lipid vesicles to give 100 or 50 ml/ml membrane and 80 mg/ml lipid and treated as above. These data are indicated by the smaller symbols and dashed line. After incubation in the Ca^{++}-uptake medium, all samples were analyzed for vesicle-trapped isotopes by sephadex filtration. This figure shows the $^{36}Cl^-$ permeable space ($^{36}Cl^-$-space/^{3}H-choline space) from these experiments. ($^{45}Ca^{++}$ uptake is shown in Fig. 7). The data from three experiments is shown. Differently shaped symbols show data from different experiments. Filled symbols: cosonicated samples. Open symbols: mixtures of separately sonicated membrane and lipid vesicles

cholesterol (Fig. 4, bottom curve). Figure 5 shows this $^{36}Cl^-$ permeability effect with higher ratios of lipid to membrane. At a lipid (dry wt)/membrane (wet wt) ratio of 0.4, the $^{36}Cl^-$-permeable space is 24 times that which would be enclosed by the membrane alone, and 80% of the total

enclosed space is $^{36}Cl^-$ permeable. At the highest lipid/membrane ratio used (1.6), the $^{36}Cl^-$-permeable space is approximately 64 times that of membrane alone.

The experiments of Figure 4 show other features that hybrid formation can explain. The more sonically susceptible the lipid vesicles, the more readily $^{36}Cl^-$ permeability is induced[3]. However, further sonication destroys $^{36}Cl^-$ permeability, and the more susceptible the lipid vesicles, the more readily $^{36}Cl^-$ permeability is destroyed. These effects are not seen if lipid vesicles and membrane are sonicated separately and then mixed. Hybrids should form more easily with the more fragmentable lipids, but once formed their sonic susceptibility should reflect that of the lipid matrix.

3) *Lipid vesicles protect glycine uptake activity from sonic damage.* We did analogous experiments using Na^+-dependent glycine uptake as the membrane property. This activity is less suitable than $^{36}Cl^-$ permeability because glycine uptake has no simple relationship to trapped space and is quite sensitive to sonic damage. Figure 6*a* shows that Na^+-dependent glycine uptake activity is more resistant to sonic damage when asolectin-cholesterol is present. Comparison of the effect of asolectin-cholesterol (Fig. 6*a*) and asolectin-egg yolk lecithin-cholesterol (Fig. 6*b*) shows effects reminiscent of $^{36}Cl^-$ permeability; the property (here protection) is easier to induce but less persistent with the more fragmentable lipid. Egg yolk lecithin-cholesterol does not protect (data not shown).

4) *$^{45}Ca^{++}$ uptake increases with increasing lipid.* If hybrids are formed, increasing the amount of lipid with a fixed amount of membrane should increase the amount of space served by membrane functions. This effect was seen with $^{36}Cl^-$ permeability (Fig. 5). It was also found with another membrane property, ATP-dependent $^{45}Ca^{++}$ uptake. As shown in Figure 7, ATP-dependent $^{45}Ca^{++}$ uptake/mg membrane increased with increasing lipid up to 12 times that of membrane alone. The dotted curve in Figure 7 shows the effects of still higher lipid/membrane ratios, a sharp drop in activity. This might result from dilution of an oligomeric Ca^{++} transport system; Vanderkooi *et al.* [32] have reported that the Ca^{++} porter of sarcoplasmic reticulum is multimeric in the membrane.

3 Both the cosonicated and mixture curves show $^{36}Cl^-$-permeable space values of approx. 0.5 at zero sonic dose. This is because the added membrane had been given only 5.4 cal/ml of sonic energy and was still in the large-vesicle form. The rapid reduction in membrane vesicle size during sonication can be seen as the step rise in $^{36}Cl^-$-impermeable space in the "mixture" curves.

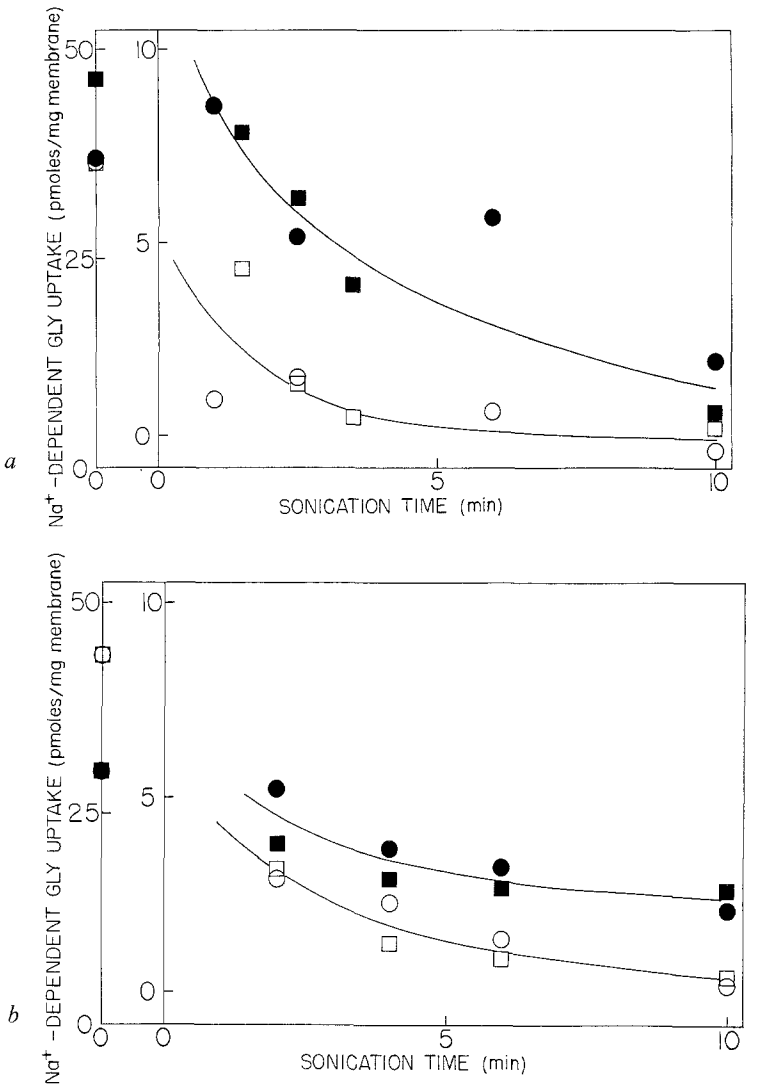

Fig. 6. The effect of sonic dose and lipid composition on Na^+-dependent glycine uptake into cosonicated and separately sonicated membrane and lipid vesicles. The experiments were done as described for Fig. 4. At the 5-min incubation in glycine uptake medium (*see* legend, Fig. 4), 0.2 mM labeled glycine was present. If the choline was ^{3}H-labeled, the glycine was ^{14}C-labeled (0.54 μCi/ml); with [^{14}C]-choline, 2.0 μCi/ml [^{3}H]-glycine was used. One portion of each membrane-lipid sample was mixed with Na^+-glycine uptake medium and a duplicate portion mixed with the K^+-medium. The rest of the procedure was described in the legend of Fig. 4. The difference, glycine uptake from the Na^+ medium minus glycine uptake from the K^+ medium, is plotted as Na^+-dependent uptake. The double ordinates are used to show on the same graph uptake from material given no second sonication and samples sonicated further. The data of two experiments (shown by differently shaped symbols) has been normalized to the same uptake at zero sonic dose. Filled symbols represent cosonicated samples and open symbols represent mixtures. (*a*): Lipid is asolectin/cholesterol, 4:1 (wt/wt). (*b*): Lipid is asolectin/egg yolk lecithin/cholesterol, 2:2:1 (wt/wt/wt)

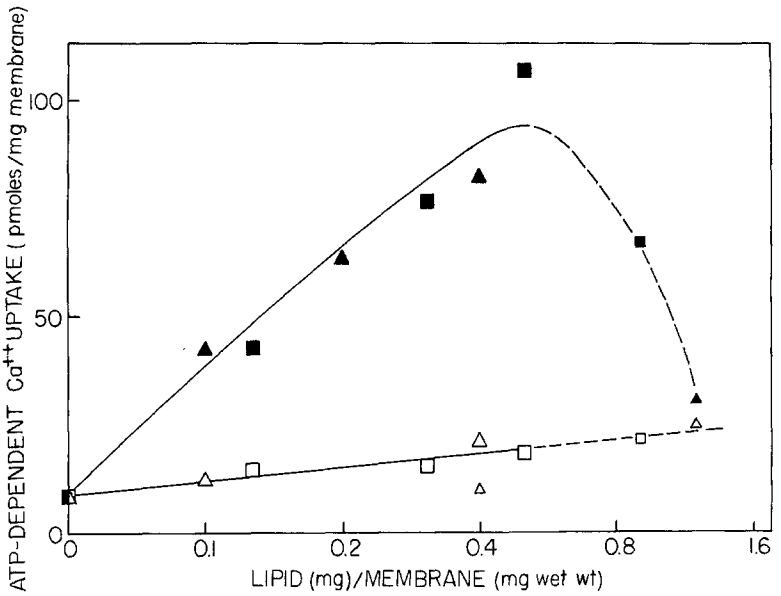

Fig. 7. The effect of increasing lipid/membrane ratio on ATP-dependent $^{45}Ca^{++}$ uptake by cosonicated and separately sonicated membrane and lipid vesicles. The data is from the experiments described in Fig. 5. The data from two experiments is normalized to the uptake from membrane alone (lipid/membrane = 0; normalization factor = 1.65). Differently shaped symbols show data from different experiments. Filled symbols: cosonicated samples. Open symbols: mixtures of separately sonicated membrane and lipid vesicles

5) *Membrane and lipid comigrate on density gradient centrifugation.* Association between membrane and lipid properties was also found by density gradient centrifugation. Lipid was labeled with ^{3}H-cholesterol, vesicle space was marked with [^{14}C]-choline and/or $^{32}P_i$, and the membrane activities measured were $^{36}Cl^-$ permeability, ATP-dependent $^{45}Ca^{++}$ uptake and Na^+-dependent glycine uptake. Two to 15% (wt/vol) bovine serum albumin (BSA) gradients were used, corresponding to a density range of 1.012 to 1.048 g/ml. Samples were loaded on top in solutions with a density of 1.007 g/ml.

If the lipid we used had a density similar to egg yolk lecithin (1.019 g/ml [11]), unsealed lipid vesicles could move at most 2 ml down the gradient. Sealed lipid vesicles trapping solution with a density of 1.007 g/ml could not move that far. If hybrids form, the lipid should be found farther down the gradient and membrane properties should be shifted upward. This was found.

Figure 8 shows the gradient positions of lipid (^{3}H-cholesterol), $^{32}P_i$-space and $^{36}Cl^-$-space of membrane and lipid vesicles sonicated separately and then mixed (Fig. 8*a*) or cosonicated (Fig. 8*b*). In the "mixed"

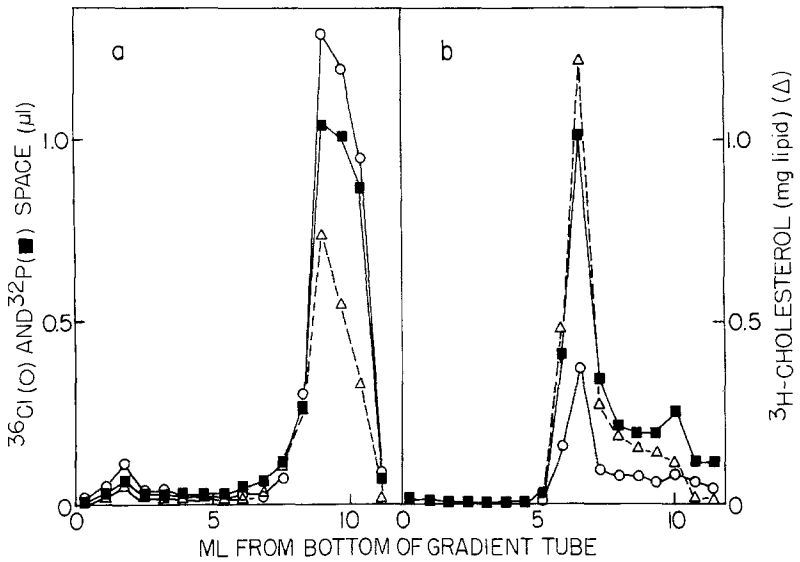

Fig. 8. Distribution on bovine serum albumin density gradient centrifugation of lipid, phosphate space, and chloride space of cosonicated and separately sonicated lipid and membrane vesicles. Lipid/membrane: 0.17. Asolectin/cholesterol, 4:1, wt/wt, containing [^{3}H]-cholesterol, 0.025 μCi/mg total lipid was sonicated (35 °C, 510 cal/ml) at 138 mg/ml in sonication buffer labeled with $^{36}Cl^{-}$ (2 μCi/ml) and ^{32}P (4 μCi/ml). Aliquots of this suspension were mixed with membrane in the same doubly labeled buffer to give 34.5 mg lipid/ml and 200 mg membrane/ml, sonicated at 35 °C and 1.7 cal·ml^{-1}·sec^{-1} for a sonic dose of 255 cal/ml and then annealed at 40° for 15 min. Cosonicated samples were then diluted twofold with the same doubly labeled buffer. The samples were then diluted fourfold with Ca^{++}-uptake medium, with or without ATP, containing 8 μCi/ml ^{45}Ca and the same [^{3}H]-choline and $^{36}Cl^{-}$ concentrations and specific activities as the sonication solution, incubated 10 min at 40° and passed through the sephadex columns. Then 3-ml aliquots of the vesicle fraction from these columns were centrifuged in the BSA gradients as described in *Methods*. The mixtures of separately sonicated membrane and lipid vesicles were prepared to match the composition of the cosonicated samples as described for the experiments of Fig. 4 for glycine uptake, except that membrane and lipid vesicle samples were not mixed until after the incubation with (Ca^{++}) uptake medium (but before passage through the sephadex columns). This figure shows the distribution of lipid (^{3}H-cholesterol), vesicle-trapped space holding P_i (^{32}P) and $^{36}Cl^{-}$. (*a*): Mixed lipid vesicles and membrane. (*b*): Cosonicated lipid vesicles and membrane. The gradients shown are of the samples incubated with $^{45}Ca^{++}$ in the presence of ATP. (Fig. 10 shows the $^{45}Ca^{++}$ uptake data from this experiment)

sample lipid remains at the top of the gradient, the lipid and space peaks coincide, and $^{36}Cl^{-}$ is there as well retained as $^{32}P_i$. In the cosonicated sample, virtually all the lipid is found further down the gradient at a density pure lipid could not reach. The lipid (^{3}H) peak again coincides with the $^{32}P_i$ and $^{36}Cl^{-}$ peaks, but now most of the space retaining $^{32}P_i$ is incapable of retaining $^{36}Cl^{-}$. This lack of $^{36}Cl^{-}$ retention shows that the lipid peak is not lipid and membrane vesicles merely stuck

together. When a lower lipid/membrane ratio was used, the "hybrid" peak was further down in the gradient (Fig. 9).

In the experiments above, no membrane property could be seen in the "mixed" samples. With ATP-dependent $^{45}Ca^{++}$ uptake activity as a membrane marker, the membrane in membrane-lipid mixtures can be located in the gradients. In the "mixed" sample (Fig. 10*a*) there is a peak of ATP-dependent $^{45}Ca^{++}$ uptake deep in the gradient while a large ^{32}P (space) and ^{3}H (lipid) peak is at the top. In the cosonicated sample (Fig. 10*b*), all three markers coincide at an intermediate position.

A similar experiment was done with Na^{+}-dependent glycine uptake as the membrane marker. Since this activity has a much poorer signal-to-noise ratio than $^{45}Ca^{++}$ uptake activity, the results were less clean than with $^{45}Ca^{++}$ uptake. We saw well separated low and high density peaks of lipid and uptake activity, respectively, in the "mixtures" and a clear-cut shift towards a common intermediate position resulting from cosonication. However, the different isotope peaks did not appear so nearly coincident (data not shown).

6) *Electron microscopic appearance is consistent with hybrid formation.* Electron micrographs were made of membrane vesicles, lipid vesicles, mixtures of separately sonicated membrane and lipid vesicles, and cosonicated membrane and lipid vesicles. The "hybridization" conditions used were those found optimum according to the properties already described; the lipid was asolectin-cholesterol, consonication was at 255 cal/ml and the lipid/membrane ratios were 0.1, 0.2 and 0.4.

We saw only a few multilamellar structures of unknown origin in the pure lipid fields (not shown). Pure lipid vesicles apparently did not survive our fixing and embedding procedures. Mixtures of separately sonicated membrane and lipid vesicles (Fig. 11*a*) resembled pure membrane. There were numerous small vesicle-like structures and a considerable amount of fragmented and/or amorphous material. Cosonication of lipid and membrane produced structures different from those in the mixtures (Fig. 11*b* *vs.* *a*). Increasing the lipid (11*b* *vs.* *c*) resulted in more, larger (up to 150–250 nm), and often multilamellar vesicles. These vesicles showed a wide range of staining intensities and appeared less regular than pure membrane vesicles of similar size, e.g., the moderately sonicated membrane shown in Fig. 1*b*. Both the small vesicle-like structures and the fragmented and/or amorphous material of pure membrane (Fig. 1*c*) or mixed membrane and lipid (Fig. 11*a*) were nearly absent (Fig. 11*b*). These observations are consistent with the formation of hy-

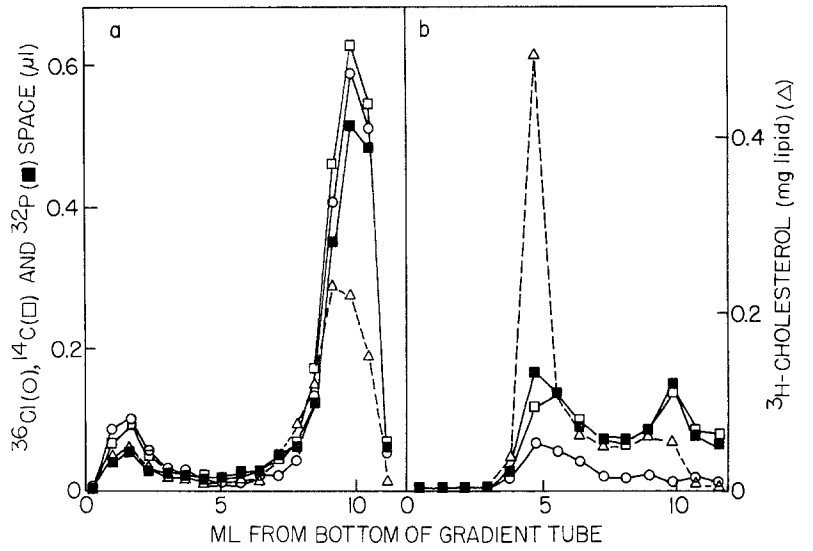

Fig. 9. Distribution on bovine serum albumin density gradient centrifugation of lipid, phosphate space, choline space, and chloride space of cosonicated and separately sonicated lipid vesicles and membrane. Lipid/membrane: 0.093. This experiment was done like that of Fig. 8 except for the lower lipid/membrane ratio and the presence of [^{14}C]-choline (8 μCi/ml, 128 μM) as a trapped space marker in addition to $^{32}P_i$

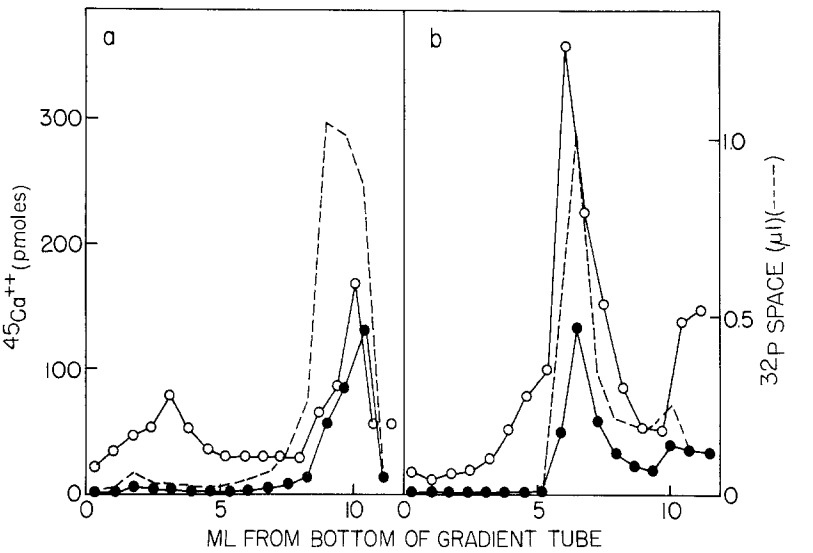

Fig. 10. Distribution on bovine serum albumin density gradient centrifugation of Ca^{++} uptake activity of cosonicated and separately sonicated membrane and lipid vesicles. This data is from the experiment described for Fig. 8. ○: $^{45}Ca^{++}$ taken up in the presence of ATP. ●: $^{45}Ca^{++}$ taken up in the absence of ATP. -----: the $^{32}P_i$ trace from the gradient of the samples incubated with $^{45}Ca^{++}$ but no ATP. (the ^{3}H, $^{32}P_i$ and $^{36}Cl^-$ traces from the samples incubated with and without ATP were very similar)

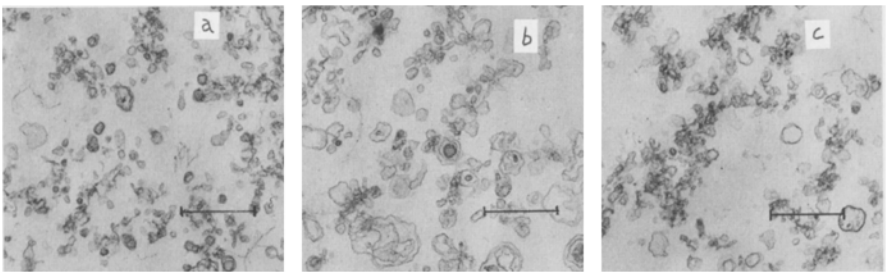

Fig. 11. Electron microscopic appearance of cosonicated and separately sonicated lipid and membrane vesicles. Samples were prepared as for Fig. 5 except without labeled solutes and the uptake incubation step. Processing for electron microscopy is described in *Methods*. Bar = 500 nm. (*a*): Mixed lipid vesicles and membrane sonicated separately. Lipid/membrane = 0.4 (dry wt/wet/wt); lipid was 80 mg/ml. (*b*): Cosonicated lipid vesicles and membrane. Lipid/membrane = 0.4; lipid was 80 mg/ml as for *a* above. (*c*): Cosonicated lipid vesicles and membrane. Lipid/membrane = 0.1; lipid was 20 mg/ml

brids. Pure membrane structures disappear, new structures appear, and the irregularities of outline and staining intensity of the new structures could plausibly arise from localized patches of membrane proteins incorporated into the lipid matrix.

Discussion

The density gradient experiments show that cosonication of lipid vesicles and membrane causes virtually all of the added lipid to become associated with membrane material, and many properties of lipid vesicles and membrane are altered by sonication of one in the presence of the other. The question is whether these effects are due to hybrid formation. All the observed properties can be accounted for by hybrid formation (*Results*).

No other hypothesis appears to fit the data. Simple absorption of membrane material onto lipid vesicles is inadequate. It could account for only one observation, the coincident density gradient positions of lipid and Ca^{++}-uptake activity. Even then the *ad hoc* assumption is required that every lipid vesicle "sticks to" approximately the same quantity of membrane material and vice versa.

Very little of the water in a pellet of highly sonicated membrane is vesicle-enclosed. If small amounts of lipid "patched" otherwise leaky vesicles, that could explain the large increase in a Ca^{++}-transport activity and the large increase in $^{36}Cl^{-}$-permeable vesicle-trapped space. ("Patching", however, is itself a limited hybrid formation.) Such "patching"

might well occur more easily with more readily fragmentable lipids. However, "patching" with small amounts of lipid does not account for (i) the nearly complete loss of $^{36}Cl^-$-retaining space even at added lipid/membrane lipid ratios of 12, (ii) the greater sonic sensitivity of $^{36}Cl^-$ permeability of "hybrid" vesicles with the more sonic-sensitive lipids, (iii) the increased resistance to sonic damage of glycine and Cl^--transport properties of the cosonicated material. Concerning points *ii* and *iii*, the sonic susceptibility of membrane vesicles "patched" with small amounts of lipid should approximate that of the most susceptible component, the membrane. "Patching" could also not explain (iv) the loss of Ca^{++} uptake activity at very high lipid/membrane ratios, nor (v) the disappearance of membrane-like structures, both vesicles and fragments (and the appearance of large amounts of new structures), in the electron micrographs.

Selective transfer to the lipid vesicles of particular proteins can be considered, but we found the three membrane functions we measured were all affected (Cl^- permeability, Ca^{++} transport, and glycine transport) and selective transfer of everything is a contradiction. Selective transfer also does not explain the disappearance of membrane-like structures. We conclude that cosonication very probably produces hybrids.

The data do not show that all the lipid and membrane is converted to hybrids, but the gradient and Cl^--permeability experiments show little material or "space" with pure membrane-like or lipid-like character, and the electron micrographs show little pure membrane-like material. This suggests that with the appropriate lipid and sonic dose, most of the added material forms hybrids.

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