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Fusion of Liposomes and Rat Brain Microsomes Examined by Two Assays

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Summary. Liposomes are prepared from rat brain microsomal lipid and loaded with either Tb^{3+} or dipicolinic acid (DPA) to test fusion with the Tb-DPA assay. They are also loaded with octadecyl Rhodamine B chloride (R_{18}) to test fusion with the R_{18} assay. The addition of either Ca^{2+} or Mg^{2+} to loaded liposomes develops fluorescence with both assays. The fluorescence elicited by Mg^{2+} is similar to that elicited by Ca^{2+} if assessed with R_{18}, but much higher if determined by Tb-DPA. The Ca^{2+}-dependent fluorescence of the Tb-DPA complex is not suppressed by the addition of EDTA, and therefore it is internal to vesicles. The contrary is true for the Mg^{2+}-dependent fluorescence. Rat brain microsomes can be disrupted by adding octylglucoside and reconstituted by removing it by dialysis. We use this procedure to load microsomes with DPA. This allows the use of the Tb-DPA assay for testing the fusion of rat brain microsomes. Reconstituted microsomes fuse with liposomes. This fusion has characteristics similar to those of liposome-liposome fusion. However, no microsome-microsome fusion could be detected with either method. The two methods give different results, owing to the chemical properties of the assays. Indeed Tb-DPA implies the retention of vesicle content, whereas this is not required by the R_{18} assay.

Key Words liposomes · microsomes · fusion · membranes · brain · octadecyl · rhodamine

Introduction

Microsomes are a mixed population of vesicles whose metabolic activities are connected to those of other cell compartments and imply the formation and the release of secretive vesicles (Poste & Allison, 1973). However, the participation of rat brain endoplasmic reticulum to events connected with membrane fusion has not yet been thoroughly investigated.

The fusion of membranes has been studied using several methods, such as vesicle aggregation, mixing of membrane components, release of vesicle contents or morphological changes. The Tb-DPA assay (Wilschut et al., 1980) is the most rigorous, implying the mixing of the aqueous vesicle content, and has become popular to detect membrane fusion in artificial systems. On the other hand, its usefulness to study natural membranes is limited by the difficulty of encapsulating Tb^{3+} and DPA. However, in previous works (Corazzi & Arienti, 1986; 1987) we reported the possibility of disaggregating rat brain microsomes by exposure to octylglucoside and of reconstituting them following detergent removal by dialysis. In this paper, the procedure is exploited to load brain microsomes with DPA or Tb^{3+}, thus allowing the investigation of the fusion of microsomal membranes with the Tb-DPA assay.

A method based on the relief of fluorescence self-quenching of octadecyl Rhodamine B chloride (R_{18}) has been proposed (Hoekstra et al., 1984) for studying the fusion of biological membranes. This assay has the advantage of permitting a ready incorporation of the probe into membrane structure. However, it might not be as rigorous as the Tb-DPA one, since it does not necessarily imply a mixing of the internal vesicle material. Therefore, we use both DPA and R_{18} assays with the aim of determining if there is an apparent difference of microsome and liposome fusion, depending on the method of determination.

Studies on membrane fusion have been performed mainly on liposomes made with acidic phospholipids, secretory vesicles and viruses (Klappe, Wilschut & Hoekstra, 1986; Ekerdt, Dahl & Gratzl, 1981) and particular relevance has been given to the role of ions (Ohki & Ohshima, 1985; Deleers, Servais & Wülfert, 1985). To this purpose, it should be noticed that Ca^{2+} is certainly involved in fusion (Chernomordik, Melikyan & Chizmadzhev, 1987), but the role of Mg^{2+} and of other cations is still under debate (Chernomordik et al., 1987; Yoshimura & Aki, 1985). For this reason, we test the ability of Ca^{2+} and Mg^{2+}, in millimolar range, to induce fusion of liposomes and of rat brain microsomes.

Materials and Methods

Materials

Pyridine-2,6-dicarboxylic acid (dipicolinic acid; DPA) and $TbCl_3 \cdot 6H_2O$ were purchased from Aldrich-Chemie (Steinheim, FRG). HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), octylglucoside (octyl-β-D-glucupyranoside), thesit (dodecylpoly(ethylenglycolether)9) and dithiothreitol (DTT) were produced by Boehringer-Biochemie (Mannheim, FRG) and nonpolar polystyrene absorbent (Bio-Beads SM-2) by Bio-Rads Laboratories (Richmond, CA). Sephadex G-50 was obtained from Pharmacia Fine Chemicals A B (Uppsala, Sweden) and octadecyl Rhodamine B chloride (R_{18}) from Molecular Probes (Eugene, OR). Other reagents, all of high purity, were purchased from Carlo Erba (Milano, Italy).

Preparation of Liposomes

Suitable amounts of lipids were extracted from rat brain microsomes (Folch, Lees & Sloane-Stanley, 1957), dissolved in chloroform/methanol (2:1 by vol) and the solvent removed under a gentle stream of N_2. The composition was the following (molar ratio): 34% cholesterol, 28% phosphatidylcholine, 20% phosphatidylethanolamine, 10% phosphatidylserine, 7% phosphatidylinositol + sphingomyelin.

Preparation of DPA- and Tb-Loaded Liposomes

To prepare small unilamellar vesicles (SUV, Cullis et al., 1985), lipids were suspended in a buffer (about 1 ml/mg lipid; pH 7.0) containing 2 mM L-histidine, 2 mM HEPES and 150 mM DPA, whereas to prepare Tb-liposomes, the buffer (pH 7.0) contained 2 mM L-histidine, 2 mM HEPES, 15 mM $TbCl_3$ and 150 mM sodium citrate. Suspensions were sonicated to clearness (about 5 min in a MSE sonicating apparatus), and aggregated material was eliminated by centrifugation at 5°C for 1 hr at 105,000 × *g*.

To remove nonencapsulated Tb^{3+}, vesicles were gel-filtered through a Sephadex G-50 column (0.5 × 25 cm) and eluted with a buffer (pH 7.0) containing 2 mM L-histidine, 2 mM HEPES, 1 mM EDTA and 100 mM NaCl. To remove EDTA, the procedure was repeated by eluting particles with the same buffer, made by omitting EDTA. Nonencapsulated DPA was removed by gel filtering vesicles through a Sephadex G-50 column (0.5 × 25 cm) and eluting with a buffer (pH 7.0) containing 2 mM L-histidine, 2 mM HEPES and 100 mM NaCl. The removal of nonencapsulated material was satisfactory, as demonstrated by the absence of fluorescent peak at 545 nm (*see Assay of Fusion*) mixing DPA liposomes with free Tb^{3+} (0.75 μM) or of free DPA (5 μM) to Tb-liposomes. Leakage of the probes from the vesicles was negligible at least for 30 min, as determined adding free Tb^{3+} (0.75 μM) to DPA-loaded liposomes or free DPA (5 μM) to Tb-loaded liposomes in the absence of either EDTA or divalent cations (Rosenberg, Düzgünes & Kayalar, 1983).

The amount of DPA entrapped into the vesicles was calculated measuring the intensity of fluorescence of a known amount of vesicles after the addition of an excess Tb^{3+} (0.75 μM) in the presence of disaggregating concentrations of thesit (0.03%) and comparing it to that of a standard solution of DPA in the same conditions. The same procedure was repeated for Tb-liposomes, which were tested against free DPA (5 μM) in the presence of detergent. Liposomes contained about 25 nmol Tb^{3+}/μmol of lipid P (about 16 nmol Tb/μmol lipid) and 360 nmol DPA/μmol of lipid P (about 234 nmol DPA/μmol lipid). The encapsulation of probes was therefore similar to the one already reported for liposomes made with acidic phospholipids (Wilschut et al., 1980).

Preparation of R_{18} Liposomes

Small unilamellar vesicles were prepared suspending lipids extracted from rat brain microsomes in a buffer (1 ml/mg lipid; pH 7.0) containing: 2 mM L-histidine, 2 mM HEPES and 100 mM NaCl. Suspensions were sonicated to clearness (about 5 min in a MSE-sonicating apparatus), and aggregated material was eliminated by centrifugation at 5°C for 1 hr at 105,000 × *g*. R_{18} was dissolved in ethanol (1 mg/ml) and 20 μl of this solution were added to 1 ml of liposomal suspension, and the mixture was kept in the dark for 1 hr at room temperature. To eliminate noninserted R_{18}, liposomes were chromatographed on a Sephadex G-50 column (0.5 × 25 cm) and eluted with the above buffer (Hoekstra et al., 1984). This preparation was stable for many hours. Surface density was calculated by measuring the fluorescence obtained from liposomal suspension in the presence of 0.03% thesit and comparing it to that of standard solutions of R_{18}. About 50–60% of the added probe was incorporated into liposomes, and by adding 20 μg of probe/ml a surface density of 2 mol% or less was obtained.

Preparation of Microsomes

Sprague-Dawley male rats (150–200 g body wt) were used to prepare brain microsomes, as described previously (Porcellati et al., 1971). Membranes were resuspended in cold 0.32 M sucrose, 2 mM DTT, and immediately used.

Preparation of DPA- and Tb-Loaded Microsomes

Microsomes were solubilized and loaded as follows: membranes (0.5 ml, about 3 mg protein) were mixed with 0.5 ml of a buffer (pH 7.0) containing 60 mM octylglucoside, 200 mM dipicolinic acid (or 60 mM $TbCl_3$ + 300 mM citrate), 4 mM HEPES and 4 mM L-histidine. The mixture was left for 15 min at room temperature to equilibrate and then dialyzed at 4°C against 150 ml of a buffer (pH 7.0) containing 0.32 M sucrose, 2 mM HEPES, 2 mM L-histidine, 2.5 mM $CaCl_2$ and 2 mM DTT. Bio-Beads were added to the external medium to increase the speed and the efficiency of detergent removal. The medium and the Bio-Beads were replaced after 2 hr, and dialysis was continued up to 16 hr. Any DPA not entrapped into vesicles was removed by gel-filtration through a Sephadex G-50 column (25 × 0.5 cm), using 0.32 M sucrose, 2 mM L-histidine and 2 mM HEPES as eluent buffer (pH 7.0). Nonentrapped Tb^{3+} was eliminated by gel filtration through a Sephadex G-50 column (25 × 0.5 cm) using 0.32 M sucrose, 2 mM histidine, 2 mM HEPES, and 1 mM EDTA buffer (pH 7.0). EDTA was subsequently removed, repeating the chromatographic procedure and eluting with the same buffer made by omitting EDTA. Microsomes contained 10 nmol of DPA/mg protein (7.8 nmol/μmol lipid) as determined by measuring fluorescence in the presence of an excess (0.75 μM) Tb^{3+} and 0.03% thesit, or 25 nmol of Tb^{3+}/mg protein (7.8 nmol/μmol lipid) as measured by determining fluorescence in the presence of an excess DPA (5 μM) and 0.03% thesit. The leakage of encapsulated material was negligible for at least 30 min (*see Preparation of Liposomes* for details on determination).

Preparation of R_{18}-Microsomes

Microsomal suspension (2 mg protein in 1 ml of 0.32 M sucrose, 2 mM HEPES and 2 mM L-histidine buffer (pH 7.0)) were mixed with R_{18} as described above (25 μg of R_{18}/ml of suspension). The mixture was permitted to stay 1 hr at room temperature in the dark. The excess R_{18} was eliminated as described for liposomes and using 0.32 M sucrose, 2 mM HEPES, 2 mM L-histidine as elution buffer (pH 7.0). Fluorescence was stable for several hours, and the surface density of the probe was calculated as reported above. About 50–60% of the added probe was incorporated into membranes, and a probe/lipid ratio of 2 mol% or less was obtained.

Assay of Fusion

Tb-DPA Assay

The fusion of liposomes was performed in a cuvette containing 2 ml of 100 mM NaCl, 2 mM L-histidine and 2 mM HEPES (pH 7.0). To test microsome fusion, NaCl was replaced by 0.32 M sucrose. Ca^{2+} or Mg^{2+} were added to stirred vesicle suspensions in small increments, utilizing 1 M $CaCl_2$ or 1 M $MgCl_2$ solutions and a Hamilton syringe.

Vesicle fusion was assayed with a method involving the encapsulation of Tb^{3+} as $[Tb(citrate)_3]^{6-}$ complex in one population of vesicles and of DPA in another (Wilschut et al., 1980). The mixing of the aqueous content of vesicles which followed fusion resulted in the formation of a fluorescent $[Tb(DPA)_3]^{3-}$ chelation complex whose fluorescence was monitored with a MPF-3 Perkin-Elmer apparatus. Excitation wavelength was set at 276 nm and emission at 545 nm. A cut-off filter (UV 31) was inserted to eliminate contribution to signal due to light scattering. Windows were set at 10 nm for excitation and 6 nm for emission.

The calibration fluorescence (100%) for the Tb/DPA assay was calculated for every preparation by determining the maximal fluorescence obtainable from Tb- and DPA-loaded vesicles in the presence of 0.03% thesit and in the absence of Ca^{2-}. Further additions of either thesit or DPA did not increase fluorescence.

The leakage of Tb^{3+} or of DPA was measured in the presence of nonencapsulated DPA or Tb^{3+}, respectively (Rosenberg et al., 1983) and, since no increase of fluorescence was detectable in 30 min in the absence of divalent cations, the leakage of encapsulated material was negligible.

R_{18} Assay

The fusion was tested in a cuvette containing the same buffers used for the DPA-Tb assay. Two vesicle populations (one loaded with the probe and the other unloaded) were added. No increase of fluorescence was detected until the addition of divalent cations. Ca^{2+} or Mg^{2+} were added to stirred vesicle suspensions in small increments, utilizing 1 M $CaCl_2$ or 1 M $MgCl_2$ solution and a Hamilton syringe.

Fluorescence was monitored at 560 nm excitation and 580 nm emission using a Perkin-Elmer MPF-3 spectrofluorometer. Windows were set at 4 nm for both excitation and emission.

The calibration of the assay was performed by taking as 100% the fluorescence obtainable upon the addition of 0.03% thesit to the mixture. Fluorescence did not increase by adding further amounts of the detergent. We investigated the linearity of fluorescence self-quenching *vs.* surface density and always used concentrations comprised in the linearity range, which was ≤2 mol% for either microsomes or liposomes.

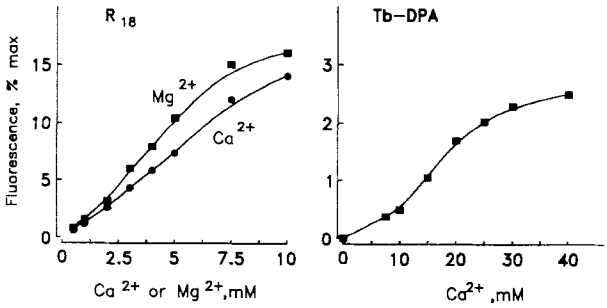

Fig. 1. Ca^{2+} and Mg^{2+}-induced fusion of liposomes made with rat brain microsomal lipids. Data are expressed as percent of maximal fluorescence (which is obtained in the presence of 0.03% thesit and in the absence of Ca^{2+}). Fluorescence was measured 60 sec after the addition of the ions. Liposomes were obtained from rat brain microsomal lipids and contained, in molar ratio: 34% cholesterol, 28% phosphatidylcholine, 20% phosphatidylethanolamine, 10% phosphatidylserine, and 7% phosphatidylinositol + sphingomyelin. Tb-DPA method: The indicated concentrations of Ca^{2+} were added to the vesicles (Tb-liposomes, 27 nmol lipid P · ml^{-1}; DPA-liposomes 37 nmol lipid P · ml^{-1}) and the fluorescence increase of 2 ml of suspension measured as described under Materials and Methods. R_{18} method: The fluorescence increase was measured as described under Materials and Methods, mixing R_{18} loaded liposomes (50 nmol lipid P · ml^{-1}) with unloaded liposomes (50 nmol lipid P · ml^{-1})

Analyses

Protein was determined as described (Lowry et al., 1951) and phospholipid phosphorus assayed following the assay of Bartlett (1959) after digestion with 70% (wt/wt) perchloric acid. Cholesterol was assessed as reported (Rudel & Morris, 1973).

Results

Fusion of Liposomes

Liposomes prepared with total lipid extracted from rat brain microsomes did not fuse until divalent cations were added. The addition of divalent cations to liposome mixtures induced fusion, as measured with Tb-DPA and R_{18} assays (Fig. 1). It should be noticed that the curve of fluorescence *vs.* cation concentration was sigmoid, as in other preparations (Ohki, 1982; Ohki & Ohshima, 1985). In our hands, the threshold cation concentration (the intercept on the concentration axis of the segment drawn from

Table 1. Effect of divalent cations on liposome fusion

Divalent cation (mM)	Tb-DPA method			R_{18} method	
	A	B	C	D	E
0	0.0	1.7	0.0	0.0	0.0
5	8.0	1.8	1.1	19.3	20.2
15	30.1	2.7	1.7	34.2	33.0
20	38.3	3.6	5.1	34.1	30.0
25	—	0.8	5.9	—	—

Tb-DPA method: Divalent cations were added to a mixture containing Tb-liposomes (25 nmol lipid P · ml^{-1}) and DPA-liposomes (25 nmol lipid P · ml^{-1}). Data are expressed as percent of maximal fluorescence (obtained in the presence of 0.03% thesit and in the absence of Ca^{2+}) and report plateau fluorescence values which were reached 8 min after the addition of the ions.

A: Mg^{2+}.

B: As in A, but the assay was performed in the presence of 15 mM Ca^{2+}.

C: Ca^{2+}.

The addition of EDTA (0.1 mM) to A before or after the development of fluorescence quenched the fluorescence completely. The addition of EDTA (0.1 mM) to B or C had no effect. Reported values are the average of three determinations. SE (omitted for clarity's sake) did not exceed 10%.

R_{18} method: Divalent cations, Mg^{2+} (D) or Ca^{2+} (E), were added to a mixture containing R_{18}-loaded liposomes (50 nmol lipid P · ml^{-1}) and unloaded liposomes (50 nmol lipid P · ml^{-1}). Data are expressed as percent of maximal fluorescence (obtained in the presence of 0.03%) and refer to plateau fluorescence values which were reached 4 min after the addition of the ions. Reported values are the average of three determinations. SE (omitted for clarity's sake) did not exceed 10%.

the sharpest slope of the curve) depended on the assay used; indeed, it was about 5 mM if measured with Tb-DPA and about 0.5 mM if assessed with R_{18}. The values reported in Fig. 1 are somewhat different from those previously described for liposomes made with acidic phospholipids (Wilschut, Düzgünes & Papahadjopoulos, 1981; Ohki, 1982; Ohki & Ohshima, 1985). Indeed, threshold Ca^{2+} concentrations of 2–3 mM have been reported for small unilamellar phosphatidylserine liposomes. The same authors also described an increase of fluorescence followed by a decrease due to the collapse of fused vesicles (Wilschut et al., 1980). However, using liposomes made with total brain microsomal lipids, we did not notice any decrease of fluorescence. The different lipid compositions would probably explain these findings (Papahadjopoulos et al., 1976).

If fusion was induced by Ca^{2+}, the presence of this ion inhibited the formation of the Tb-DPA fluorescent complex outside the vesicles, which, however, was not inhibited by Mg^{2+}; therefore, upon the addition of Mg^{2+}, the DPA-Tb complex could be located either outside or inside the vesicles. It can be observed that using Tb-DPA, the fluorescence elicited by Mg^{2+} was much higher than that elicited by Ca^{2+} (Table 1). However, 0.1 mM EDTA quenched almost completely the Mg^{2+}-dependent fluorescence, although leaving practically unaffected Ca^{2+}-dependent fluorescence. The occurrence of a Mg^{2+}-induced leakage or of a leaky fusion might explain this result. The addition of both ions (increasing amounts of Mg^{2+} added to 15 mM Ca^{2+}) showed a synergism at low concentrations of Mg^{2+}, which could be in agreement with the data of Portis et al. (1979). However, Ca^{2+} and Mg^{2+} were less effective than Ca^{2+} alone at concentrations of Mg^{2+} higher than 15 mM, as a result of the leakage produced by Mg^{2+}. The R_{18} assay showed liposome fusion in the presence of either Ca^{2+} or Mg^{2+}. No large differences could, however, be seen in this case, indicating a similar ability of the two ions to mix lipid phases of different liposomal populations.

Experiments were also performed with different DPA-liposome/Tb-liposome ratios. To increase the ratio, increasing amounts of DPA-liposomes were added to a fixed amount of Tb-liposomes (Fig. 2*A*), and to decrease it increasing amounts of Tb-liposomes were added to a fixed amount of DPA-liposomes (Fig. 2*B*). The increase of fluorescence due to the increase of the ratio is connected to the probability of obtaining productive (Tb-DPA) *versus* unproductive (Tb-Tb or DPA-DPA) fusion (Wilschut et al., 1981). On the other hand, the decrease of fluorescence produced by a decrease of the ratio (Fig. 2*B*) is explained by the fact that the limiting factor in the development of fluorescence is the availability of Tb^{3+} (liposomes contain less Tb than DPA/nmol lipid P) and the formation of unproductive Tb-Tb liposomes would diminish available Tb^{3+}. These results also demonstrate that multiple fusion events were not important in our experimental conditions; indeed, multiple fusion would have produced vesicles containing both Tb^{3+} and DPA, eventually decreasing the probability of obtaining vesicles containing Tb^{3+} or DPA alone (Wilschut et al., 1981), and this would have eliminated any effects due to the ratio.

Fusion of Microsomes with Liposomes

In previous works (Corazzi & Arienti, 1986, 1987) we demonstrated the possibility of disrupting rat brain microsomes with octylglucoside and of reforming vesicles through the dialytic removal of the detergent. Reconstituted vesicles were characterized elsewhere (Corazzi & Arienti, 1986) in terms of size, lipid composition and sidedness and could

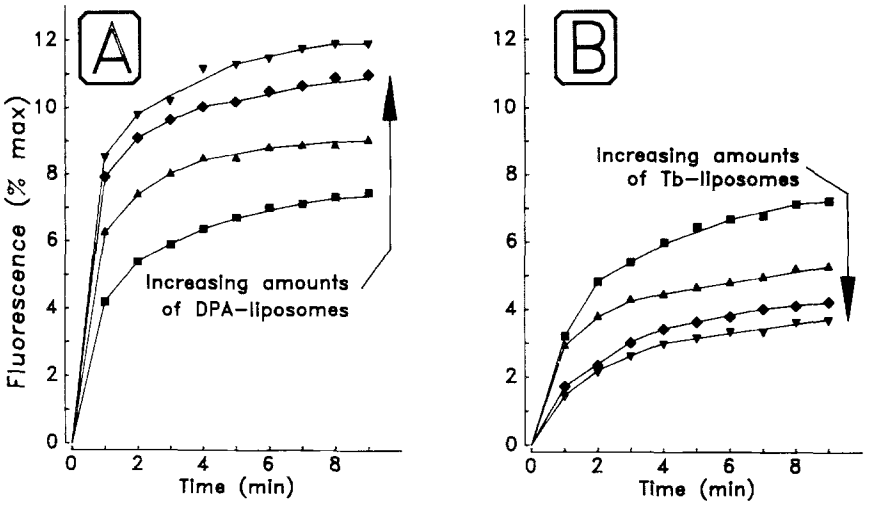

Fig. 2. The effect of Ca^{2+} and of DPA-liposome Tb-liposome ratio on liposome fusion as tested by Tb-DPA. Data are expressed as percent of maximal fluorescence (obtained in the presence of 0.03% thesit and in the absence of Ca^{2+}) and report plateau values obtained 6 min after the addition of the ions. The ratio was increased by adding various amounts of DPA liposomes to fixed amounts (27 nmol lipid P · ml^{-1}) of Tb-liposomes in the presence of 25 mM Ca^{2+}. DPA liposomes added: ■–■–: 37 nmol lipid P · ml^{-1}; ▲–▲–: 56 nmol lipid P · ml^{-1}; ◆–◆–: 75 nmol lipid P · ml^{-1}; ▼–▼–: 91 nmol lipid P · ml^{-1}. (*B*) The ratio was decreased by adding various amounts of Tb-liposome to fixed amounts of DPA-liposome (37 nmol lipid P · ml^{-1}) in the presence of 25 mM Ca^{2+}. Tb-liposomes added: ■–■–: 27 nmol lipid P · ml^{-1}; ▲–▲–: 41 nmol lipid P · ml^{-1}; ◆–◆–: 55 nmol lipid P · ml^{-1}; ▼–▼–: 68 nmol lipid P · ml^{-1}.

aggregate similarly to native microsomes in the presence of divalent cations (results *not shown*). Moreover, the addition of DPA or of Tb^{3+} to microsome-octylglucoside mixtures produced, after detergent removal, DPA-loaded or Tb-loaded vesicles, and this made possible the use of the Tb-DPA method for testing microsome fusion.

The fusion of microsomes showed properties similar to those already described for liposome-liposome fusion, although with some differences, the most striking one being the impossibility of demonstrating any microsome-microsome fusion with either R_{18} or Tb-DPA. For this reason the reported results refer only to liposome-microsome fusion.

When Tb-liposomes were mixed with DPA-microsomes (or vice-versa) in the absence of divalent cations no fluorescent peaks were observed. This result indicated that encapsulation was good. On the other hand, the presence of 0.03% thesit produced a fluorescent peak as a consequence of membrane solubilization and the addition of free DPA did not further increase the fluorescence, indicating that the amount of DPA entrapped into microsomes was enough to reveal all possible Tb^{3+} fluorescence. The preparation of R_{18}-loaded membranes was also stable until the addition of divalent cations.

The addition of cations to mixtures containing liposomes and microsomes produced an increase of fluorescence with both assays. The threshold cation concentration (either Ca^{2+} or Mg^{2+}) measured with R_{18} method was in the range of 0.5–1.5 mM, whereas, the Ca^{2+} threshold concentration determined by Tb-DPA was about 2.5 mM.

The variation of DPA-microsomes/Tb-liposomes ratio influenced fluorescence. Indeed, the percent of maximal fluorescence increased, increasing the ratio, and decreased in the opposite case (results *not shown*), indicating that also for liposome-microsome fusion, multiple fusion events were not important. The effect of Mg^{2+} on the liposome-microsome interaction as measured with the Tb-DPA method was similar to the one observed for liposome-liposome interaction; indeed, Mg^{2+} produced a peak of fluorescence whose intensity was quenched by the addition of EDTA (Table 2). The amount of fluorescence obtained with Ca^{2+} was much lower than that obtained with Mg^{2+}, indicating that the latter ion produced leakage of the probes from vesicles or a leaky fusion. On the other hand, the addition of both ions produced similar extents of fusion, if tested with the R_{18} method.

Discussion

Fusion of Liposomes

Although liposomes used in this work are prepared with lipids extracted from rat brain microsomes, they can trap Tb^{3+} or DPA similarly to phosphati-

Table 2. Effect of divalent cations on microsome-liposome fusion

Divalent cation, mM	Tb-DPA method		R_{18} method	
	A	B	C	D
0	0.0	0.0	0.0	0.0
2.5	—	—	4.5	3.5
5	7.0	1.1	9.7	9.1
7.5	—	—	13.0	11.5
10	22.3	3.0	13.9	12.0
15	30.7	4.5	14.6	12.2
20	35.2	5.5	15.3	—

Tb-DPA method: Divalent cations Mg^{2+} (A) or Ca^{2+} (B) were added to a mixture containing Tb-liposomes (25 nmol lipid P · ml^{-1}) and DPA—microsomes (0.06 mg protein · ml^{-1}). Data are expressed as percent of maximal fluorescence (obtained in the presence of 0.03% thesit and in the absence of Ca^{2+}) and refer to plateau fluorescence values which were reached 8 min after the addition of the ions.

The addition of EDTA (0.1 mM) to A before or after the development of fluorescence, quenched the fluorescence completely. The addition of EDTA (0.1 mM) to B had no effect. Reported values are the average of three determinations. SE (omitted for clarity's sake) did not exceed 10%.

R_{18} method: Divalent cations, Mg^{2+} (C) or Ca^{2+} (D), were added to a mixture containing R_{18}-loaded microsomes (0.06 mg protein · ml^{-1}) and unloaded liposomes (25 nmol lipid P · ml^{-1}). Data are expressed as percent of maximal fluorescence (obtained in the presence of 0.03% thesit) and refer to plateau fluorescence values which were reached 4 min after the addition of the ions. Reported values are the average of three determinations. SE (omitted for clarity's sake) did not exceed 10%.

dylserine liposomes (Wilschut et al., 1980) and can be used for studies on fusion with the Tb-DPA method. However, their composition may explain why the threshold Ca^{2+} concentration and other properties are different from those reported for liposomes made with acidic phospholipid (Bentz, Düzgunës & Nir, 1983); these are indeed responsible for the binding with Ca^{2+} (Lansman & Haynes, 1975) but represent not more than 10% of total membrane lipid. The threshold cation concentration is different depending on the method used to test fusion; indeed, R_{18} gives values some 10 times as high as those obtained with Tb-DPA. We can exclude diffusion of the probe from one population of vesicles to another since no increase of fluorescence can be observed without the addition of cations.

The fluorescence of the DPA-Tb complex is quenched completely by 5 mM Ca^{2+}. For this reason the Ca^{2+}-induced fluorescence is a measure of the mixing of internal aqueous content of the vesicles, excluding any contributions due to the leakage of probes, either spontaneous or fusion induced. Indeed, the Tb-DPA assay might underestimate the extent of fusion because any loss of probes to the external medium during fusion and/or the penetration of Ca^{2+} into vesicles are not taken into account.

Mg^{2+} is a divalent cation whose ability to induce fusion is still debated. For example, it does not make large unilamellar vesicle phosphatidylserine liposomes fuse, the contrary being true for small unilamellar vesicle phosphatidylserine liposomes (Wilschut et al., 1981). Therefore, the size and the composition of liposome preparations is important in this connection. In our hands (Table 1), Mg^{2+} develops fluorescence very well (40% of maximal fluorescence is obtained with 20 mM Mg^{2+}); however, the addition of 0.1 mM EDTA before or after Mg^{2+} suppresses Mg^{2+}-dependent fluorescence. Therefore, the Mg^{2+}-dependent fluorescence is due to the formation of the complex outside the vesicles. On the contrary, R_{18} shows a similar rate of mixing of vesicle lipid adding either Mg^{2+} or Ca^{2+}. If the mixing of the lipid phases can be assumed to be fusion, it should be admitted that Mg^{2+} makes liposomal vesicles fuse as well as Ca^{2+} does. Therefore, the finding obtained with microsomal lipid would agree with those reported by Wilschut et al. (1981) who used small unilamellar vesicles of phosphatidylserine.

The idea that Mg^{2+} induces fusion is also supported by the finding that Mg^{2+} adds to Ca^{2+} to cause an increase of fluorescence (Table 1, Tb-DPA). Indeed, the effect reverses only at concentrations of Mg^{2+} exceeding 15 mM, indicating that the ion may produce an important leakage of liposomal content.

Fusion of Microsomes

The study of the interaction of biological membranes with liposomes through the Tb-DPA method is hindered by the difficulty of encapsulating the probes inside natural vesicles. However, the solubilization of microsomes with octylglucoside followed by the dialytic removal of the detergent produces vesicles which can trap either DPA or Tb^{3+}.

The fusion of Tb-liposomes and DPA-microsomes is demonstrated in this work. Most properties of microsome-liposome fusion are similar to those already described for liposome-liposome fusion, such as the curve *vs.* Ca^{2+} concentration, the absence of multiple fusion events and the effects of cations. All these facts suggest that lipid is very much important for fusion to occur.

The size of reconsituted microsomes was similar to that of native microsomes, as examined by electron microscopy (Corazzi & Arienti, 1987) and therefore, microsomal diameter is about 10 times as large as liposomal diameter (Cullis et al., 1985). Since the size of the particles has been demonstrated to be important to determine fusion extent

(Wilschut et al., 1981), this could explain why microsomes do not fuse among themselves.

Conclusions

Tb-DPA and R_{18} assays give different results when used to test the fusion of either liposomes or microsomes. Indeed, Mg^{2+} and Ca^{2+} behave almost identically if fusion is tested with the R_{18} method, but they do not if fusion is tested with Tb-DPA. It should be kept in mind that, using Tb-DPA, Mg^{2+} reveals the mixing of the probes either inside or outside the vesicles, whereas Ca^{2+} reveals only the inside complex, since this ion is a strong inhibitor of fluorescence development. Moreover, if the fusion process permits some entering of Ca^{2+} inside the vesicles, this would decrease the apparent extent of fusion. All these considerations indicate that Tb-DPA may underestimate fusion. The chemical characteristics of the Tb-DPA method imply the retention of vesicle content, and this process can be demonstrated only for the Ca^{2+}-induced fusion, because the fluorescence induced by Mg^{2+} is suppressed by EDTA.

On the other hand, the R_{18} method does not show differences between the two ions. The two assays may therefore reveal different types of interaction, one consisting in the complete mixing of vesicle material with the retention of vesicle content and the other in a mixing of lipid phases accompanied by a leakage of the material inside the vesicles.

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