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STRUCTURE OF MOLECULAR AGGREGATES OF 1-(3-*sn*-PHOSPHATIDYL)-L-*myo*-INOSITOL 3,4-BIS(PHOSPHATE) IN WATER

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Key words: Phospholipid structure; Lamellar phase; Lipid aggregation; Micelle formation

Summary

The molecular organization of 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 3,4-bis-(phosphate)/water systems is investigated over a wide range of lipid concentrations using X-ray diffraction, calorimetry, analytical ultracentrifugation, densitometry and viscometry.

At high lipid concentrations, the lipid molecules are found to form a lamellar phase. The repeat distance increases from 60 to 120 Å with increasing water content to 70 wt% and the surface area per lipid molecule increases from 41.7 Å^2 to a limiting value of 100 Å^2.

On the other hand, at very low lipid concentrations the molecules are found to form not vesicles but micelles, the total molecular weight of which takes a value of 93 000.

This finding revises the prevalent view that lipids containing two (or more) hydrocarbon chains form extended bilayers or vesicles, whereas single chained lipids form micelles. (Tanford, C. (1972) J. Phys. Chem. 76, 3020—3024).

Introduction

1-(3-*sn*-Phosphatidyl)-L-*myo*-inositol 3,4-bis(phosphate) is a constituent of myelin and plasma membranes and is considered to be involved in membrane permeability changes related to nerve conduction [1]. PtdIns(3,4)P_2 is an acidic lipid with a maximum of five negative charges.

There are studies on the interactions of PtdIns(3,4)P_2 with proteins and divalent cations including Ca^{2+} [2,3]. The interpretation of such studies involving PtdIns(3,4)P_2 depends on a knowledge of its state of aggregation in water.

Abbreviation: PtdIns(3,4)P_2, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 3,4-bis(phosphate).

Although the physical properties and behavior of many kinds of phospholipids have been well studied by a variety of physicochemical method, the physicochemical properties of PtdIns(3,4)P_2 have received little attention, probably because of the difficulty in obtaining large amounts of this phospholipid.

In 1969, Hendrickson [3] reported the physical properties and micellar structures of this lipid, and obtained a value of 78 100 for the total molecular weight of the micelle.

Studies on the physical properties of PtdIns(3,4)P_2 over a wide range of lipid concentration are reported in this paper.

Materials and Methods

PtdIns(3,4)P_2 used in the present study was generously supplied by Dr. K. Hayashi, Gunma University, Japan. The lipid was isolated from bovine brain according to the procedures described previously by Hayashi [4]. The purified PtdIns(3,4)P_2 was subsequently converted to the ammonium salt. The ammonium salt of PtdIns(3,4)P_2 was a white powder and its aqueous solution was completely clear and highly viscous.

X-ray diffraction

PtdIns(3,4)P_2 in water was sealed between mica windows of a variable temperature sample holder and its small-angle and wide-angle X-ray diffraction patterns were recorded photographically as a function of temperature within the range 20—80°C.

CuK_α radiation was obtained by the use of a nickel filter. A Rigaku-Denki camera was used for small-angle diffraction patterns and a special film holder was used for wide-angle diffraction patterns.

Calorimetry

Calorimetric measurements were carried out using a scanning calorimeter of the conduction type (Daini Seikosha Co. Ltd., Tokyo). This instrument is provided with sample vessels that can be tightly sealed to prevent any loss of volatile component. Silver vessels charged with about 70 μl PtdIns(3,4)P_2 solution were sealed and weighed before and after each measurement.

The thermograms were run from room temperature to 120°C at a heating rate of 0.6 K/min.

Ultracentrifugal analysis

All the ultracentrifugal studies were performed in a Beckman-Spinco Model E analytical ultracentrifuge equipped with an RTIC temperature control unit, controlling the rotor temperature to within ±0.1°C, and an electronic speed control system.

Sedimentation velocity measurement. A single-sector, capillary-type synthetic boundary cell with a 12-mm optical path was used in all experiments. Rotor speed was maintained at 64 000 rev./min. Measurements of Schlieren pattern peak positions were made with a travelling microscope.

Molecular weight measurements. The molecular weight determinations were

carried out by the meniscus-depletion sedimentation equilibrium method of Yphantis [5]. Rotor speed was maintained at 20 000 rev./min. Interference fringe patterns were read on a two-dimensional microcomparator.

Density measurement

The densities of the aqueous PtdIns(3,4)P_2 solutions were measured with a digital density measuring device DMA-02 from Anton Paar (Graz).

The system was calibrated using NaCl solutions and the experiments were performed at 20.0°C.

Viscosity measurements

The steady flow viscosity of PtdIns(3,4)P_2 solutions was measured by a coaxial cylindrical viscometer as shown in Fig. 1 [6], the inner cylinder being suspended from the torque measuring device and the outer cylinder being rotated by means of a gear box and driving motor. The measurement was carried out in a range of shear rate from 0.3—30 s^{-1} at 25.0°C.

Results

X-ray diffraction

In all cases examined, in the low-angle region of the X-ray diffraction pattern a series of diffraction lines in a ratio of 1 : 1/2 : 1/3 : 1/4 was observed, corresponding to a one-dimensional lamellar organization. A plot of the repeat distance of the lamellar phases (d) as a function of $(1 - c)/c$ being directly proportional to the number of water molecules per lipid molecule, where c is the weight fraction of lipid in the sample, is shown in Fig. 2. The repeat distance of the lamellar structure increases from 60 to 120 Å with increasing water content of 70 wt%.

Assuming a molecular weight for PtdIns(3,4)P_2 of 1132 (see below) and

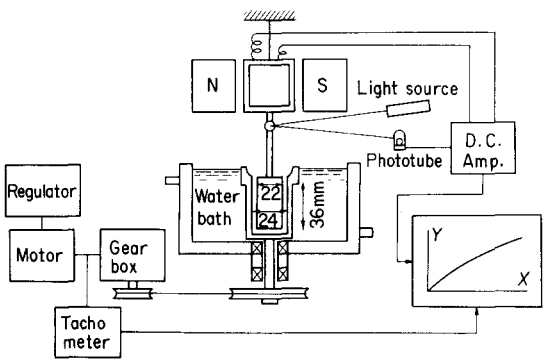


Fig. 1. A schematic diagram of a low-shear-rate viscometer.

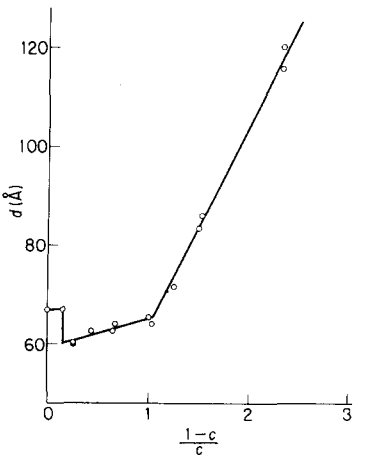


Fig. 2. A plot of the repeat distance of the lamellar phases (d) as a function of $1 - c/c$; where c is the weight fraction of the lipid in the sample at 22.0°C.

using a ratio of the partial specific volume of the two components $\bar{\nu}_w/\bar{\nu}_l$ of 1/0.742 (see below), the surface area per lipid molecule, S, was calculated for each mixture from the following equations [7]:

$$S = \frac{2M\bar{\nu}_l}{d\phi N 10^{-24}} \quad \text{in Å}^2$$

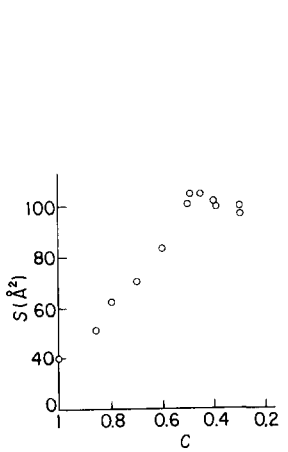
$$\phi = \left[1 + \frac{\bar{\nu}_w}{\bar{\nu}_l}\,\frac{1-c}{c}\right]^{-1}$$

where ϕ is the volume fraction of lipid, M the molecular weight and N Avogadro's number. These data are plotted as a function of lipid concentration in Fig. 3. As the hydration increases (or c decreases), S increases from 41.7 Å^2 to a limiting value of 100 Å^2.

The wide-angle region of the diffraction pattern consisted of a single, fairly sharp diffraction at 4.2 Å, suggesting an ordered configuration for the hydrocarbon region of the lamellar structure.

A thickening of the phospholipid bilayer results from decreasing the thermal motion of the hydrocarbon chains by lowering their temperature, i.e. there is a negative thermal coefficient of bilayer thickness of approx. $2 \cdot 10^{-3}$ [7,8]. Changes in bilayer thickness also give a similar effect on the repeat distance of the lamellar phase. Fig. 4 shows a plot of the repeat distance of the lamellar phase against temperature for PtdIns(3,4)P_2 and for phosphatidylethanolamine from *Escherichia coli*. As the temperature increased, there was a monotonic decrease in the repeat distance for phosphatidylethanolamine while no decrease was observed for PtdIns(3,4)P_2. This is additional evidence of the hydrocarbon chains of PtdIns(3,4)P_2 molecules being solid-like within the temperature range examined.

For PtdIns (3,4)P_2/water systems, the first four order diffractions in the low-angle region were obtained. If the correct phases are chosen for the lamellar dif-



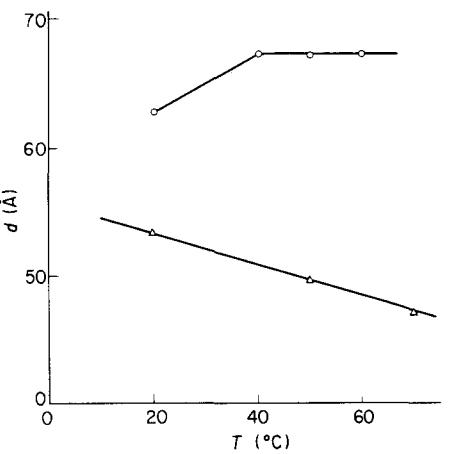


Fig. 3. A plot of the surface area per lipid molecule as a function of lipid concentration.

Fig. 4. A plot of the repeat distance of the lamellar phase against temperature for PtdIns(3,4)P_2 (○) and for phosphatidylethanolamine (△).

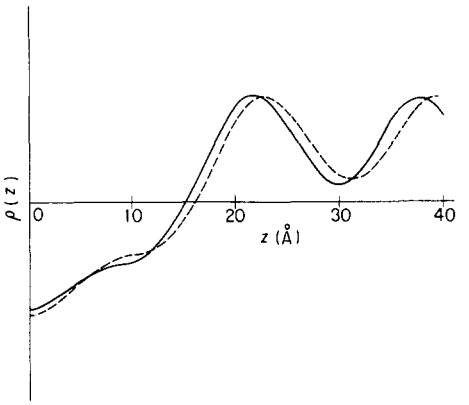


Fig. 5. Electron density profiles along the perpendicular to the plane of the bilayer of PtdIns(3,4)P_2 containing 20 (———) and 40% (- - - - -) water.

fraction amplitudes, a Fourier synthesis may be derived which corresponds to a low-resolution picture of the variation of the average electron density along the perpendicular to the plane of the bilayer [9].

The method of phasing by swelling was tried, but it failed to define the correct phase combination.

Then all phase combinations (2^3 sets) were tested. Six phase choices out of eight gave anomalous results, such as large positive electron-density peaks in the center of the bilayer. Therefore, these six phase combinations were ruled out.

The remaining two phase combinations (——+—) and (—++—) were further examined. The phase assignment —++— gave the electron-density profile of lipid bilayer with isotropic hydrocarbon region, that is, uniformly distributed methyl groups. The other phase assignment (——+—) gave the electron density profile of lipid bilayers with the terminal methyl groups of hydrocarbon chains localized in the center of the bilayer (Fig. 5). This electron-density profile seems physically reasonable and is similar to that of phospholipid, such as lecithin.

Calorimetry

Scanned upward from room temperature PtdIns(3,4)P_2 suspended in water underwent a very broad endothermic transition, as seen in Fig. 6. The onset temperature of the transition from heating runs was about 80°C for 10% suspension. A thermogram of PtdIns(3,4)P_2, which was heated at 80°C for 15 min and subsequently cooled down to room tenperature, is shown in Fig. 7. With this sample, no endothermic transition was observed in any temperature range examined.

Determination of partial specific volume

The density of the solution was found to depend linearly on the lipid concentration within the concentration range examined. The partial specific volume of PtdIns(3,4)P_2, $\bar{\nu}_1$, was calculated from the density data using the

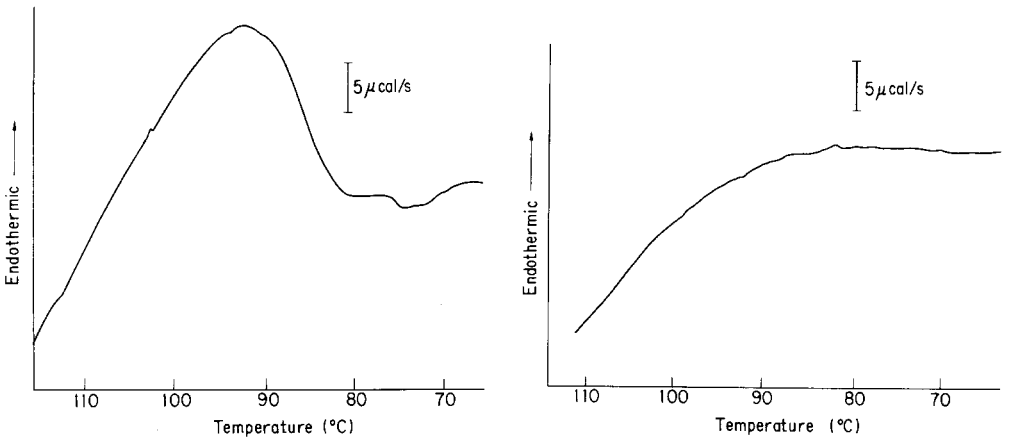


Fig. 6. Thermogram of a 10% PtdIns(3,4)P_2 aqueous solution.

Fig. 7. Thermogram of a 10% PtdIns(3,4)P_2 solution heated at 80°C for 15 min and subsequently cooled down to room temperature.

following relationship [10]:

$$\bar{\nu}_1 = \frac{1}{\rho_0}\left[1 - \frac{(\rho - \rho_0)}{c_1}\right]$$

where ρ_0 is the density of the solvent (water) and ρ is the density of the aqueous PtdIns(3,4)P_2 solution at concentration c_1 in g/ml. A value of 0.742 ml/g was obtained at 20.0°C.

Viscosity

When PtdIns(3,4)P_2 was dispersed in water or a 0.1 M NaCl solution and kept standing at room temperature overnight, a completely clear and highly viscous solution was obtained. The samples prepared thus exhibited non-Newtonian viscosity with a very high viscosity value. These samples were heated up to a given temperature and kept at that temperature for 15 min. After cooling to 25°C, viscosity measurements were made. Such procedures were repeated with increasing treatment temperature to the maximum temperature of 80°C. Fig. 8 and 9 show the relationship between the stress of shear and the rate of shear. Non-Newtonian viscosity was obviously seen below 55°C for both samples, whereas the viscosity was Newtonian above the temperature.

Fig. 10 shows the relative viscosities to water at a very low rate of shear as a function of the treatment temperature, indicating that the viscosity dropped to a high degree with the increase in treatment temperature.

After the samples, once heated to 80°C, had been kept standing in a freezer at −20°C, viscosity measurements were made at 25°C. Non-Newtonian viscosity again appeared, and the value of viscosity increased to some extent (Figs. 8 and 9, dashed lines). After the samples were heated to 80°C, the solution remained very clear. These observations provided evidence that no decomposition of PtdIns(3,4)P_2 molecules occurred, because the decomposition of the molecules

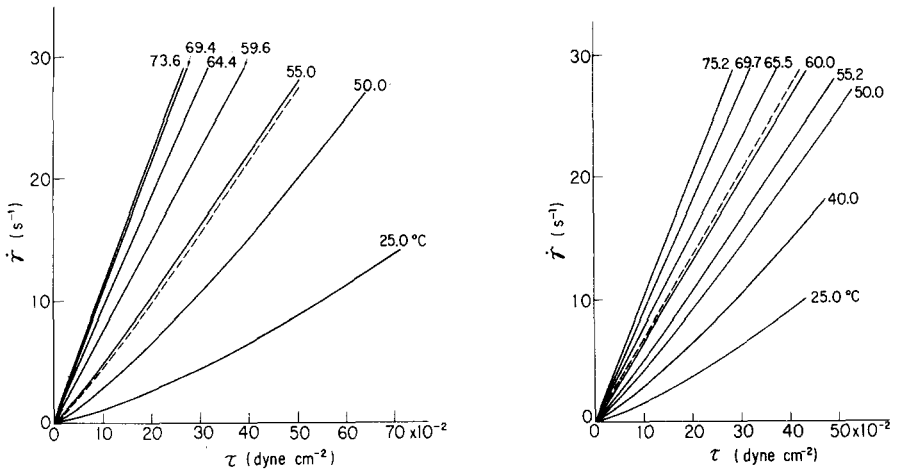


Fig. 8. The relation between the stress of shear (τ) and the rate of shear ($\dot{\gamma}$) for PtdIns(3,4)P_2 in water. Lipid concentration, 0.1%. The dashed line represents the sample which had been heated to 80°C and subsequently kept standing in a freezer at −20°C.

Fig. 9. The relation between the stress of shear (τ) and the rate of shear ($\dot{\gamma}$) for PtdIns(3,4)P_2 in 0.1 M NaCl. Lipid concentration, 0.1%. The dashed line represents the sample which had been heated to 80°C and subsequently kept standing in a freezer at −20°C.

makes the PtdIns(3,4)P_2 solution turbid due to the very low solubility of fatty acids in water.

Accordingly, such a large decrease in viscosity of the PtdIns(3,4)P_2 solution upon heating is due to the dissociation of large molecular aggregates to small aggregates.

Sedimentation

PtdIns(3,4)P_2 (0.5%) dispersed in a 0.1 M NaCl solution was heated at 80°C for 15 min and subsequently cooled down to room temperature. The PtdIns-(3,4)P_2 solution thus treated was thoroughly dialyzed against a 0.1 M NaCl

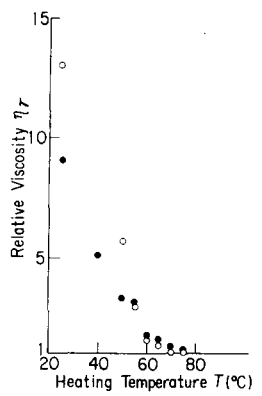


Fig. 10. A plot of the relative viscosities to water at a very low rate of shear as a function of the treatment temperature. ○, water; ●, 0.1 M NaCl.

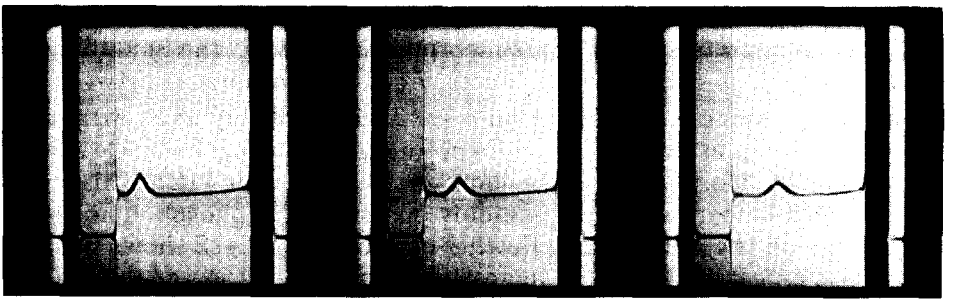


Fig. 11. (Upper) Sedimentation patterns of PtdIns(3,4)P_2 in 0.1 M NaCl. Lipid concentration, 0.5%. Left, 0 min; middle, 8 min; right, 16 min.

Fig. 12. (Lower) Interference fringe patterns of PtdIns(3,4)P_2 in 0.1 M NaCl. Lipid concentration, 0.5%.

solution. Sedimentation patterns are shown in Fig. 11. It is seen from the figures that the PtdIns(3,4)P_2 solution prepared thus by heat-treatment shows a single peak. PtdIns(3,4)P_2 molecular aggregates formed by heat-treament were homogeneous in size and shape. A value of 6.1 S for sedimentation velocity was obtained at a lipid concentration of 0.5% and 25°C.

As the PtdIns(3,4)P_2 solution exhibited a single peak in sedimentation patterns, the total molecular weight of the lipid aggregate was measured by a sedimentation equilibrium method. Interference fringe patterns of the PtdIns(3,4)-P_2 solution are shown in Fig. 12. The total molecular weight of the lipid aggregate was calculated from the following equation [11]:

$$M = \frac{2RT}{(1 - \bar{\nu}_l \rho_0)\,\omega^2} \cdot \frac{2.303(d \log C)}{d(r^2)}$$

where R is the gas constant, $T = 295.7$ K, $\bar{\nu}_l$ the partial specific volume of the lipid. ρ_o the density of the solvent, C the concentration and r the radial distance, corrected for camera lens magnification. The total molecular weight of the lipid aggregate took a value of 93 000.

Discussion

The structure of PtdIns(3,4)P_2 molecular aggregates at low hydration range

X-ray diffraction and calorimetry studies indicate that at low water content from 0—70 wt% and at below 80°C, PtdIns(3,4)P_2 exhibit a lamellar phase structure, in which water intercalated between sheets of the lipid molecules in the bilayer fashion.

The hydration characteristics of PtdIns(3,4)P_2 are different from the phos-

pholipids with zwitterionic polar head groups, such as phosphatidylcholine, in that only limited amounts of water are incorporated between the lipid bilayers. With PtdIns(3,4)P_2, as seen in Fig. 2, water added is incorporated between the lipid bilayers at least to a concentration $c = 0.3$, and probably to an extremely dilute concentration, as evidenced by the production of an optically clear solution. Bovine brain phosphatidylserine shows similar behavior [12]. This continuous swelling behavior has been commonly observed with phospholipids bearing net charges on the polar group. Further inspection of Fig. 2 shows that the increase of the repeat distance with increasing hydration consists of three parts. In the absence of water, $c = 1.0$, PtdIns(3,4)P_2 took the β-type structure, in which the hydrocarbon chains pack with rotational disorder in a hexogonal lattice, the chain axes being normal to the surface of the lipid bilayer because the surface area per molecule occupied by anhydrous PtdIns(3,4)P_2 was 41.7 Å^2 (see Fig. 3). At $c = 0.8$, a decrease of the repeat distance was observed. This decrease of 5 Å is not due to the order-disorder transition of the hydrocarbon chains but may be due to the configurational change occurring in the polar group. As the hydration increased or c decreased to 0.5, the repeat distance increased only slightly (see Figs. 2 and 5) although the polar group must be ionized to a fair degree. These changes may be accounted for by a structure in which the hexagonally packed, ordered hydrocarbon chains tilt progressively with respect to the normal to the bilayer plane [13], and by incorporation of water added into a free volume associated with the polar groups. On increasing hydration further, c less than 0.5, the repeat distance continued to increase by incorporating water added between the opposed bilayers.

Surface area per molecule is an important physical quantity because it is a measure of the separation between amphiphile head groups. As seen in Fig. 3, on increasing the hydration or decreasing the lipid concentration the surface area per lipid molecule increases from 41.7 Å^2 to a limiting value of 100 Å^2. This high limiting value must be associated with the negative charges on the polar head, which can be 5 at complete dissociation.

The PtdIns(3,4)P_2 thermal transition starts at about 80°C and is very broad. The transition arises from melting of the hydrocarbon chains within the phospholipid bilayers and also probably from the effect of dissociation of the lipid aggregates to the smaller aggregates. The temperature at which this endothermic phase transition occurs has been shown [14] to be dependent upon the head group, the hydrocarbon chain length and the degree and type of unsaturation present. It is noted that with bovine brain PtdIns(3,4)P_2 up to a fairly high temperature (80°C) a stable crystalline form is present. Of particular interest is the much higher temperature, 80°C, at which the order-disorder transition takes place for this naturally occurring and, in terms of its fatty acid composition, structurally inhomogeneous PtdIns(3,4)P_2. Cerebroside from bovine brain has also a high transition temperature of 70°C [15].

The structure of PtdIns(3,4)P_2 molecular aggregates at high hydration range

When PtdIns(3,4)P_2 was dispersed in water or in a 0.1 M NaCl solution, it became an optically clear and highly viscous solution. These solutions were heated at 80°C for 15 min and subsequently cooled down to room temperature. Viscosities of the solution thus treated were very low, indicating dissocia-

tion of large bilayer aggregates to smaller micellar aggregates of which the total molecular weight was 93 000. This value is comparable with the value of 78 100 obtained by Hendrickson [3]. Although most naturally occurring phospholipids are structurally inhomogeneous in terms of their fatty acid composition, i.e., the chain length, the number and the position of unsaturation, inositol lipids from animal sources have a very characteristic fatty acid pattern in which a large proportion of the molecules possess a stearyl (C18;0) residue at position 1 and an arachidonyl (C20;4) residue at position 2 [16]. Therefore, assuming that PtdIns(3,4)P_2 has a stearyl residue at position 1 and an arachidonyl residue at position 2, the molecular weight and the volume of the lipid molecule were calculated as 1132 and as 1395 Å^3, respectively (Table I).

Using a molecular weight of 1132 and a volume of 1395 Å^3 for PtdIns(3,4)-P_2 molecule, several physical quantities were calculated as described in Table I. These values, the aggregation number of 82 and the volume of $1.2 \cdot 10^5$ Å^3, indicated that the molecular aggregate prepared by the heat treament is not a vesicle but a micelle. On the other hand, egg lecithin prepared by sonication under proper conditions was reported to form vesicles of which the aggregation number was 2678 and the vesicle weight was $2.1 \cdot 10^6$ [18].

Although the shape of the micelle cannot be determined, assuming a spherical shape the radius of the micelle takes a value of 30 Å, this value being not larger than half of the repeat distance of the lamellar phase determined by X-ray diffraction. Stokes radius is also calculated as 39 Å.

It was believed that lipids containing two (or more) hydrocarbon chains form extended bilayers or vesicles, whereas single chained lipids form micelles [17]. However, PtdIns(3,4)P_2 is found to exist as micelles in solution in spite of a two-hydrocarbon-chained lipid. According to the theory of Israelachvili et al. [19,20] a value of v/al is a very important quantity describing the mode of lipid aggregation, where v is the hydrocarbon chain volume, a the hydrocarbon/water interface area and l the hydrocarbon thickness. Fig. 13 shows a schematic representation of the relationship between the mode of lipid aggregation and

TABLE I

PHYSICAL PROPERTIES OF PtdIns(3,4)P_2

Molecule		
Molecular weight	1 132	Ammonium salt $C_{18:0}$—$C_{20:4}$
Volume	1 395 Å^3	
V_{hc}	1 023 Å^3	$V = 27.4 + 26.9\ n_c$[17]
V_{pol}	372 Å^3	
Partial specific volume	0.742 ml/g	
Hydrocarbon chain length (max)	24.3 Å	$l_{max} = 1.5 + 1.26\ n_c$[17]
Micelle		
Weight	93 000	
Aggregation number	82	
Sedimentation constant	6.1 S	
Stokes radius	39 Å	
Volume	$115 \cdot 10^3$ Å^3	
V_{hc}	$84 \cdot 10^3$ Å^3	
V_{pol}	$31 \cdot 10^3$ Å^3	
Radius	30 Å	

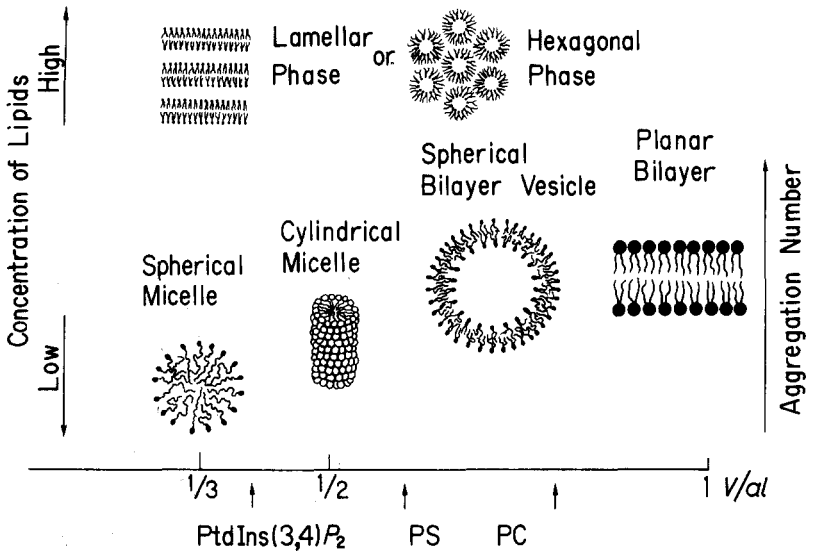


Fig. 13. The schematic representation of the relationship between the mode of lipid aggregation and *v/al*. PC, phosphatidylcholine; PS, phosphatidylserine.

v/al. It is also schematically show that the aggregation number, i.e., the number of molecules in an aggregate, increases in going from spherical micelle → cylindrical micelle → spherical bilayer vesicle → planar bilayer. Furthermore, in this figure, two main phases, i.e., a lamellar phase and an inverted hexagonal phase taken by phospholipids in high lipid concentrations, are drawn. The value of *v/al* was calculated as 0.4 for bovine brain PtdIns(3,4)P_2 and 0.6—0.8 for phosphatidylserine and phosphatidylcholine, assuming that the surface area per molecule, S, be equal to the hydrocarbon/water interface area, a. The value of *v/al*, 0.4, for PtdIns(3,4)P_2 explains that this lipid can form micelles in solution. The origin of the unusual characteristics of this lipid is ascribed mainly to the maximum five negative charges on the polar head group, which is apparently enormous due to the mutual repulsive interactions when compared with, for example, the phosphocholine head group of phosphatidylcholine.

As gangliosides bear the enormous carbohydrate head groups, this class of lipid can form micelles also, although it is a two-hydrocarbon-chained lipid [21].

The present study reveals that bovine brain PtdIns(3,4)P_2 possesses physical properties which are significantly different from those of other classes of membrane phospholipid.

The study of the physical properties of PtdIns(3,4)P_2 alone and in combination with other membrane lipids is a prerequisite for the detailed understanding of the complex membrane phenomena.

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