

Topological properties of two cubic phases of a phospholipid:cholesterol:diacylglycerol aqueous system and their possible implications in the phospholipase C-induced liposome fusion**

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Abstract Water dispersions of phospholipid:cholesterol:diacylglycerol may, under certain conditions, originate either the lipid- and water-permeable Q^{224} cubic phase, or the lipid-permeable but water-impermeable Q^{227} cubic phase. These results are discussed within the framework of the phospholipase C-induced fusion of liposomes [Nieva et al. (1993) *Biochemistry* 32, 1054]. It is suggested that the cubic phases Q^{224} and Q^{227} represent two classes of lipid organization, one promoting, the other hindering the mixing of aqueous contents that is characteristic of membrane fusion. In this context, inverted micelles appear to be the end point of the fusion process, rather than fusion intermediates.

Key words: Cubic phase; Non-lamellar phase; Phospholipid; Diacylglycerol; Membrane fusion

1. Introduction

The generation of diacylglycerols (DG) in cell membranes is a key event in metabolic processes involving cell activation and membrane fusion [1–3]. DG is believed to act by destabilizing the lamellar organization of membranes [4]. In a X-ray scattering study of model systems, Das and Rand [5,6] demonstrated that the addition of DG to egg-PE induces the formation of phase H_{II} , and that high proportions (70 mol%) of DG in aqueous dispersions of PC lead to the formation of a cubic phase that they did not identify. This cubic phase was later observed in a monolein:fatty acid:water system (phase Q^{227}) and its space group (Fd3m) identified [7]. This phase was also described in other systems, often in the presence of DG [8,9]. After some hesitations, the structure of this phase was determined [9].

Previous biochemical studies [10] have shown that the enzymatic action of phospholipase C induces the fusion of large unilamellar liposomes containing PC, PE and CHOL and that the fusion process is modulated by DG (a product of the enzymatic degradation). More precisely [11] vesicle aggregation was

shown to occur only during the enzymatic degradation (and the accompanying generation of DG), and fusion (assayed by the mixing of the aqueous contents) was observed only beyond a threshold DG concentration in the lipid bilayer. Besides, at still higher concentrations DG has a dramatic inhibitory effect on the fusion process.

The experimental section of this paper describes the temperature-dependent phase behaviour of a lipid mixture PC:PE:CHOL containing variable amounts of DG, in excess water. Two techniques were used to identify the phases: ^{31}P NMR and X-ray scattering. Lamellar, hexagonal and cubic (Q^{224}) phases were observed with small amounts of DG; the cubic phase Q^{227} was observed at higher DG concentration. It is worthwhile to point out that phase Q^{224} has not been previously reported in the presence of DG.

The structures of those two cubic phases are remarkably different from each other [12]. Phase Q^{224} belongs to the bi-continuous type: it consists of two polar labyrinths, continuous throughout the three-dimensional space, separated from each other by an apolar septum (Fig. 1A). This structure may be described in terms of a three-dimensional sponge, highly permeable to both water- and hydrocarbon-soluble substances. In contrast, Q^{227} is micellar: it consists of two types of disjointed polar micelles embedded in a continuous hydrocarbon matrix (Fig. 1B) and it is impervious to water-soluble substances.

These remarkable properties suggest a model mechanism whereby a transition between these two cubic phases is invoked to explain the modulation of membrane permeability that follows the enzymatic degradation of the lipid moiety. According to this 'patch-the-puncture' model, first outlined in [12], the initial stage of the enzymatic attack, that generates DG (or FA and MG), has the effect of (locally) destabilizing the bilayer and making the membrane water-permeable. Subsequently, the concentration of DG (or FA) increases and the water-permeable patches transform into lumps of water-tight phase Q^{227} ; this eventually stops the mixing of aqueous contents.

The experimental results agree with the model. Moreover, the fact that small amounts of DG (like MG in previous experiments, [12]) induce the formation of phase Q^{224} suggests that the leaky patches may well be lumps of this cubic phase.

2. Experimental

Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was supplied by Boehringer-Mannheim. 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS), *p*-xylenebis (pyridinium bromide) (DPX) and octadecylrhodamine B (R_{18}) were purchased from Molecular Probes (Eugene, OR). Egg PC, egg PE, and a 1,2-diacylglycerol derived from egg PC

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**This paper is dedicated to the memory of Juan José Iturralde.

Abbreviations: CHOL, cholesterol; MG, monoacylglycerol(s); DG, diacylglycerol(s); PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; FA, fatty acid; PL, phospholipids.

were grade I from Lipid Products (South Nutfield, UK); cholesterol was from Sigma (St. Louis, MO). DG contained a trace of the 1,3-isomer. All lipids were used without further purification. The required amounts of lipids were dissolved in organic solvents, the solutions mixed, freeze-dried and then hydrated with double-distilled water. The basic lipid mixture was PC:PE:CHOL (2:1:1). When required, some of the phospholipid was replaced by DG: thus, the composition of a sample containing e.g. 10% DG was PC:PE:CHOL:DG (43:22:25:10). Both the NMR and the X-ray scattering experiments were performed on fully hydrated systems, in the presence of excess water; in these conditions the amount of water in the lipid–water phases was unknown. When required, thermal cycling of the samples was performed in a programmed water bath, originally designed for the 'polymerase chain reaction' procedure.

^{31}P NMR spectra were recorded in a KM360 Varian spectrometer, operating at 300 MHz for protons. Spectral parameters were: 45° pulses (10 μs), pulse interval 3 s, sweep width 16 kHz, full proton decoupling. 1000 FID were routinely accumulated from each sample; the spectra were plotted with a line broadening of 80 Hz. Samples were equilibrated for 10 min at each temperature before data acquisition.

The X-ray scattering experiments were performed as described previously [7] and references therein.

For phospholipase C-induced fusion of liposomes, large unilamellar vesicles (≈ 100 nm in diameter) were prepared by the extrusion method from a PC:PE:CHOL (2:1:1) mixture. Vesicle aggregation, mixing of vesicular aqueous contents and mixing of the lipid molecules induced by the enzyme were detected as in [10]. Total lipid concentration in studies of enzyme-induced fusion was 0.3 mM.

3. Results and discussion

3.1. Phase behaviour

Aqueous suspensions of the lipid mixture of PC:PE:CHOL (2:1:1 mol ratio), containing various amounts of DG (between 0 and 50%), were examined by ^{31}P NMR in the temperature range 20–80°C. Representative series of temperature- and (DG concentration)-dependent spectra are shown in Figs. 2 and 3. A pseudo-phase diagram has also been constructed, that summarizes the ^{31}P NMR phase data (Fig. 4).

Spectra were recorded at eight temperatures, between 20 and 70°C; DG concentrations were varied with 5% increments between 0 and 30%, and with 10% increments afterwards.

The spectra recorded in the absence of DG, and over the whole temperature range explored in this work, are typical of lamellar phases [13] (data not shown). In the presence of DG, hexagonal and isotropic ^{31}P NMR signals [13] are detected, in addition to the lamellar ones, in proportions that vary with temperature and DG concentration (Fig. 4). Similar temperature-induced isotropic NMR signals have been observed in other lipid systems [14,16–22]. In the wide DG concentration range over which an NMR isotropic signal is observed above 50°C (either alone or accompanied by other signals), the isotropic signal is preserved if the sample is annealed at 20°C. Moreover, when the DG content is larger than 5% repeated cycling across the transition temperature leads to the progressive reinforcement of the isotropic signal and the attenuation of all the other signals.

The same system was studied by X-ray scattering techniques. For samples devoid of DG two reflections were observed over a wide temperature range, that can be ascribed to a lamellar phase ($d = 62.3$ Å). Samples containing 5 or 10% DG display a complex behaviour that depends on temperature and concentration, and also on the thermal treatment of the sample. Following Shyamsunder et al. [14], the 10% DG sample was cycled some 600 times between 45 and 70°C and studied at different temperatures. After cycling, the sample became optically isotropic at all temperatures. Above 65°C several sharp reflections were observed, whose spacing ratios ($\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{8}:\sqrt{9}$, $a = 179$ Å) are consistent with space group Pn3m [7]. At room temperature another family of reflections sets in, suggesting the presence of another cubic phase that was not properly identified.

At higher DG content (30% or more) X-ray scattering experiments reveal a (hexagonal-to-cubic) phase transition centred at about 45°C. In samples containing 50% DG the same cubic phase is observed over a wide temperature range. This phase is characterized by a family of reflections whose spacing ratios ($\sqrt{3}:\sqrt{8}:\sqrt{11}:\sqrt{12}:\sqrt{16}:\sqrt{19}:\sqrt{24}:\sqrt{27}$, $a = 152$ Å) are typical of a cubic phase Q 227 (space group Fd3m). The formation of a cubic

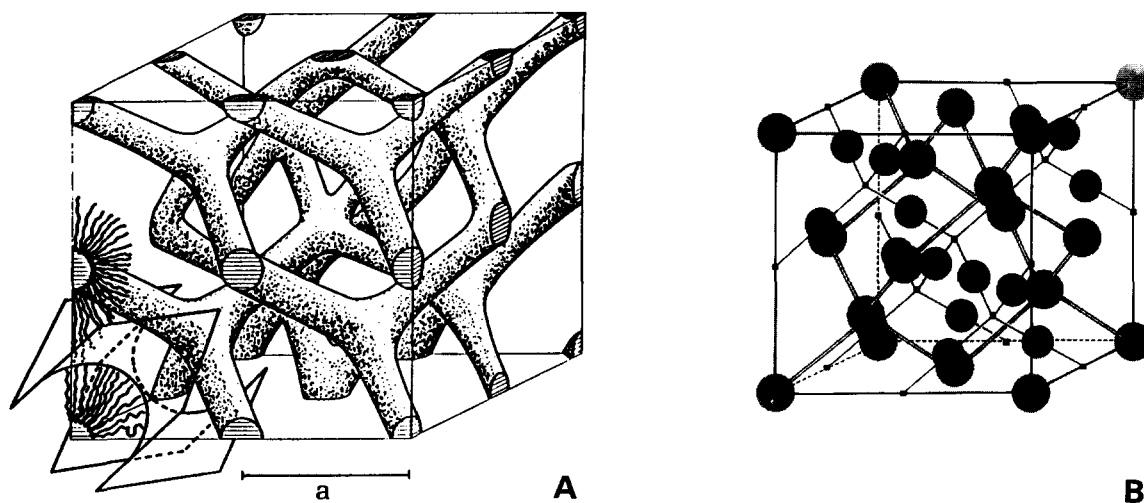


Fig. 1. Schematic representation of the structure of the two cubic phases. (A, at left) Phase Q 224 (space group Pn3m). The structure consists of two polar labyrinths, mutually intertwined and unconnected, separated by an apolar septum. Each labyrinth is represented by a 3-D network of rods, tetrahedrally joined 4 by 4. The rods are filled by water, their surface is lined by the polar headgroups of the lipid molecules; the interstices are occupied by the hydrocarbon chains. (B, at right) Phase Q 227 (space group Fd3m). The cube represents one face-centred cubic cell. Each cell contains 8 'large' (light grey) and 16 'small' micelles (heavy grey). The micelles are disjointed, filled of water and embedded in a hydrocarbon matrix; the surfaces of the micelles are lined by the polar headgroups. 'a' is the unit cell parameter.

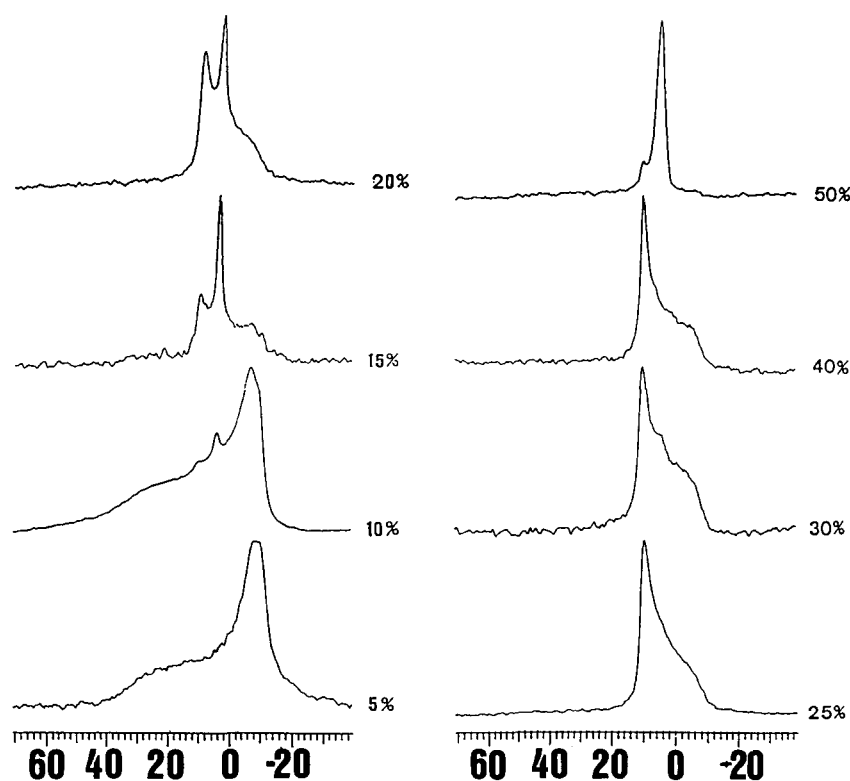


Fig. 2. ^{31}P NMR spectra of aqueous dispersions of PC:PE:CHOL (2:1:1 mole ratio) containing various proportions of diacylglycerol at 37°C . Diacylglycerol proportions (mol%) are indicated by each curve. Samples were 0.2 M in lipid. Chemical shifts (in ppm) relative to inorganic phosphate.

phase Q^{227} has been previously observed in other lipids [8,9], although at higher DG concentrations (60 to 70%).

3.2. The structure of the cubic phases

The size of the unit cell and the relative intensity of the reflections of phase Q^{224} are similar to those observed in other systems [7,15]; the structure may thus be presumed to be the same. This structure (see Fig. 1 and section 1) belongs to the bicontinuous class, and it may be visualized as a sponge-like object highly permeable to both polar and apolar substances.

The size of the unit cell and the relative intensity of the reflections that we ascribe to phase Q^{227} are very similar to those reported for other examples of the same phase (see Table 1 in [9]). It may thus be presumed that the structure, like that of the other examples of phase Q^{227} [9] is micellar of type II (water-in-oil) and impervious to water and to water-soluble substances (see Fig. 1 and section 1).

3.3. Phospholipase C-induced liposomal fusion

When fusion is assayed (in dilute vesicle suspensions) by the mixing of the liposomal water compartments, an optimum enzyme-to-vesicle ratio is found [10]. When, moreover, fusion is studied as a function of DG content an optimum is found over the concentration range 5 to 20 mol% [11]. In order to correlate these observations with the nature and structure of the cubic phases observed in the concentrated lipid dispersion, the effect of enzyme concentration was studied in more detail, looking separately at DG generation, vesicle aggregation, and the mixing of the aqueous and lipid media. The results are shown in Fig. 5. As soon as some enzyme is added, the rate of vesicle aggregation increases steeply and stays near its maximum value

irrespective of further increases in enzyme concentration (Fig. 5A, open triangles). At low enzyme concentration, the mixing of both the aqueous and the lipid media is enzyme-limited (Fig. 5B); beyond the optimum enzyme-to-lipid ratio, lipid mixing decreases (by about 30%) and then proceeds at a measurable rate irrespective of enzyme concentration. Aqueous contents mixing, in contrast, is virtually nil when the enzyme concentration exceeds approximately 4 times the optimum value. Therefore, enzyme concentrations sufficiently high to inhibit the mixing of the water compartments have hardly any effect on the mixing of the lipids. This observation suggests that under optimal fusion conditions the lipid matrix becomes water-permeable, much like the bicontinuous structure of phase Q^{224} . At higher enzyme concentrations the DG content increases and the lipid matrix is again impervious to water, in keeping with the micellar structure of phase Q^{227} . As expected from previous work [10,11] the data of Fig. 5 show that optimum fusion corresponds to a DG concentration in the range 10–20% and that increasing the DG concentration leads to an inhibition of the mixing of the aqueous compartments.

In conclusion, the physico-chemical data summarized in the pseudo-phase diagram (Fig. 4), the structure of the cubic phases and the results of the fusion experiments performed on the dilute vesicular system all display remarkable correlations.

4. Hypothesis

Two aspects of the vesicle fusion events in our system should be stressed [23]: (i) enzymatic hydrolysis and the accompanying mixing of the lipid compartments take place at all DG concentrations; (ii) mixing of the aqueous compartments takes place

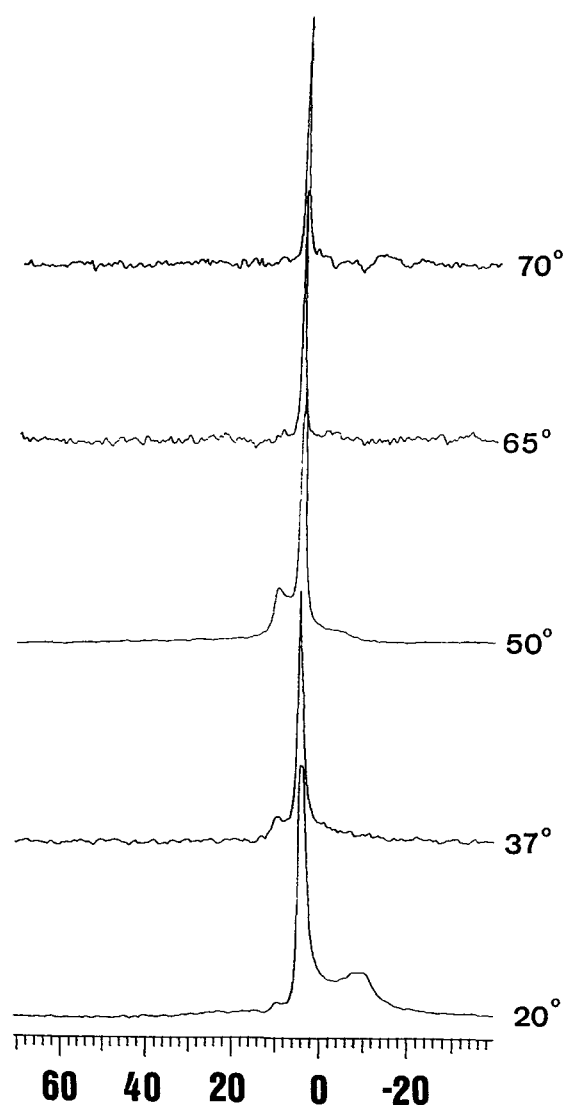


Fig. 3. ^{31}P NMR spectra of aqueous dispersions of PC:PE:CHOL:DG (40:20:25:15), equivalent to 15% phospholipid substituted by DG. Samples were 0.2 M in lipid. The spectra were recorded at increasing temperatures, as shown by each curve in $^{\circ}\text{C}$.

only at low DG concentration, and is fully inhibited when the DG-concentration exceeds 20%. The overwhelming role of DG concentration on the fusion events, in contrast with the negligible role of enzyme concentration is documented in Fig. 5.

In comparing the biochemical observations with the results of the NMR and the X-ray scattering studies one must keep in mind the idiosyncrasy of the two types of experiments. Structural studies are performed on bulk systems, ideally in the form of well defined phases, possibly in the presence of excess water. The biochemical experiments, instead, are aimed at testing the properties of the two-dimensional lipid septa that enclose the vesicles. From the thermodynamic viewpoint, a vesicle is not a phase: at best the structural studies can provide some indication regarding the organization of the lipid septa that are involved in the biochemical events.

A remarkable result of the NMR and the X-ray scattering studies is that the system displays a variety of phases (lamellar, hexagonal, isotropic) precisely in the DG-concentration and

temperature range – 5 to 20% and at 37°C (shaded in Fig. 4) – over which fusion is observed [11]. One of these phases is the cubic Q^{224} , in equilibrium with other phases (lamellar and hexagonal) at 37°C , pure at higher temperature (and after thermal cycling). It is thus tempting to presume that the small amounts of DG liberated early by phospholipase C induce the formation of patches whose organization is reminiscent of that of phases Q^{224} . In these patches both the hydrocarbon and the water matrices are continuous throughout the structure: the vesicles can thus fuse and the water compartments mix. At higher DG concentration the organization of these non-lamellar patches would transform into a structure reminiscent of that of phase Q^{227} , formed by an apolar continuum enclosing disjointed water-containing micelles. This structure would explain why the lipid molecules of different vesicles mix, but not their water compartments.

Another question that arises is the role of the enzyme, since both the lipid- and the water-mixing processes take place only as a result of the enzymatic degradation of phospholipid. Most likely, this problem involves the balance between thermodynamically stable and metastable states. In the bulk systems used in the structural studies the phases are ideally in thermodynamic equilibrium (although, in this respect, the role of the thermal cycling used to generate phase Q^{224} is not quite clear). On the contrary, the dilute liposome suspensions used in the biochemical studies are better described as metastable. Enzyme activity may well upset this metastable state via the local, and possibly asymmetric generation of DG. When the DG concentration reaches the appropriate value interspersed contacts may be stabilized, with the formation of patches of non-lamellar structure (see in [24] the relations between aggregation and fusion in a different system).

It is worthwhile to note that lipid particles, usually described as inverted micelles and thought to represent fusion intermediates, have been often observed in freeze-fractured lipid samples [25]. The fact that the samples used in these studies are often taken a long time after the putative fusion process is completed [26], and the structural similarity with the micellar phase Q^{227} make us wonder whether the particles correspond in fact to fusion end-products, much like phase Q^{227} in the system studied

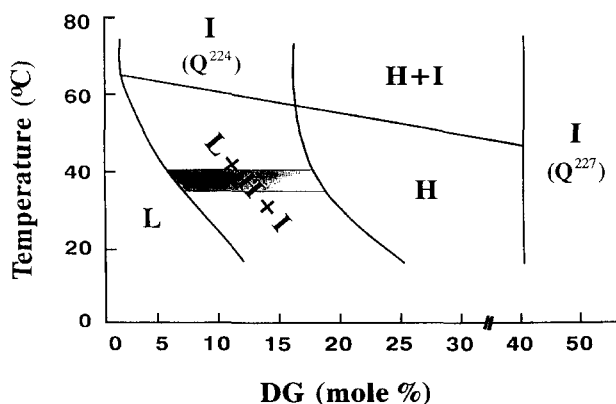


Fig. 4. A pseudo-phase diagram of PC:PE:CHOL:DG in excess water, constructed from ^{31}P NMR data. L, lamellar; H, hexagonal; I, isotropic. In parentheses the nature of the cubic phases, as identified by the X-ray scattering experiments (sample concentration 50% w/w). The shaded area corresponds to the region of temperature and composition at which optimum liposome fusion induced by phospholipase C is observed.

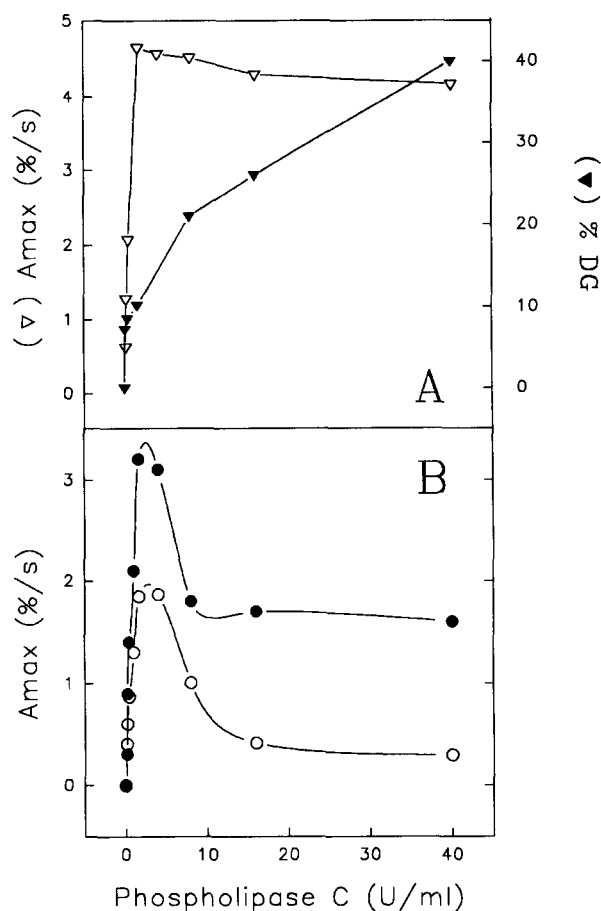


Fig. 5. Phospholipase C-promoted DG formation and liposomal fusion as a function of enzyme concentration. Lipid concentration was 0.3 mM in all cases. (A) (Δ) Maximum rates of vesicle aggregation detected as changes in light scattering. (\blacktriangledown) Amounts of DG in the vesicles, measured at the point of maximum fusion rate for each enzyme concentration. (B) Maximum rates of fusion detected as mixing of contents (\circ) or mixing of lipids (\bullet) are plotted against enzyme concentration.

in this work, rather than to fusion intermediates [27]. These ideas are consistent with a recent theoretical study by Siegel [28] that casts doubts on the hypothetical role of the inverted micelles in the fusion process.

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