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Specific volumes of lipids in fully hydrated bilayer dispersions

M.C. Wiener, S. Tristram-Nagle, D. Allan Wilkinson *, L.E. Campbell ** and J.F. Nagle

Departments of Physics and Biological Sciences, Carnegie Mellon University, Pittsburgh, PA (U.S.A.)

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The neutral buoyancy method of obtaining absolute specific volumes of lipid in multilamellar dispersions is critically investigated. Control experiments show that there is no preferential partitioning of ^2H_2O vs. H_2O into the liposomes, and several thermodynamic properties of the samples, such as the enthalpy change and the volume change of the main transition, are changed very little with deuteration of the solvent. The assumption that the molecular volume of the solvent in the interlamellar space is essentially the same as in bulk solution is discussed; and it is shown to introduce rather small corrections. Previous procedures have been modified to avoid possible kinetic limitations in phases with low water permeability. It is concluded that the molecular volume of lipid in bilayers can be obtained to an accuracy better than 0.002 nm^3 (2Å^3) which is less than 0.2% of typical molecular volumes of lipids.

Introduction

The volume occupied by lipid molecules is an important quantity for understanding and characterizing biomembranes. Comparing volumes for different systems is a way of organizing our understanding of large classes of lipids and alkanes, as has recently been emphasized by Small [1]. In this laboratory volumetric data have been essential in order to obtain the Van der Waals cohesive interaction energy, which is a major contribution to the enthalpy of the main chain-melting transition [2–4].

It is customary and most appropriate in physical chemistry, when dealing with solutions, to report the partial specific volume which is defined as the incremental change in volume per g added solute [5]. The less fundamental alternative is to report the apparent specific volume which is just the total volume of the solution minus the volume

* Present address: Medical Physics, Allegheny Singer Research Institute, Pittsburgh, PA 15212, U.S.A.
** Present address: Department of Physics, Hobart and William Smith Colleges, Geneva, NY 14456, U.S.A.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DLPE, dilaurylphosphatidylethanolamine; DSC, differential scanning calorimetry; v_M, the specific volume measured by the neutral buoyancy technique; v_s, the specific volume of the $H_2O/^2H_2O$ solvent; V_L, the volume of a single lipid molecule in the bilayer; V_X, the volume of a single lipid plus its associated n_W waters; V_S, the volume/molecule of the excess solvent; V_W, the volume/molecule of the solvent between the bilayers; m_L, the molecular weight of the lipid; m_S, the molecular weight of the $H_2O/^2H_2O$ excess solvent; m_W, the molecular weight of the solvent between the bilayers; n_W, the number of waters per lipid molecule between the bilayers; C phase, the $L_{C'}$ or crystalline subgel phase; G phase, the $L_{\beta'}$ or gel phase; R phase, the $P_{\beta'}$ or ripple phase; F phase, the L_α or fluid chain-melted phase.

Correspondence: J.F. Nagle, Departments of Physics and Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213, U.S.A.

of the pure solvent [6]. However, for bilayer dispersions in excess water there is no essential difference between partial and apparent specific volumes. For lipids the critical concentration for bilayers is only approx. 10^{-9} M [7] so that for even modest concentrations of lipid of order 0.1% by weight nearly all the lipid molecules are in bilayers. As more lipid is added, some of the excess water is incorporated between the bilayers, but properties of the bilayers remain the same until all the excess water is removed [8–11]. Accordingly, the volumes that are reported and discussed in this paper will be called just specific volumes rather than partial or apparent specific volumes. It may also be noted that, as more lipid is added, eventually there is no excess water and one again has to be concerned with partial specific volumes. While such systems without excess water are simpler in so far as it is precisely known how many water molecules there are per lipid molecule in single-phase regions, those systems are further removed from biological interest in which excess water is present because the chemical potential of water in systems with no excess water is altered from its bulk value. This, in turn, must affect the hydrophobic forces responsible for forming bilayers. Furthermore, by Gibbs phase rule, phase transitions in such systems, even with only one lipid component, will take place over a range of temperatures, making it more difficult to guarantee that one is working in a single-phase region. Therefore, this paper deals exclusively with lipid systems in excess water.

This paper will focus upon the technique of neutral buoyancy centrifugation of lipid dispersions in $H_2O/^2H_2O$ mixtures that was first employed for small unilamellar vesicles by Huang and Charlton [12] and has been extended to multilamellar vesicles [2,13]. This technique should not be confused with the differential dilatometric instrument that has been developed in this laboratory [14]. The differential dilatometer measures relative volume changes accurately as a function of temperature or time, but it does not measure absolute specific volumes. Conversion of results from the differential dilatometer to absolute volumes has required a supplementary technique which in this laboratory has been the neutral buoyancy method. The reason for focussing this paper on the neutral buoyancy method is that it has not been previously thoroughly analyzed in the literature and certain controls, that we now realize to be crucial, were either not performed or not well documented.

Materials and Methods

DPPC and DLPE were purchased from Avanti Polar Lipids, Inc. and used without further purification. The sharpness of the main transition to the chain-melted (F) phase is the best assay of sample quality, with a broadened half-width indicative of degradation or contamination. Calorimetric scans yielded half-widths comparable to those previously seen in this and other laboratories, e.g., 0.09–0.14 C° for DPPC. H_2O was deionized and distilled; ^2H_2O was 99.8 atom% (Aldrich).

Phospholipids were dried overnight in a vacuum oven at 65 °C prior to dispersion and appropriate amounts of H_2O and/or ^2H_2O (1–10 ml total solvent) were added to weighed amounts (5–10 mg for neutral buoyancy measurements) of the dried lipids. Visually homogeneous dispersions were formed in one of two ways. DPPC samples were taken five times through the following thermomechanical cycle: suspension in a water bath 10–15 C° above the main phase-transition temperature for several minutes followed by brief vortexing followed by suspension in an ice-water slurry for several minutes followed by brief vortexing. DLPE samples, more obdurate to hydration, were suspended in a water bath 30–40 C° above the main transition temperature for 45 min, then slowly cooled to room temperature. DPPC samples hydrated in this second way showed no differences in measured properties from the first way.

Neutral buoyancy experiments were performed in several ways. For temperatures below 25 °C a refrigerated ultracentrifuge was employed with an acceleration of about $100\,000 \times g$. When the centrifuge was not spinning, its thermometer was carefully calibrated with an external thermistor probe (which itself had been calibrated by comparison to certified reference Hg thermometers). Heating effects due to spinning were determined to be negligible by measuring the sample temperature with this same external thermistor probe im-

mediately after an experiment and comparing to the centrifuge thermometer. Other experiments were carried out with a small desktop centrifuge (acceleration about $1000 \times g$) equilibrated in various constant temperature rooms to ± 0.4 C° over the course of the experiment. Heating corrections due to spinning were made by measuring the temperature of the samples immediately following centrifugation. Finally, some experiments were performed using a constant-temperature water bath (± 0.2 C°) with no centrifugation. It may be noted that rather coarse temperature regulation is all that is required to produce the accuracy reported in this paper because the coefficient of expansion of most lipids is less than 0.001 ml/g per degree.

A vibrating-tube densimeter (Sodev Inc. Model 02D) with a Fluke 1900 A multi-counter was employed in the $H/^2H$ partitioning control experiment. The densimeter output is the resonant frequency of a tube containing the sample solution, which is uniquely related to the absolute density of the solution [15]. Calorimetry was done on a Microcal MC-1 differential scanning calorimeter, scanning at 10 C°/h, interfaced to an Apple II^+ computer for real-time digital data acquisition. Enthalpies were obtained by computer integration. The dilatometer, previously described [14], was scanned at 4.8 C°/h.

Theory of the neutral buoyancy method

The neutral buoyancy method is based upon the simple principle that an object that neither sinks nor floats (nor swims) has the same density or specific volume, v_m, as the fluid in which it is immersed. Obtaining the condition of neutral buoyancy (neither sinking nor floating) of a given object requires changing the specific volume of the excess solvent, v_S, to achieve $v_S = v_M$. Neutral buoyancy can be achieved for many lipids by dispersing them in the appropriate mixture of ^2H_2O and H_2O. It should be noted that it has been shown that ^2H_2O and H_2O mix very nearly perfectly, so v_s is easily determined by knowing the mass fractions of light and heavy water used [16,17]. (Incidentally, there is not just ^2H_2O and H_2O but ^2HHO in the solvent, so it is best to refer to ^2H and H concentrations.) Since it is unlikely that exactly the correct ratio for neutral buoyancy will be found, v_M of the lipid is determined to lie between the specific volume of the densest solvent in which the lipid sinks and the least dense solvent in which the lipid floats. In practice, the determination of sinking or floating of a sample that is near neutral buoyancy is expedited by spinning in a centrifuge. The density gradient in the solvent is only about 0.0004 g/cm^4 for an acceleration of $100\,000 \times g$, and about 100-times smaller for acceleration of $1000 \times g$, so most samples either completely sink or float. Sensitivity is also limited by temperature fluctuations in the sample. In this laboratory the typical sensitivity for the determination of neutral buoyancy is ± 0.001 ml/g.

Liposomes of many neutral lipids such as DPPC essentially consist of stacks of bilayers separated by interlamellar water. On average each lipid molecule with volume V_L and molecular weight m_L is associated with n_W solvent molecules, each with molecular volume V_W and molecular weight M_W. It then follows with no assumptions from the principle of neutral buoyancy that

$$v_M = 0.6023 \frac{V_L + n_W V_W}{m_L + n_W m_W} \tag{1}$$

The factor 0.6023 is Avogadro's number times 10^{-24}, which converts from the units cm^3/g, in which v_M and all other volumes with a lower case v will be given, to the units Å^3 per molecule, in which V_L, V_W and all other volumes with a capital V symbol will be given.

The assumption has been made [2] that the interlamellar solvent is likely to have the same density as the excess bulk solvent with molecular volume V_S and molecular weight m_S. In symbols this assumption is written

$$v_M \equiv 0.6023\, V_S/m_S = 0.6023\, V_W/m_W \tag{2}$$

where the first equivalence is just the definition of v_M in terms of the excess solvent. Combining Eqn. 2 with Eqn. 1 yields

$$v_M = 0.6023\, V_L/m_L \tag{3}$$

which has the advantage over Eqn. 1 of determining V_L directly from the measurement of v_M and

not involving interlamellar water terms that may be difficult to measure.

In this paper we wish to investigate the transition from Eqn. 1 to Eqn. 3 more thoroughly. To facilitate this we will divide the assumption that the density of the interlamellar water equals that of the bulk water into the next two assumptions.
(1) The water between the bilayers has the same deuterium concentration as the excess solvent, i.e., $m_W = m_S$.
(2) The volume per molecule of the interbilayer solvent is the same as that of the excess solvent, i.e., $V_W = V_S$.

We will also investigate two additional assumptions that bear upon the reliability of the neutral buoyancy method for obtaining absolute specific volumes.
(3) The replacement of H_2O by ^2H_2O does not affect the specific volume of the membrane.
(4) The interbilayer solvent equilibrates quickly upon the time-scale of the measurements with the excess solvent.

Results and Discussion

It is possible that there may be preferential partitioning of deuterium/hydrogen into the interbilayer aqueous space, so that $m_W \neq m_S$ in contradiction to assumption (1). To test assumption (1) 1 g of DPPC was dispersed in a mixture consisting of 1 g ^2H_2O and 1 g H_2O. This amount of solvent suffices to hydrate fully any phase of DPPC, since hydration levels consist of about 17% water for the C phase of DPPC and less than 50% water for the F phase [18]. If there were preferential partitioning of ^2H or H between the bilayers, the composition of the excess solvent would differ from that of the original 1/1 mixture used to disperse the lipid, and this difference would be larger if less solvent were used to disperse the lipid. Following brief centrifugation of the lipid dispersion into the lower portion of the tube, approx. 0.4 ml of excess solvent supernatant was extracted from the upper portion of the sample tube, loaded into the vibrating-tube densimeter, and its resonant frequency measured. The error of the densimeter experiment is $\pm 1.8\%$ mass fraction of $H/^2H$, which, for a dispersion of lipid that consists of 50% interlamellar water, converts to a maximum (and probably highly overestimated) error in v_M of 0.002 ml/g. $H/^2H$ partitioning in the G phase of DPPC was investigated in detail in this fashion with the result that the supernatant was identical in $H/^2H$ composition within the above-quoted error to the original dispersing solvent. Therefore, assumption (1) ($m_W = m_S$) appears to be a good one.

Already, without requiring assumption (2), an important relation can be obtained by replacing m_W by m_S in Eqn. 1 and using the definition of v_M (only the first part of Eqn. 2) to yield

$$v_M = 0.6023 \frac{V_X - n_W V_S}{m_L} \tag{4}$$

where $V_X = V_L + n_W V_W$ is the unit cell volume. Of the three experimental quantities, n_W, V_X and v_M (m_L is known), the easiest to measure is V_M. For a few well-ordered phases [18–20] V_X has been obtained by diffraction methods and then Eqn. 4 can be used to obtain n_W from v_M and V_X. This is useful because hydration numbers, n_W, have often been reported, but results have not been very consistent, as will be discussed in some detail in a subsequent paper. In most cases it has not been possible to obtain V_X directly from diffraction. Then, Eqn. 4 may be used with measured values of v_M and n_W to obtain V_X.

We now turn to discuss the possibility that the volumes per solvent molecule are different between bilayers than in excess solution, contrary to assumption (2). Let us assume, for the moment, that $V_W \neq V_S$, and show how this complicates the interpretation of the measurement of v_M by replacing V_X by $V_L + n_W V_W$ in Eqn. 4 to obtain

$$V_M = 0.6023 \frac{V_L + n_W (V_W - V_S)}{m_L} \tag{5}$$

Clearly, Eqn. 5 is equivalent to Eqn. 2 if and only if $V_W = V_S$.

Determination of V_W is not straightforward even if one had a detailed static structure of the interfacial region because it is somewhat arbitrary where one draws the dividing surface between the headgroups and the interbilayer water. Any modifications of the dividing surface will simply be reflected in altered values of the lipid volume, V_L, with the constraint that $V_X = n_W V_W + V_L$ be conserved. Under these circumstances, $V_W = V_S$ might

be considered to be a convention rather than an assumption. In discussing this it is useful to make some estimates of the magnitude of the volumes under consideration and to determine the consequences of this assumption/convention on other results.

Let us first obtain some estimate of the magnitude of the residual uncertainty in V_W associated with drawing the dividing surface. Since the area per lipid molecule in bilayers is of the order of 50 $Å^2$, an uncertainty of 0.5 Å in drawing the interface amounts to at least 25 $Å^3$ uncertainty in V_L. If $n_W = 25$, this amounts to an uncertainty of 1 $Å^3$ in V_w compared to a value of $V_S = 30$ $Å^3$ at 20 °C.

Next, we will estimate how much volume change is likely in the headgroup region due to changes in the effective concentrations of the headgroups in the interfacial water which occur when there are phase changes which change n_W. Such changes might be expected to be small, since the headgroups are fully hydrated and the water would be expected to fill up any voids between headgroups, but electrostrictive and hydrogen bonding effects are certainly concentration dependent. Of course, it is impossible to separate the volume changes due to headgroups from those due to chain packing in lipids. However, it is possible to obtain reasonable estimates of the headgroup effect by considering volumetric data for headgroup-like molecules in water as a function of concentration. First, let us recall earlier estimates [2] which used measurements of the volume of solutions of ethanol, fructose, glycerol, and phosphoric acid at concentrations typical of the concentrations of the headgroups in the interfacial water. Per lipid molecule the volume change of the headgroup from this concentration effect was estimated to be about -1 to -4 $Å^3$ upon going from the gel phase to the fluid phase of DPPC, for which n_W goes from about 15 to 25. The calculation has been repeated with the solution data previously utilized, along with data [21] from additional organic solutes, among them formic acid, lactic acid, glucose and sucrose. Furthermore, if the hydration values of Ruocco and Shipley [18] for G and F phases, 19 and 25 water molecules/DPPC, respectively, are used, the volume change would be approx. -1 ± 1 $Å^3$ per lipid through the transition. This is much smaller than the residual uncertainty in drawing a dividing surface. It is also much smaller than the typical volume change at the chain melting transition, which is 45 $Å^3$ for DPPC. Nevertheless, one may wish to keep this in mind as a possible secondary effect which may become even more important for lipids and phases with smaller n_W. Keeping such an effect along with the convention that $V_W = V_S$ would mean that the headgroup volume V_H would change with different hydration levels, becoming slightly smaller in higher temperature phases that have more water. If this effect is ignored, then V_H would be required to be the same value in all phases. In either case, Eqn. 3 is recovered.

A different kind of concern with the buoyancy method of obtaining specific volumes (as well as with many NMR experiments) is that the bilayer properties may change with deuteration of the water, contrary to assumption (3). If this were true, then, while one would indeed measure V_L using the buoyancy method, V_L itself could depend upon the degree of deuteration of the solvent. While it is conventional wisdom that deuteration of the solvent is a very mild perturbation, it is true that the bulk properties of ^2H_2O are measurably different from those of H_2O, though most differences are rather small. In addition, a recent calorimetric study [22] reported that the properties of the chain-melting phase transition in lecithins change upon deuteration of the solvent. In particular, the main transition temperature of DPPC dispersed in ^2H_2O was reported to increase by 0.4 degrees and the enthalpy of transition, ΔH, to decrease by about 8.4 kJ/mol. The percentage increase in the transition temperature is only 0.1% on a Kelvin scale which appears consistent with only a very small perturbation due to deuterating the solvent. However, the percentage change of ΔH is nearly 25%, which would indicate a very substantial perturbation. Due to the potential significance of this result, DSC on DPPC in ^2H_2O has been performed in this laboratory independently by two different workers with a 2-year interval between measurements. We confirm the increase in transition temperature T_M but find that the difference ΔH in ^2H_2O vs. H_2O is 0.0 ± 1.68 kJ/mol for the main transition. We have also performed differential dilatometry on DPPC in ^2H_2O and again find the small increase in T_M but

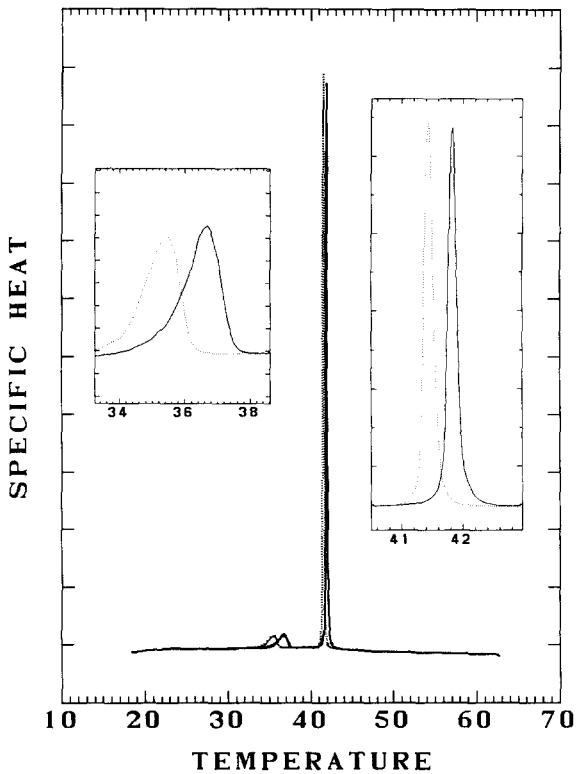

Fig. 1. Relative specific heat (J/g per degree) of DPPC as a function of temperature (° C). The solid curves were obtained for DPPC in ^2H_2O; the dotted curves for DPPC in H_2O. One tick mark equals 21 J/g per degree for the primary plot and the main transition insert; for the pre-transition insert one tick mark equals 0.84 J/g per degree.

negligible difference in the volume change of the main transition.

Our DSC results on the pre-transition agree within experimental error with the previous results [22], namely the transition temperature of DPPC in ^2H_2O is increased by 1.2 C°, with ΔH unchanged. These results on the pre- and main transitions are presented in Fig. 1. Also, the enthalpies of the subtransition are the same for DPPC dispersions in H_2O or ^2H_2O, with the final C phase taking roughly twice as long to form in ^2H_2O [23].

Our most direct control to test assumption (3) is to compare the results obtained from our differential dilatometer with the results from the buoyancy method, as has already been reported before [2,14] and this control has been repeated for this report. This involves measuring the difference in absolute specific volumes at two different temperatures using the buoyancy method and comparing to the measured difference in specific volumes using the differential dilatometer. In the high-temperature fluid phase of DPPC less than 15% ^2H_2O is required to achieve neutral buoyancy, so any perturbative deuteration effect should be small. In contrast, in the gel and crystal phases, the solvent must be over 60% and 90% ^2H_2O, respectively, for neutral buoyancy, so the putative deuteration effect should be much larger. However, when the volume differences are compared to what is measured directly with the differential dilatometer on samples dispersed in ordinary H_2O, the results agree to within 0.001 ml/g when the total volume change is 0.107 ml/g as seen in Fig. 2. For convenience the data in Fig. 2 are also given in more precise tabular form in Table I which is in good agreement with the earlier data for DPPC given in Ref. 2. We conclude that

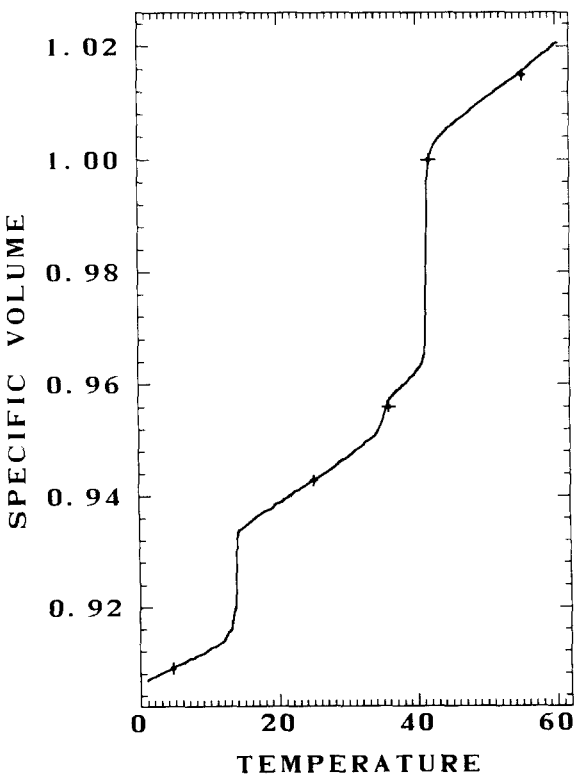

Fig. 2. Specific volume (ml/g) of DPPC as a function of temperature (° C). The solid dots are the values obtained using the neutral buoyancy technique at five different temperatures. The solid curve was obtained for a sample in H_2O using the differential dilatometer; the solid curve was pinned at 25.1° C.

TABLE I

VOLUMETRIC PROPERTIES OF DPPC

ΔV is the volume change at the midpoint temperatures; α is the coefficient of thermal expansion; $\Delta T_{1/2}$ is the width of transition for half ΔV.

Phase	Transition	T [a] (°C)	$T_{midpoint}$	v_M [a] (ml/g)	ΔV (μl/g)	α [a] (per degree)	$\Delta T_{1/2}$
Subgel (C)		7		0.911		$(67 \pm 10) \cdot 10^{-5}$	
	sub (CG)		13.8		18 ± 1		0.3
Gel (G)		24		0.942		$(90 \pm 7) \cdot 10^{-5}$	
	pre (GR)		35.3		4 ± 1		1.2
Ripple (R)		38		0.960		$(140 \pm 15) \cdot 10^{-5}$	
	main (RF)		41.4		38 ± 2		0.16
Fluid (F)		55		1.015		$(94 \pm 7) \cdot 10^{-5}$	

[a] Representative values.

assumption (3) is a good one and that V_L obtained via Eqn. 3 does not depend significantly upon the degree of deuteration of the solvent.

It is sometimes convenient, if one has limited sample available, to disperse only one lipid sample for neutral buoyancy and to add successively ^2H_2O or H_2O until neutral buoyancy is nearly achieved. From the known permeability of lipid bilayers to water, $P = 6.3 \cdot 10^{-4}$ cm/s in the fluid phase [24], reasonable estimates of liposome sizes $z \approx 10$ μm, and the formula $\langle z^2 \rangle = 2(PD_B)t$, where D_B is the bilayer thickness and PD_B is the effective diffusion constant for water transport across stacks of bilayers, an equilibration time of approx. 30 min is obtained, suggesting that assumption (4) is reasonable for the fluid phase. Since the permeability is about 100-times smaller in the gel or crystal phases [24] the equilibration time could be comparable to the time between buoyancy measurements, although cracks in these phases of the liposomes would probably develop and this would speed up equilibration. Fortunately, assumption (4) is not essential to the neutral buoyancy method of obtaining specific volumes of lipids because a different procedure is now followed that completely avoids requiring this assumption. Many sample tubes are prepared with different ratios of $H_2O/^2H_2O$, sealed, and never altered [13]. Besides avoiding the equilibration problem posed by subsequent additions, this procedure has the advantage that the solvent does not evaporate or exchange with water vapor in the outside air.

Some additional precautions to take in using the neutral buoyancy method to obtain specific volumes are illustrated by the lipid DLPE which presents two special problems. The first problem is that the gel phase sinks in ^2H_2O at temperatures below 25 °C. In order to compare our v_M to X-ray results at 20 °C we first measured the relative specific volumes as a function of temperature on the differential dilatometer. We then performed a buoyancy measurement at 37 °C at which temperature DLPE does not sink in ^2H_2O (nor float in H_2O). As shown in Fig. 3, the absolute specific volume at this temperature suffices to convert the results from the differential dilatometer to absolute specific volumes, including those results at 20 °C. The second problem with DLPE is that, when incubated at low temperature for extended periods of time, it forms a C phase that is thermodynamically stable up to approx. 43 °C [25–27]. Other workers observe an additional C phase melting at 35 °C [28,29]. We were initially concerned that the DLPE sample might spontaneously convert into the C phase(s) and that buoyancy measurements at 37 °C would then be for a poorly characterized mixture of C and F phases. Studies using the differential dilatometer showed that this was not a problem for our samples of DLPE. Starting from above 45 °C, at which temperatures the fluid phase is absolutely stable, the temperature was scanned at −4.8 C°/h to 15 °C. No transition was seen at 43 °C or 35 °C. The usual F-to-G (main chain ordering) transition at 30 °C was observed. Also, no volume change was observed as a function of time when the

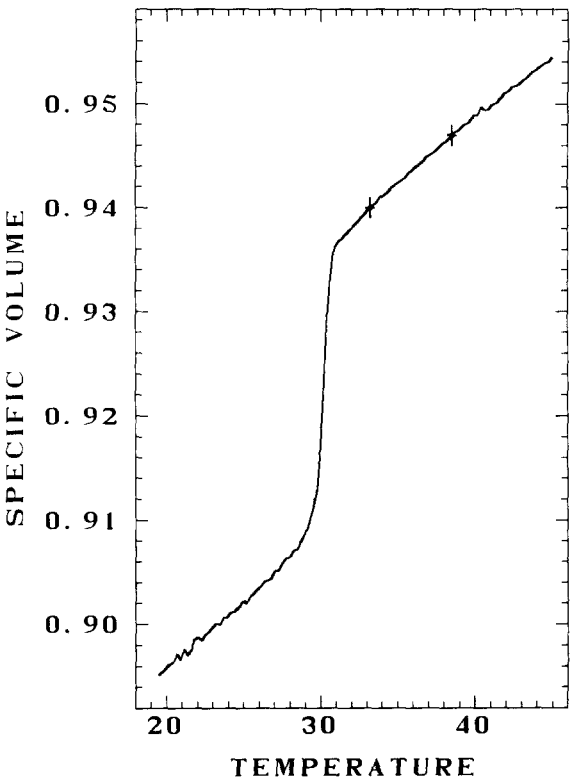

Fig. 3. Specific volume (ml/g) of DLPE as a function of temperature (°C). The solid dots are the values obtained using the neutral buoyancy technique at two different temperatures. The solid curve was obtained for a sample in H_2O using the differential dilatometer; the solid curve was pinned at 38.5°C.

sample was held at 15°C. Subsequent heating scans revealed only the G-to-F transition, with no other transitions discernible. Therefore, we find for DLPE that the value of $v_M = 0.942$ ml/g at 35°C pertains to the F phase and the value $v_M = 0.896$ ml/g at 20°C pertains to the G phase.

In conclusion, it appears that the neutral buoyancy method of obtaining the absolute specific volume V_L of lipids via Eqn. 3 is valid. The experimental errors in V_L are directly related to the errors in v_M. Since v_M for most lipids are in excess of 0.900 ml/g, the experimental error of 0.001 ml/g results in an error of 0.11% in V_L. Since V_L for most lipids are less than 1500 Å^3, the error in V_L is typically less than 2 Å^3.

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