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Ca^{2+}-induced phosphatidylcholine vesicle aggregation in the presence of ferricyanide

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The titration of sonicated vesicles of egg phosphatidylcholine with ferricyanide in the presence of Ca^{2+} results in the formation of aggregates. The turbidity increase caused by these aggregates cannot be reversed by EDTA treatment. In addition, no rearrangement of the bilayer structure has been found in this process, either measuring leakage of vesicle content or exchange of lipids among the bilayers themselves. The aggregation is dependent on the Ca^{2+} content of the vesicles, the outer Ca^{2+} and $Fe(CN)_6^{3-}$ concentration and the order of addition of Ca^{2+} and ferricyanide. The results can be explained by a specific adsorption of $Fe(CN)_6^{3-}$ to bilayers of sonicated vesicles, in contrast to other multivalent anions. In contrast to the stability found with sonicated vesicles, the aggregation causes a leakage of the internal solution when multilamellar liposomes are titrated with $Fe(CN)_6^{3-}$.

Introduction

The interaction of ions with lipid interfaces has received considerable attention due to its importance in physiological processes. In particular, among divalent cations, Ca^{2+} has been investigated thoroughly as a consequence of its unique properties of adsorption on neutral and acid phospholipid bilayers [1–6].

Ca^{2+} promotes aggregation and fusion of phosphatidic acid and phosphatidylserine bilayers, a fact that has been related with the properties of Ca^{2+} to trigger cell fusion, secretory and permeation processes in physiological systems [7–11].

The presence of negative charges, as phosphate groups, at the lipid interface promotes the chelation of Ca^{2+} ions inducing structural rearrangements of the phospholipid and water molecules, changes in membrane stability and permeability and surface potentials [12–15].

The interaction of Ca^{2+} with neutral phospholipid membranes, such as phosphatidylcholines, is weak and it does not promote membrane aggregation or fusion [7,10,13,15].

On the other hand, anions such as ClO_4^-, I^-, SCN^-, Br^-, NO_3^-, Cl^- and SO_4^{2-} bind in that order to phosphatidylcholine liposomes inducing a pronounced zeta potential [16]. This anion adsorption exhibits a complex behavior in relation to Ca^{2+} binding. However, none of them promotes aggregation or fusion of neutral phospholipid membranes in the presence of Ca^{2+} [16,17].

In contrast, multivalent anions such as molybdate, ferricyanide and hexaciano cobaltate have been shown to form strong complexes with phosphatidylcholines and Ca^{2+} [18]. This effect has been used satisfactorily to fix lipid bilayers of

Abbreviations: ANS, 1-anilinonaphthalene-2-sulfonic acid; PC, phosphatidylcholine.

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saturated and nonsaturated lipids for electron microscopic studies [19].

In this regard, a tricomplex theory has been formulated in which the interaction of the colloidal zwitterion, such as PC, Ca^{2+} and the anion play a decisive role in the flocculation process [18].

The possibility of adsorbing anions on neutral phospholipid bilayers opens a way to confer negative net charges to a lipid membrane different from the usual method of including acidic phospholipids in the bilayer. In this way, the effect of Ca^{2+} on the aggregation of PC bilayers with negative charges on it can be studied independently on the effect induced by the fatty acyl chains of the phosphatidic acids. This might result in a better understanding of the role of the charge groups in the process of aggregation and fusion.

As a first stage in this direction, we have studied the effect of ferricyanide on the aggregation of multilamellar and sonicated vesicles of phosphatidylcholines induced by Ca^{2+}. The aggregation appears related with the specific adsorption of ferricyanide on the PC bilayer and it is dependent on the Ca^{2+} concentration both inside and outside the vesicle. The reversibility, the integrity of the lipid particles and the mixing of lipid molecules in aggregation process was studied in vesicles and liposomes of egg phosphatidylcholine by means of spectrophotometric and fluorometric methodologies.

Materials and Methods

Egg yolk phosphatidylcholine was obtained and purified by standard methods [23]. Purity was checked by chromatography using chloroform/methanol/amonia/water (90 : 54 : 5.5 : 5.5, v/v).

All chemicals were reagent grade from Mallinckrodt (AR). Water was twice-distilled (Millipore systems).

The solutions of the desired solutes were prepared in a 10 mM Tris-HCl (pH 7.4); 5(6)-carboxyfluorescein was from Kodak-Eastman and purified according to Ralston et al. [22]. Octadecylrhodamine and ANS were obtained from Molecular Probes Inc.

Multilamellar liposomes were prepared dispersing a dry film in aqueous solution. Small unilamellar vesicles were obtained by sonication of these coarse lipid dispersions.

Sonication was carried out in a bath sonicator at controlled temperature (15 °C) under N_2 for 30 min. After this time the solution was transparent. Control experiments in order to check the homogeneity of the sample were performed running unsonicated liposomes and sonicated vesicles loaded with carboxyfluorescein in a Sepharose 2B column. It was observed that after the sonication procedure described above a negligible fractions of coarse liposomes was present.

Peroxidation index before and after sonication was checked by the absorption ratio 210 : 234 nm.

Turbidity assays were performed measuring the absorbance difference $(A - A_0)$ at 450 nm with a double-beam Hitachi spectrophotometer. A is the sample absorbance obtained after the addition of an aliquot of ferricyanide solution to the vesicle suspension. A_0 corresponds to the absorbance of the control sample without vesicle when the same amount of ferricyanide was added. The general procedure was as follows.

An aliquot of 0.2 ml of PC vesicles (4 mg/ml in all cases except in Fig. 1) prepared in 50 mM $CaCl_2$ or 100 mM NaCl was dispersed in about 2 ml of the desired suspending solution. Titrations with a 20 mM $Fe(CN)_6K_3$ were performed. Final turbidity values were obtained after stirring the solution and letting it equilibrate in the cuvette thermostatically controlled holder. All the assays were performed at 25 ± 0.1 °C.

For the kinetic assay of Fig. 7, the values were taken immediately after the stirring. No influence on the stirring procedure was apparent.

The assays with carboxyfluorescein were done using vesicles loaded with 50 mM 5-carboxyfluorescein solution. At concentrations higher than 10 mM, carboxyfluorescein is self-quenched and liberation or dilution would give place to a net increase in fluorescence [22]. Vesicles were prepared by sonicating a coarse liposome dispersion loaded with the carboxyfluorescein solution. Outer carboxyfluorescein was eliminated by passing an aliquot of the vesicle dispersion through a Sephadex G-50 column [22]. Fluorescence measurements were done with a M2000 Perkin-Elmer spectrofluorometer at an excitation wavelength of 472 nm and emission of 520 nm.

Two types of assay were done with vesicles

loaded with carboxyfluorescein. In one of them the 100% population consisted of vesicles containing carboxyfluorescein. Thus, if an increase in fluorescence was observed upon titration with ferricyanide, it was an indication that vesicles were disrupted or leak during the aggregation.

In the second assay of Fig. 3, only a 10% of the vesicle population was loaded with carboxyfluorescein. These assays were performed after checking that, in the experiment in which all the vesicles were loaded, no increase in fluorescence was observed. Thus, if a fluorescence increase was found in the second assay it could be attributed to the dilution of carboxyfluorescein when passing from loaded to unloaded vesicles.

The assays with octadecylrhodamine were performed mixing an aliquot of vesicles containing octadecylrhodamine at two different ratios, 8% and 10% mol/mol, with vesicles without octadecylrhodamine. At these ratios, octadecylrhodamine is self-quenched and mixing of lipids of the two vesicle populations would lead to an increase in fluorescence [24].

The fluorescence of octadecylrhodamine was measured in the above-mentioned instrument at 500 nm for excitation and 570 nm for emission.

Vesicles containing octadecylrhodamine were prepared following the method described by Hoekstra et al. [24].

The binding of ANS to Na^+ or Ca^{2+} containing vesicles in the presence of ferricyanide or ferrocyanide was studied by titrating vesicle dispersions with a 20 mM ferro- or ferricyanide solutions in the presence of $2.5 \cdot 10^{-5}$ M ANS. The fluorescence was measured at 372 nm (excitation wavelength) and at 470 nm (emission wavelength).

Results

The changes in turbidity obtained by titrating different lipid concentrations with a ferricyanide solution are shown in Fig. 1. The turbidity increases are dependent not only on the lipid concentration but also on the type of particle they constitute. Curves obtained with multilamellar liposomes (Fig. 1A) are markedly different from those obtained with unilamellar sonicated vesicles (Fig. 1B).

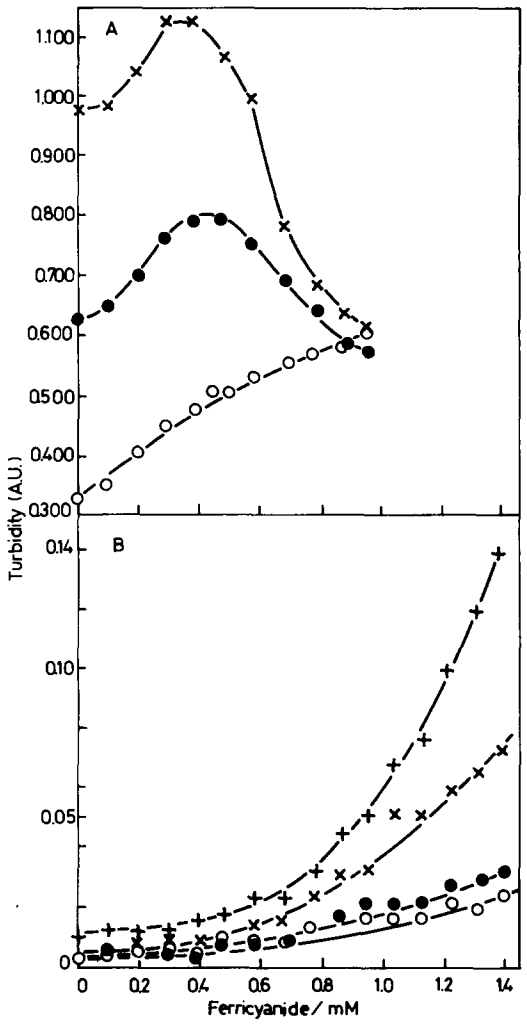

Fig. 1. Effect of the addition of ferricyanide on the turbidity of lipid dispersions. (A) Multilamellar liposomes: ○, 0.1 mg/ml; ●, 0.2 mg/ml; ×, 0.4 mg/ml. (B) Sonicated vesicles: ○, 0.05 mg/ml; ●, 0.1 mg/ml; ×, 0.25 mg/ml; +, 0.5 mg/ml. In all cases the Ca^{2+} concentration inside and outside the lipid particles was 0.1 N.

In Fig. 2 it can be observed that the ferricyanide effect is strictly dependent on the presence of Ca^{2+} in the outer solution (Table I). In addition, in the same figure, a steeper increase in turbidity at lower ferricyanide concentration is found for Ca^{2+}-containing vesicles.

The phenomenon of flocculation of lipid vesicles is completely irreversible at different stages, as shown by the dilution of the floccules with EDTA.

No reversal of the turbidity was obtained after

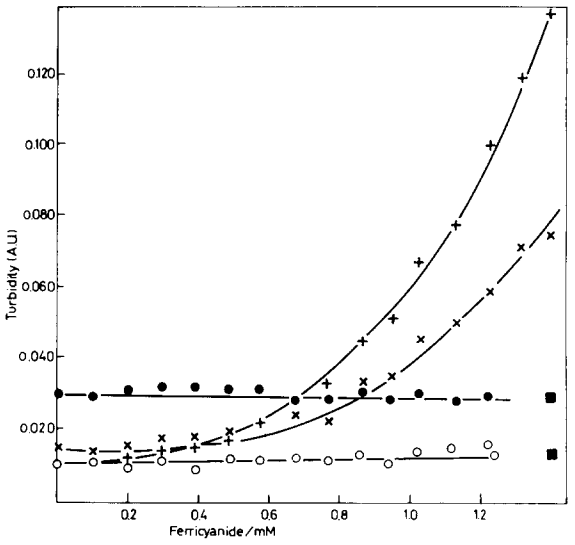

Fig. 2. Effect of ferricyanide on vesicles containing Ca^{2+} or Na^{+} solutions. ○, vesicles containing 0.1 N Na^{+} dispersed in 0.1 N NaCl; ●, vesicles containing 0.1 N Ca^{2+} dispersed in 0.1 N NaCl; ×, vesicles containing 0.1 N Na^{+} dispersed in 0.1 N $CaCl_2$; + vesicles containing 0.1 N Ca^{2+} dispersed in 0.1 N $CaCl_2$; ■, Turbidity obtained after the addition of Triton X-100 to the aggregates.

the addition of 0.1 N EDTA to aggregates formed at either 0.5 or 1.5 mM ferricyanide concentrations.

Note that, according to Fig. 2, with 0.5 mM ferricyanide, aggregation is just starting, while at 1.5 mM its extent has been significantly increased. Control experiments were done diluting the aggregates at those stages with 0.1 N $CaCl_2$. In these cases, results similar to those obtained with EDTA were found.

In appearance, the aggregates formed by adding ferricyanide to a vesicle dispersion look like a coarse liposomal dispersion. However, when starting with multilamellar liposomes, further addition of ferricyanide (Fig. 1A) promotes a turbidity decrease. This effect is not found when an excess of ferricyanide is added to vesicle dispersions. This fact indicates that the phenomenon is not due to the fraction of lipids initially available for the interaction with $Ca^{2+}/Fe(CN)_6^{3-}$.

The addition of Triton completely disrupts these floccules, which is an indication that the particles are not a salt precipitate (see Fig. 2).

Fig. 3A and Table II, we present results showing that the process of aggregation does not involve vesicle disruption. Meanwhile, a significant leakage was observed when $Fe(CN)_6^{3-}$ was added to a multilamellar liposome dispersion (Fig. 3B). The concentration at which liberation of carboxyfluorescein appears is coincident with that found in Fig. 1, in which turbidity begins to increase. The maximum leakage is obtained at a ferricyanide concentration at which turbidity reaches a maximum. The leakage of the vesicle content during the aggregation was found to be negligible in the light of experiments with carboxyfluorescein of Fig. 3A. In the same figure it is also shown that no significant fluorescence increase was found when aggregation was produced in a vesicle sample in which only the 10% of the population was loaded with carboxyfluorescein.

These two types of assay indicate that during the process of aggregation no leakage of the vesicle content occurs. In the assays in which all the population of vesicles contained carboxyfluorescein, it would be expected that an increase in fluorescence would appear if vesicles disrupted by lysis rearrange in another type of structure. The other possibility was that aggregation would result in an exchange of material between the vesicles' inner aqueous content without leakage to the suspending solution.

The results obtained with a 10% vesicle population loaded with the fluorophore indicate that this is not the case in the present conditions. Moreover, the results with octadecylrhodamine shown in Table II confirm that there is no phospholipid exchange among vesicles during the aggregation. The integrity of the particles, however, is not

TABLE I

EFFECT OF EXTERNAL Ca^{2+} ON THE CRITICAL FERRICYANIDE CONCENTRATION FOR THE AGGREGATION OF Ca^{2+}-CONTAINING VESICLES

The critical ferricyanide concentration was taken from the intersection of the two straight lines in the absorbance vs. ferricyanide plot shown in Fig. 2.

External Ca^{2+} concentration (N)	Critical ferricyanide concentration (mM)
0.0	0.60
0.01	0.54
0.05	0.52
0.10	0.46

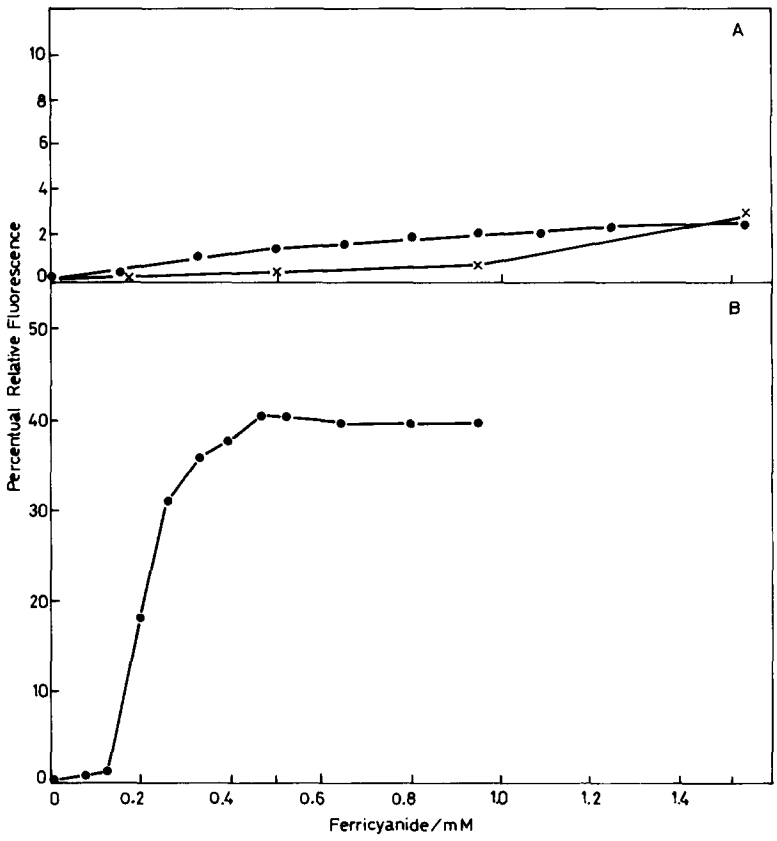

Fig. 3. Effect of ferricyanide on the leakage of sonicated vesicles and liposomes. (A) Fluorescence changes obtained during the process of aggregation of vesicles containing 5-carboxyfluorescein. ●, The whole population of vesicles was loaded with carboxyfluorescein at a self-quenching concentration. ×, Only 10% of the vesicle population contained 50 mM (For details see Materials and Methods.) (b) Fluorescence increase obtained by the titration of multilamellar liposomes with ferricyanide in the presence of Ca^{2+}. The percentual fluorescence was calculated by the relation $((F - F_0)/(F_\infty - F_0)) \cdot 100$, where F is the fluorescence obtained at each ferricyanide concentration, F_0, the fluorescence before adding ferricyanide and F_∞, the fluorescence obtained after 10 μl of a 10% (w/w) Triton X-100 solution.

maintained when multilamellar liposomes are titrated, according to the results in Fig. 1A and 4B.

However, the changes in turbidity are also a function of the concentration of Ca^{2+} in which the vesicles were prepared. In these cases, vesicles prepared in increasing Ca^{2+} concentration, indicated in the abscissa of Fig. 4, were dialyzed against Ca^{2+} which was maintained constant during the titration with ferricyanide. The data of Fig. 4 show the turbidity increase obtained for a given ferricyanide concentration as a function of the Ca^{2+} vesicle content.

As vesicles do not leak during aggregation, as shown in Fig. 3A, the enhancement of aggregation can not be ascribed to the propagation of the effect due to the entrance of ferricyanide or the exit of inner Ca^{2+}. The possibility that Ca^{2+} containing vesicles may have structural differences in comparison to those with Na^+ was next investigated. In other words, it might be expected that vesicles prepared in different Ca^{2+}/Na^+ ratios would differ in its surface properties, as far as ferricyanide adsorption concerns.

As shown in Fig. 5 a slight difference in adsorption of ferricyanide on Na^+-containing or Ca^{2+}-containing vesicles is observed by means of a decrease of ANS fluorescence. In the same fig-

TABLE II

EFFECT OF FERRICYANIDE ON THE TRANSFERENCE OF LIPID MOLECULES BETWEEN AGGREGATED VESICLES AS MEASURED BY OCTADECYLRHODAMINE

Vesicles of column 1 contained on 8% and those of column 2 a 10% (mol/mol) octadecyl rhodamine/phosphatidylcholine ratio. In both cases, an aliquot of vesicles containing octadecylrhodamine was dispersed in a vesicle suspension of the same nature without the fluorophore. Final ratio of loaded to unloaded vesicles was 1:4. Results are expressed as the percentage of quenched rhodamine remaining after the treatment with ferricyanide, 100% dequenching was measured by the disruption of vesicles with Triton X-100 (for details see Materials and Methods).

Ferricyanide (mM)	Percentage of octadecylrhodamine quenching	
	1	2
0	73.8	86.9
0.4	77.5	85.4
0.8	76.3	82.8
1.2	74.2	87.0

ure, the competence of ferrocyanide (an anion which does not promote aggregation) with ANS is several times lower that that found for ferricyanide. This indicates that the ferricyanide effect on aggregation is related to a specific adsorption rather than to an electrostatic interaction.

In order to gain insight into the sequence of the adsorption of Ca^{2+} or ferricyanide triggering the aggregation, the titrations of Fig. 6 were performed.

In the presence of an excess of ferricyanide, the critical Ca^{2+} concentration for Na^{+}-vesicles aggregation amounts 12 mN, which is very close to that found for Ca^{2+} vesicles (Figs. 6A and C). In an excess of Ca^{2+} (100 mN) ferricyanide concentration is in the order of 0.52 mM for Na^{+}-vesicles and 0.47 mM for Ca^{2+}-vesicles.

Finally, in Fig. 7, the kinetic assays show that vesicles dispersed in 1.5 mM ferricyanide aggregates rapidly when Ca^{2+} is added in order to achieve a 10 mN concentration. However, when the same type of vesicle is dispersed in 10 mN Ca^{2+} and ferricyanide is added to achieve a 1.5 mM concentration, no effect is found. Similar results were obtained with Na^{+}-vesicles, although the course of the turbidity increase differs.

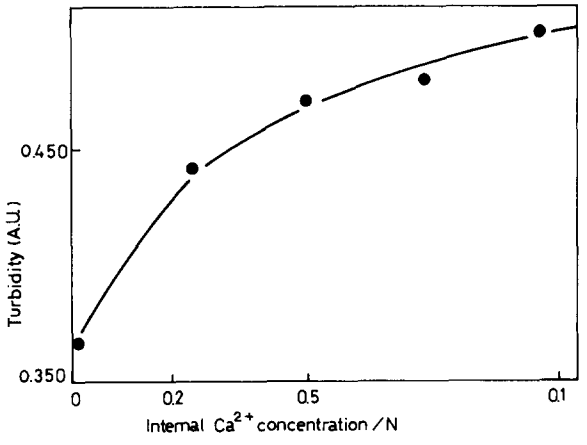

Fig. 4. Dependence of the extent of the aggregation induced by 1.5 mM ferricyanide as a function of the Ca^{2+} concentration inside the vesicle. The turbidity values correspond to the increase in absorbance obtained when equal aliquots of vesicles with different Ca^{2+} content are dispersed in a given concentration of ferricyanide.

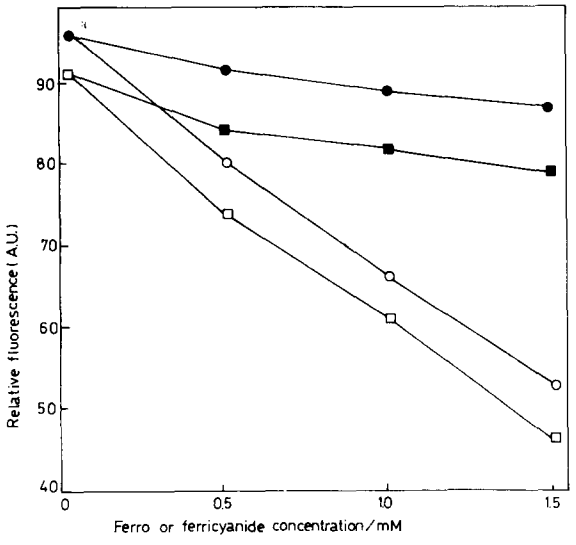

Fig. 5. Adsorption of ferri- or ferrocyanide as measured by the fluorescence decrease of 1-anilinonaphthalenesulfonic acid. Titration of Na^{+}-vesicles (■, □) or Ca^{2+}-vesicles (○, ●) with ferrocyanide (full symbols) or ferricyanide (open symbols), was done in the absence of Ca^{2+} in the external solution to avoid turbidity interferences. In all cases, vesicles prepared in Na^{+} or Ca^{2+} solution were dialyzed against 0.1 NaCl until no Ca^{2+} was present as tested with the Ca^{2+} murexide complex formation.

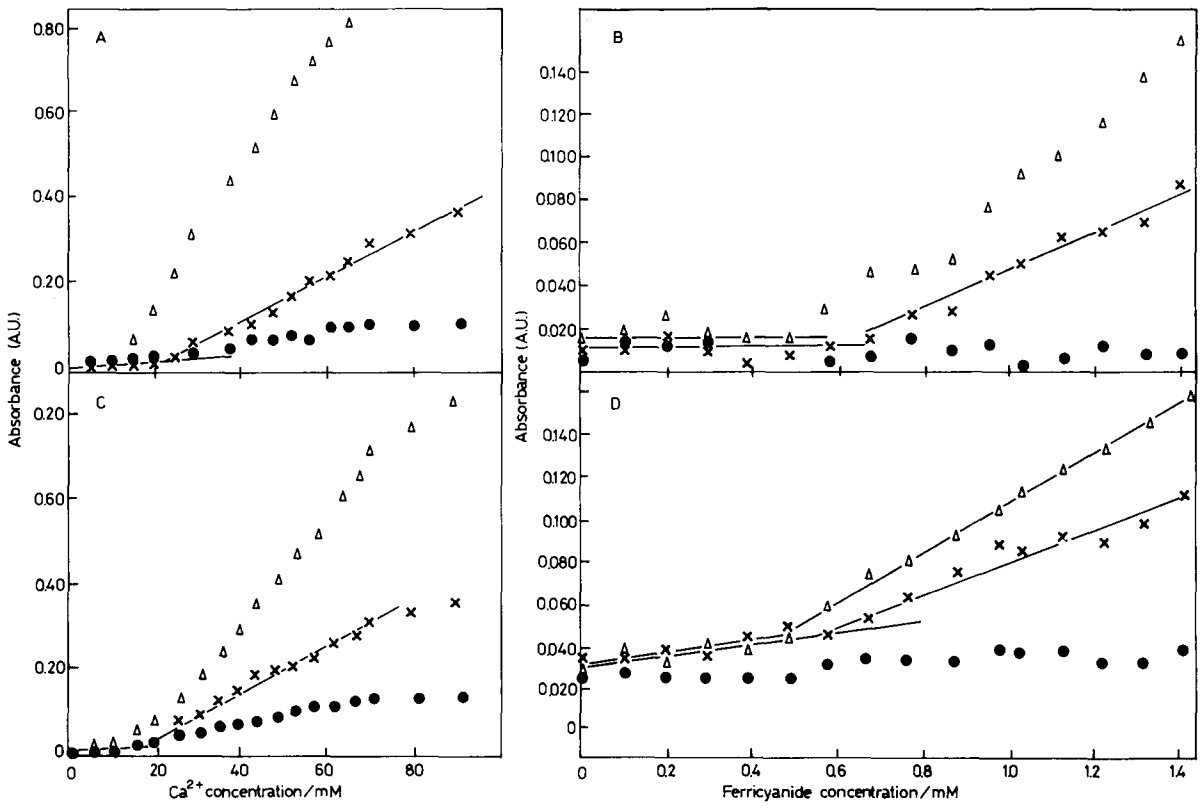

Fig. 6. Effect of the sequence of Ca^{2+} and ferricyanide addition on the Ca^{2+} or Na^{+} vesicle aggregation. (A) Titration of Na^{+}-vesicles with Ca^{2+} in the presence of $Fe(CN)_6^{3-}$ at 0.5 mM (●), 1.0 mM (×) and 1.5 mM (△). (B) Titration of Na^{+}-vesicles with ferricyanide in the presence of Ca^{2+} at 10 (●), 50 (×) and 100 mN (△). (C) Titration of Ca^{2+}-vesicles with Ca^{2+} in the presence of $Fe(CN)_6^{3-}$ at 0.5 (●), 1.0 (×) and 1.5 mM (△). (D) Titration of Ca^{2+}-vesicles with ferricyanide in the presence of Ca^{2+} 10 (●); 50 (×) and 100 mN (△).

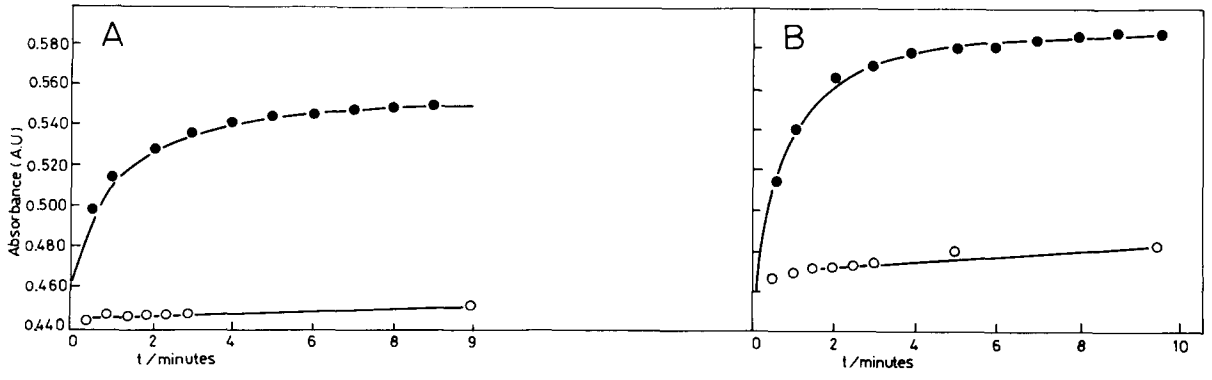

Fig. 7. Time-dependence of the Ca^{2+}- (A) and Na^{+}- (B) containing vesicles. ○, Aggregation of vesicles dispersed in 10 mN Ca^{2+} triggered by the addition of $Fe(CN)_6^{3-}$ (final ferricyanide concentration, 1.5 mM). ●, Aggregation of vesicles dispersed in 1.5 mM ferricyanide triggered by the addition of Ca^{2+} (final concentration 10 mN).

Discussion

There are several features of the action of ferricyanide on lipid bilayers which distinguish it from that occurring with other mono- and multivalent anions.

First, it induces the aggregation of neutral phospholipid vesicles in the presence of Ca^{2+}. Second, it adsorbs more strongly on the lipid interface in comparison with anions of higher negative charge as ferrocyanide. In third place, regardless of the strong association, ferricyanide does not disrupt the lipid vesicle.

The monovalent anion species affect the magnitude of the electrostatic forces between bilayers. In these cases, screening would be the dominant role played. However, as pointed out elsewhere, multiatom anions such as nitrate and acetate exhibit a complex behavior which is consistent with anion binding and screening [16,17]. An indication that the effect of ferricyanide on aggregation is due not only to electrostatic interactions comes from the comparison with the action of ferrocyanide. Ferricyanide appears, according to experiments of Fig. 5, to bind to the surface of egg PC bilayers, whereas the ferrocyanide screens the surface potential and thus it is not able to promote aggregation.

A possible reason for the different effects of these anions may be the different charge density. Ferricyanide would more adequately fit in the interface by interacting with a couple of phosphatidylcholine molecules. It would bind to them using two valence charges to the choline groups of the adjacent phospholipids. This is the group affected principally by anionic adsorption [20]. In this way, ferricyanide would confer one net negative charge per two PC molecules to the originally neutral surface bilayer. Taking into account the number of phospholipid molecules exposed to the outer solution, it can be calculated that for 1.5 mM ferricyanide there are two phospholipids per $Fe(CN)_6^{3-}$. In consequence, the addition of Ca^{2+} (see Fig. 7) would trigger the aggregation, as occurs with vesicles composed of acidic phospholipids [15]. The aggregation would be facilitated by the fact that Ca^{2+} has to interact with charges in apposite bilayers to saturate its two positive charges.

Although this complex is not reversed by EDTA, it is not strong enough to cause a reorganization of the original arrangement of the lipid molecules in the bilayer. Thus, no leakage or exchange of lipid molecules between the bilayers of the aggregated vesicle can be observed (Fig. 3A and Table II).

It is interesting that ferricyanide effect can be compared in certain extent to that produced by Ca^{2+} in phosphatidic acid vesicles. Previous studies have shown that Ca^{2+} binds to phosphatidic acid containing membranes causing aggregation [15]. This effect has been interpreted as a consequence of a combination of the ability of the Ca^{2+} ion to neutralize membrane surface charges and to participate in ionic binding which bridges the two membranes.

Two distinctions must be made. Salt bridging requires the adsorption of ferricyanide on the neutral phospholipid bilayer. Then, the presence of Ca^{2+} between the two opposing membranes brings about the aggregation. This can be inferred from the plots of Figs. 6 and 7.

Anion adsorption and apposition of the two membranes are not sufficient to cause aggregation. In fact, a delicate charge balance would be necessary. This can be inferred from the results with ferrocyanide, which does not cause aggregation. There, one net charge in excess would introduce a repulsive force between adjacent bilayer surfaces which could not be counterbalanced by the presence of Ca^{2+}.

The other difference to point out with Ca^{2+} effect on acidic phospholipid membranes is that in this case aggregation is the first step for membrane fusion [15].

It seems that the aggregation reaction occurring over a time-course of seconds is the primary determinant of the fusion reaction observed for incubation times of about 1 h [7,10,11].

The possible mechanism would involve organization of the lipid molecules in structures different from that of a bilayer in at least some stages of the process [21,22]. This phenomenon would occur in bilayers which, due to their molecular constituents, tend to expand and to allow the diffusion of lipid molecules in the plane of the membrane [22].

Sonicated vesicles are tightly packed structures.

For that reason, lateral diffusion would be improbable and therefore fusion would not be obtained, at least on the time-scale in which these experiments were performed.

It may be possible that the binding of the ferricyanide to the outer surface would promote a higher tightness, in addition to that conferred by the curvature, due to the crosslinking concerted by the anions between adjacent phosphatidylcholines. Therefore, the packing constraints in the hydrocarbon chain region might be inhibiting the fusion process.

However, in planar bilayers where lipids have a significant mobility, the formation of the complex $Ca^{2+}/Fe(CN_6^{3-}$ promotes reaccommodation of the structure, as can be inferred from the turbidity and leakage assays shown in Figs. 1A and 3B. This is in accordance with the reorganization of lipid molecules in multilamellar dispersions reported elsewhere [18,19].

The differences observed between multilamellar liposomes and sonicated vesicles cannot be ascribed to the smaller fraction of the lipid available in the outer bilayer of the liposomes. This can be inferred from the fact that addition of an excess of ferricyanide to a vesicle dispersion does not produce a decrease in the absorbance, as is found for a similar ferricyanide/lipid ratio in multilamellar liposomes.

Therefore, the differences between sonicate vesicles and multilamellar liposomes could be due to their differences in size or are the result of the presence of one versus several bilayers.

However, it is well known that sonicated vesicles, due to their small radius, present a tightly packed structure in comparison to large multilamellar or unilamellar liposomes. The point is thus to compare this effect of the Ca^{2+}-$Fe(CN)_6^{3-}$ complex on planar (loose) or curved (packed) bilayers.

The observation that only 40% maximal dye release occurs from the liposomes (Fig. 3B) suggests that the disruption is mostly on the outer bilayer. This possibility may be visualized considering that the inner lamellae due to their reduced radius, would have increased curvature.

Therefore, according to the explanation given above, they would be more difficult to disrupt. In addition this may be taken as another indication that differences between liposomes and vesicles would not depend on whether the liposomes are uni- or multilamellar particles.

These results are in concordance with those reported for the crystallization of lipid particles containing phosphatidylserine [25]. The vesicle chain packing influences the acyl crystallization, which is coincident with vesicle rupture and precipitation.

In addition, the rate of crystallization has been found to be a function of the size of the vesicle. The greater disorder in the hydrocarbon acyl chain packing in the small vesicles as compared to the large ones [26] results in structural differences related to surface/volume ratio. As reported elsewhere, Ca^{2+}-containing vesicles are larger and more packed than those containing Na^+ [27]. Thus, this should also be accounted for by the different effect of ferricyanide on liposomes and vesicles and, to a lesser extent, between Na^+- and Ca^{2+}-containing vesicles. The slight difference in surface properties observed in Fig. 4 in relation to ferro- and ferricyanide adsorption on Ca^{2+}- and Na^+-vesicles may be ascribed to a positive surface potential induced by the curvature and the inner $[Ca^{2+}]$ [28].

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