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Specific volumes of unsaturated phosphatidylcholines in the liquid crystalline lamellar phase

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Abstract

The specific volumes of seven 1,2-diacyl-*sn*-glycero-3-phosphocholines with symmetric, unbranched acyl chains containing one, four, or six cis double bonds per chain, or with a saturated *sn*-1 chain and one, four, or six cis double bonds in the *sn*-2 chain were determined by the neutral buoyancy method. Experiments were conducted in the liquid crystalline lamellar phase over the temperature range from 5 to 35 °C. It is demonstrated that the molecular volume of phosphatidylcholines can be well approximated as the sum of a constant volume of the polar lipid head region and the temperature-dependent volumes of hydrocarbon chain $-CH_2$, $=CH$, and terminal CH_3 groups. A linear dependence of chain segment volumes on temperature was observed. A self-consistent set of partially temperature-dependent volumes is obtained that allows prediction of phosphatidylcholine molecular volumes within very tight error margins.

Keywords: Specific volume; Lipid; Polyunsaturated fatty acid; DHA; Neutral buoyancy method

1. Introduction

Lipids with polyunsaturated hydrocarbon chains are important constituents of biological membranes. For example, high levels of docosahexaenoic acid (22:6ω3, DHA) are found in phospholipids of the nervous system, the retina, and sperm [1,2]. Deficiencies in ω-3 polyunsaturated acyl chain content, in particular in DHA have been related to impaired visual acuity [3,4], and delayed mental development [5]. In adult mammals, high consumption of DHA was associated with a reduced risk of cardiovascular disease [6], and Alzheimer's disease [7], and a lower risk for developing depression [8].

In biological membranes, phospholipids with saturated *sn*-1 and unsaturated *sn*-2 fatty acid chains are prevalent, but highly unsaturated membranes, as found in the retina and brain synapses, also contain significant fractions of phospholipids with two polyunsaturated chains. It is still debated if the particular health effects of polyunsaturated lipids result from modifications of membrane biophysical properties, from specific interactions of polyunsaturated lipids with membrane proteins, or from signalling molecules that are synthesized from polyunsaturated lipids. In recent years, the biophysical properties of membranes composed of polyunsaturated hydrocarbon chains received a lot of attention. There is firm experimental evidence for a dependence of important membrane properties on hydrocarbon chain unsaturation, e.g., the phase transition temperatures [9], membrane thickness and area per lipid [10,11],

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Abbreviations: 16:0, 18:1 PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 18:0, 18:1 PC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 18:0, 20:4 PC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; 18:0, 22:6 PC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; 18:1, 18:1 PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 20:4, 20:4 PC, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine; 22:6, 22:6 PC, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid

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elastic membrane properties and hydration [12,13], and packing characteristics of the hydrocarbon chains [14–16].

The molecular volume of lipids plays a pivotal role in structural studies of liquid crystalline membranes [17], including interpretation of X-ray and neutron scattering data that yield membrane thickness and area per molecule as well as interpretation of ^{2}H NMR order parameter profiles. Molecular volumes are also important for development of force fields for molecular simulations of polyunsaturated lipid membranes that reproduce structural and motional features of lipids with atomic resolution [18–20].

The most direct way to determine the volume of a lipid molecule in the biologically relevant liquid crystalline lamellar phase is to study neutral buoyancy of liposomes in $H_2O/^2H_2O$ mixtures by centrifugation. Within the range from ~0.9 to ~1.0 cm^3/g, this technique allows to determine specific volumes of lipids with very high accuracy (±0.002 cm^3/g) [21,22]. The technical aspects of this method have been discussed in detail [23]. Lipid-specific volumes have also been determined using differential vibrating tube densitometers [24], differential dilatometers [25], and capillary-pycnometers [26].

A common approach to estimate lipid specific volume is to represent the volume of a lipid, V_L, as the sum of volumes of its molecular segments [27]. Lipid volume, V_L, was split into the volume of the lipid head region, V_H, representing the phosphocholine head group, the lipid glycerol moiety and the two carboxylic groups of the hydrocarbon chains, and the volume of the remainder of the hydrocarbon chains. The chain volume has been further divided into the volume of the methylene groups ($-CH_2$), the volume-per-carbon in double bonds ($=CH$), and the volume of terminal methyl groups (CH_3). It was assumed that the volumes of the chain segments are close to the corresponding values in liquid hydrocarbons ($V_{CH2}=27.0$ Å^3, $V_{CH}=20.5$ Å^3, and $V_{CH3}=54.0$ Å^3 at 25 °C) [27]. Volumetric data of phosphatidylcholines with saturated hydrocarbon chains confirmed that the $-CH_2$ volume in the liquid crystalline phase is very close to V_{CH2} in liquid alkanes [22,28]. The latter approach requires knowledge of V_H, which was estimated from X-ray diffraction analysis of phosphatidylcholines in the gel and crystalline phases. Earlier attempts yielded a $V_H=348$ Å^3 for DPPC [28], while a more recent analysis [29] gave $V_H=(319\pm6)$ Å^3. It was reported that the volume of the lipid head region changes very little at the main phase transition and that it has negligible temperature dependence [23,28].

Although the composite volume concept is widely used, there is some variability between laboratories in the volumes (V_H, V_{CH}, V_{CH2}, V_{CH3}) used for the estimates, and the temperature dependence of chain segment volumes is rarely accounted for. Some of the uncertainty stems from the lack of experimental data for lipids with unsaturated hydrocarbon chains. Here, the experimentally determined specific volumes of seven unsaturated phosphatidylcholines with grossly different numbers and location of double bonds are reported over the temperature range from 5 to 35 °C. A self-consistent set of temperature-dependent chain segment volumes was derived from the data that allows to reproduce the measured specific volumes with very high precision. This set of segment volumes can be used to predict specific volumes of phosphatidylcholines in the liquid crystalline lamellar phase over the physiologically relevant range of temperatures.

2. Materials and methods

Phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Quartz-glass distilled deuterium oxide (^2H_2O) with a degree of deuteration of 99.9% was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). BHT and DTPA (purity ≥99%) were obtained from Aldrich (Milwaukee, WI).

Samples were prepared in a glove box under argon gas to prevent lipid oxidation. The antioxidant BHT was added to the lipid solutions in chloroform or methylene chloride at a BHT-to-lipid molar ratio of 1/100. All phospholipids were checked for chromatographic purity by HPLC as well as for composition and state of oxidation of the hydrocarbon chains by gas chromatography. The content of oxidized hydrocarbon chains was below 1% for all lipids at the beginning of experiments and less than 3% after completion. Lipid aliquots of ~2 mg were transferred to glass test tubes. For efficient drying, the small amount of lipid was spread over the surface of the tube. The organic solvent was removed in a stream of argon followed by subjecting the samples to vacuum for at least 2 h. By ^{1}H MAS NMR, it was confirmed that not even trace amounts of organic solvent were left in the samples. Lipid dispersions were obtained by adding ~3 ml of $H_2O/^2H_2O$ mixtures containing 50 μM DTPA to the dry lipid. The chelator DTPA was added to minimize lipid oxidation induced by trace amounts of divalent ions.

Vigorous vortexing of the samples at ambient temperature in the glove box resulted in visually homogeneous samples with a milky appearance.

Lipid dispersions were transferred to 3.2 ml ultra clear centrifuge tubes which were then loaded into air-tight screw-cap buckets of a swinging bucket Beckman TLS-55 centrifuge rotor. The loaded rotor was temperature equilibrated for ~2 h followed by an overnight spin in a temperature controlled Beckman TLX-100 centrifuge. Immediately after the centrifugation, the temperature of each sample was measured and the lipid solution was visually inspected. In most cases, the dispersion had cleared and the lipid layer was either floating on top of the aqueous phase or was forming a pellet at the bottom of the tube, indicating that the specific volume of the lipid was higher or lower, respectively, than that of the aqueous phase.

The specific volume of $H_2O/^2H_2O$ mixtures was calculated assuming ideal mixing of protonated and deuterated water. The temperature-dependent mass densities of H_2O and ^2H_2O, respectively, were derived from spline interpolations of tabulated density data [30]. The specific volume of the water phase was incremented in steps of ~0.002 cm^3/g around the point of lipid neutral buoyancy using freshly prepared samples for each centrifugation experiment, resulting in a precision of the measured specific volumes of ±0.001 cm^3/g.

3. Results and discussion

The specific volumes of seven unsaturated 1,2-diacyl-*sn*-glycero-3-phosphocholines were determined in the liquid crystalline lamellar phase as a function of temperature from 5 to 35 °C. The main phase transition temperature of investigated lipids is below 5 °C [9,31], with the exception of 18:0, 18:1 PC which has a phase transition temperature of 6 °C [32]. The measured specific volumes are shown in Fig. 1. The specific volumes of all investigated phosphatidylcholines depend linearly on temperature ($r^2 \geq 0.99$). The intercept of the regression line with the *y*-axis yields an extrapolation of the specific volume to 0 °C while the slope is the coefficient of thermal lipid expansion. Regression parameters for each lipid are reported in Table 1.

Very few volumetric data of unsaturated lipids were reported previously. Our experimental data for 18:1, 18:1 PC are in excellent agreement with specific volume measurements by Tristram-Nagle et al. [33,34]. Earlier we had reported a molecular volume of 18:0, 22:6 PC at 30 °C that is 8 Å^3 lower than the value calculated from the specific volume given in this paper [15]. Because this difference is slightly outside the very low experimental error limits, we repeated the measurements for 18:0, 22:6 PC with a new batch of lipid and verified that the values reported here are accurate. The predicted molecular volume of 18:0, 22:5 PC, using segmental volumes from the analysis below, is also slightly higher than our previously reported value at 30 °C [15], suggesting existence of a small systematic error (−0.6%) of unknown origin in our previous specific volume measurements.

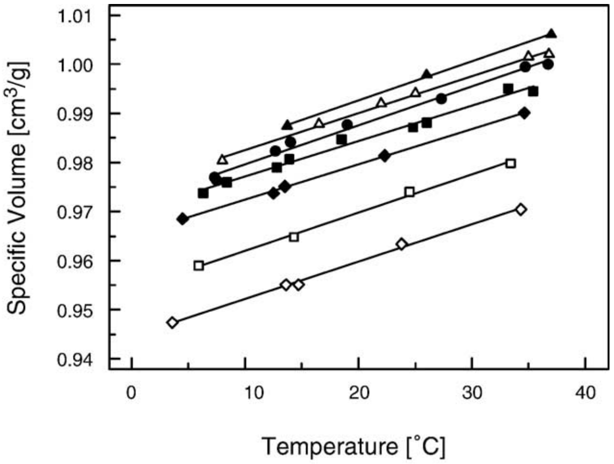

Fig. 1. Specific volumes of unsaturated phosphatidylcholines in the liquid crystalline lamellar phase as a function of temperature. Symbol height reflects the experimental uncertainty of the data (±0.001 cm^3/g). Solid lines represent a linear regression to the data. The following lipids are shown: 16:0, 18:1 PC (solid circles), 18:0, 18:1 PC (solid triangles), 18:1, 18:1 PC (open triangles), 18:0, 20:4 PC (solid squares), 20:4, 20:4 PC (open squares), 18:0, 22:6 PC (solid diamonds), and 22:6, 22:6 PC (open diamonds).

Table 1
Specific volumes of phosphatidylcholines in the L_α phase

Lipid	MW [g mol^{-1}]	n_{CH2}	n_{CH}	Specific volume[a]	
				Intercept at 0 °C [cm^3 g^{-1}]	Slope [cm^3 g^{-1} deg^{-1}]
16:0, 18:1 PC	760.09	28	2	0.9717	0.00079
18:0, 18:1 PC	788.14	30	2	0.9766	0.00080
18:1, 18:1 PC	786.15	28	4	0.9750	0.00075
18:0, 20:4 PC	810.15	26	8	0.9700	0.00072
20:4, 20:4 PC	830.14	20	16	0.9542	0.00078
18:0, 22:6 PC	834.17	24	12	0.9651	0.00072
22:6, 22:6 PC	878.18	16	24	0.9446	0.00076

[a] Linear regression of measured specific volume vs. temperature (cf. Fig. 1).

The volume of a lipid molecule is $V_L(T) = v_i(T) \times MW_i / N_{Avogadro}$, where $v_i(T)$ is the specific lipid volume. The seven investigated lipids had 16 to 30 $-CH_2$ and 2 to 24 $=CH$ groups per lipid, permitting to fit segmental lipid volumes to the $V_L(T)$ data that were derived from the measured specific volumes. Lipid volumes are related to the segmental volumes by

$$V_L(T) = V_H + n_{CH}V_{CH}(T) + n_{CH2}V_{CH2}(T) + n_{CH3}V_{CH3}(T), \quad (1)$$

where V_H is a constant volume of the polar head region (phosphocholine headgroup, glycerol, and lipid carbonyl groups), n_{CH}, n_{CH2}, n_{CH3} are the number of carbons in double bonds, methylene-, and methyl groups, respectively, and V_{CH}, V_{CH2}, and V_{CH3} the respective segmental volumes. Since all lipids had the same polar heads and two terminal methyl groups in their lipid hydrocarbon chains, the V_H and V_{CH3} parameters did not vary between lipids. During a fit their values were fixed. It was assumed that $V_{CH3}(T) = 2 \times V_{CH2}(T)$, in good agreement with $-CH_2$ and CH_3 segmental volumes of liquid alkanes (Fig. 2). Molecular dynamics simulations of phosphatidylcholine bilayers suggested a very similar segmental volume ratio of 1.9 [35]. The fitting procedure was repeated with several different values of V_H, based on data reported in literature (see below). The two free parameters $V_{CH2}(T)$ and $V_{CH}(T)$ were determined by minimizing the root mean square deviation between experimentally determined and calculated lipid volumes based on the Levenberg–Marquardt algorithm. The fit was repeated at seven temperatures

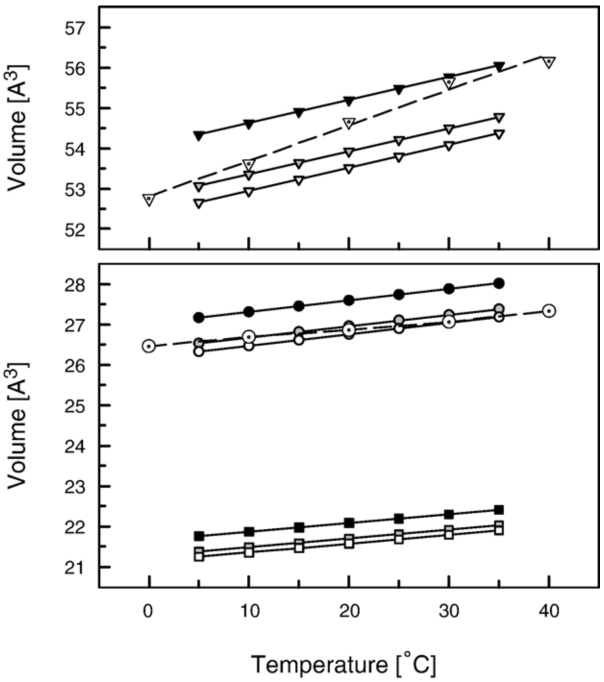

Fig. 2. Temperature dependence of volume per ═CH (squares), −CH$_2$ (circles), and CH$_3$ group (triangles) in liquid crystalline phosphatidylcholine bilayers and liquid n-alkanes (dotted symbols). Lipid values were obtained by fitting the experimental data presented in Fig. 1 assuming a volume of the lipid head region of 319 Å^3 (black symbols), 341 Å^3 (gray symbols), or 348 Å^3 (open symbols) and a fixed V_{CH3}/V_{CH2} ratio of 2 (see text for details). Alkane values are derived from literature data [36].

covering the range from 5 to 35 °C. The specific volumes of the lipids at these seven temperatures were taken from the linear regression of the experimental data (Fig. 1). The resulting component volumes are presented in Fig. 2. Three data sets are shown based on volumes of the polar head region of 319 Å^3, 341 Å^3, and 348 Å^3, respectively.

It was suggested that the volumes per −CH$_2$ and CH$_3$ groups of phosphatidylcholines with unbranched hydrocarbon chains in lamellar liquid crystalline bilayers are close to the corresponding volumes in liquid n-alkanes [27,28]. For comparison with the lipid data, we determined the segmental volumes of liquid n-alkanes at five temperatures from published density data (Appendix 1b in D.M. Small [36]) following the fit procedure suggested by Nagle and Wiener [28]. The plot of the volume of C_nH_{2n+2} vs. the number of carbon atoms, n, revealed an almost perfect linear dependence of alkane volume, V, on n at all temperatures ($r^2 > 0.999$). Linear regression of the data to $n = 2$ yielded the volume of terminal methyl groups, $V = 2 \times V_{CH3}$, while V_{CH2} was determined from the slope (cf. Table 2). The fragmental volumes of n-alkanes as a function of temperature are also shown in Fig. 2 (dotted symbols).

Table 2
Methylene and methyl volumes in liquid n-alkanes[a]

Temperature [°C]	V_{CH2} [Å^3]	V_{CH3} [Å^3]
0	26.46	52.76
10	26.69	53.61
20	26.86	54.65
30	27.06	55.64
40	27.33	56.15

[a] Based on linear regression of literature data [36].

Table 3
Volumes per CH$_2$ and CH group in liquid crystalline phosphatidylcholines[a]

Fragment	Fragment volume[b]	
	Volume at 0 °C [Å^3]	Temperature coefficient [Å^3 deg^{-1}]
−CH$_2$	27.03	0.028
═CH	21.65	0.022

[a] Assumptions: $V_{CH3} = 2 \times V_{CH2}$; $V_H = 319$ Å^3.

[b] Linear regression of fragment volume vs. temperature (cf. Fig. 2).

For lipid polar head volumes, V_H, in the range from 341 to 348 Å^3, we obtained a very close match between V_{CH2} in alkanes and lipids. At 5 °C, agreement between lipid and alkane data was best at $V_H = 341$ Å^3, while at 35 °C $V_H = 348$ Å^3 resulted in a perfect match. This confirms earlier reports [28] that the volume of the polar head region of lipids is nearly temperature independent. However, the apparent slope of V_{CH3} vs. temperature of n-alkanes is somewhat steeper than for the lipid-derived data. Over the temperature range of 30°, the volume expansion per methyl group in n-alkanes exceeded the predicted expansion of lipid methyl groups by 1 Å^3 (cf. Fig. 2).

Alternatively, a V_H of 319 Å^3 was used to fit the lipid volume data. This value was derived by the Nagle laboratory from an analysis of X-ray diffraction data of DPPC in the gel phase [29] and later reproduced in molecular dynamics simulations of several liquid crystalline phosphatidylcholines [35]. The resulting temperature dependence of lipid segmental volumes is shown in Fig. 2 (solid symbols), linear regression parameters of these data are given in Table 3. The self-consistent set of fragmental

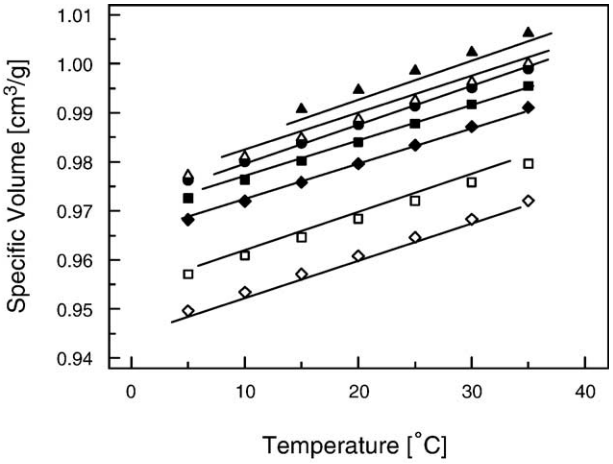

Fig. 3. Predicted specific volumes of unsaturated phosphatidylcholines in the liquid crystalline lamellar phase as a function of temperature. Data of the following lipids are shown: 16:0, 18:1 PC (solid circles), 18:0, 18:1 PC (solid triangles), 18:1, 18:1 PC (open triangles), 18:0, 20:4 PC (solid squares), 20:4, 20:4 PC (open squares), 18:0, 22:6 PC (solid diamonds), and 22:6, 22:6 PC (open diamonds). Solid lines represent linear regression to experimental data and are taken from Fig. 1.

volumes $V_{CH}(T)$, $V_{CH2}(T)$, and $V_{CH3}(T)=2\times V_{CH2}(T)$ allows prediction of molecular volumes of phosphatidylcholines with high accuracy. Fig. 3 shows predicted specific volumes together with the experimental data taken from Fig. 1. Although use of different V_H values resulted in systematic differences in V_{CH}, V_{CH2}, and V_{CH3} segmental volumes, i.e., a specific set of self-consistent segmental volumes is obtained for each V_H value, the deviation between predicted and measured specific volumes was less than 0.2% for all lipids studied independent of the choice of a V_H of 319 Å^3, 341 Å^3, or 348 Å^3.

The segmental volumes specified in Table 3 were used to predict the specific volume of 14:0, 14:0 PC (DMPC) at 30 °C for which experimental data are available. Published values vary from 0.968 to 0.978 cm^3/g [22,37–39]. Our predicted value of 0.977 cm^3/g agrees nicely with our measured value of 0.975 cm^3/g, and the value of 0.978 cm^3/g reported by the Nagle laboratory [22,39].

The concept of partially temperature-dependent segmental volumes that are valid for all phosphatidylcholines in the lamellar liquid crystalline phase appears to be a very good approximation. This conclusion is non-trivial because one might expect some minor changes of the volume of $=$CH and $-$CH$_2$ segments with hydrocarbon chain length, degree of unsaturation, and location of double bonds. However, the accurate reproduction of a large number of experimental lipid specific volumes indicates that such differences are smaller than the low error margins of the experiments. An analysis of data from molecular dynamics simulations of three different phosphatidylcholines also failed to reveal any dependence of the segmental volumes on location in the hydrocarbon chains [35]. Apparently, in the liquid crystalline state, hydrocarbon chains adapt to considerable packing strain without changing their volume. Taking this one step further, one could speculate that composite volumes of $=$CH, $-$CH$_2$ and CH$_3$ groups may be uniform across different classes of phospholipids in the lamellar liquid crystalline phase at a given temperature. Of course, this hypothesis needs to be verified experimentally.

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