# EFFECTS OF LIPOPHILIN ON DIMYRISTOYLPHOSPHATIDYLCHOLINE BILAYERS

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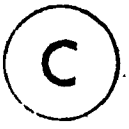

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# EFFECTS OF LIPOPHILIN ON DIMYRISTOYLPHOSPHATIDYLCHOLINE BILAYERS

To my wife Barbara,
whose love, patience and
understanding has been an
inspiration throughout this work.

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Abstract

In this study the technique of scanning densimetry was used to investigate the effects of lipophilin, an integral myelin protein, on the packing and phase transitional properties of dimyristoylphosphatidylcholine bilayers. The technique was shown to give both precise and accurate measurements of the volume properties of the system.

The phase transition of phospholipids from gel to liquid crystalline state is accompanied by a 3% increase in volume. Lipophilin does not significantly alter the phase transition temperature of 24°C of the pure lipid. However, the phase transition is broadened when the lipophilin concentration is greater than 10% by weight. This can be explained by a decrease in the cooperativity of the lipid transition induced by the protein.

As the percent lipophilin incorporated into the lipid is increased, there is a reduction in the magnitude of the change in partial specific volume which occurs at phase transition temperature. From this data, the number of lipid molecules surrounding each lipophilin molecule was calculated. The calculated value of 19 lipid molecules per molecule of protein agrees well with estimates based on calorimetric results.

The partial specific volume of lipophilin has never been reported. In this study it was determined in a number of solvents at 20°C. The partial specific volume of lipophilin in water was 0.715 ± 0.004 ml/g while the partial specific volume in the buffer system employed was 0.732 ± 0.003 ml/g. These results are explained in terms

of differences in the solvation of the protein.

Assuming no large change in the partial specific volume of the protein upon incorporation into lipid, the results demonstrate that lipophilin causes a reduction in the volume of dimyristoylphosphatidylcholine bilayers below, above and near the phase transition temperature. This may be a fundamental property of boundary lipid that surrounds protein .

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## List of Abbreviations

| | |
|---|---|
| Ala | alanine |
| $\alpha$ | coefficient of expansion |
| Arg | arginine |
| ATP | adenosine 5'-triphosphate |
| BSA | bovine serum albumin |
| $\Delta\bar{v}$ | change in partial specific volume |
| CD | circular dichroism |
| Cys | cysteine |
| ($^3$H)-DIDS | 4,4'-diisothiocyano-2,2'-ditritiostilbene disulfonic acid |
| DMPC | dimyristoylphosphatidylcholine |
| DMPG | dimyristoylphosphatidylglycerol |
| EDTA | ethylenediaminotetraacetic acid |
| ESR | electron spin resonance |
| EYPC | egg yolk phosphatidylcholine |
| f | frequency |
| Glu | glutamic acid |
| Gly | glycine |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| Leu | leucine |
| MS | multiple sclerosis |
| NMR | nuclear magnetic resonance |
| ORD | optical rotatory dispersion |

| | |
|---|---|
| Phe | phenylalanine |
| PLA | proteolipid apoprotein |
| POPC | 1-palmitoyl-2-oleoylphosphatidylcholine |
| Pro | proline |
| $\rho$ | density |
| SDS | sodium dodecyl sulfate |
| Thr | threonine |
| TLC | thin layer chromatography |
| Val | valine |
| $\bar{v}$ | partial specific volume |

## Introduction

Multiple sclerosis (MS) is a disease affecting the mature central nervous system in which more than one lesion is found, spread out in time and space. The course of the disease can be remarkably variable in each affected individual, but there are usually frequent successive relapses with periods of remission. The damage is largely restricted to the white matter of the central nervous system where lesions consist of fibrous plaques of demyelinated nerve tract, in which the axons are frequently spared. Even though the disease is widespread throughout the central nervous system, there seem to be preferred sites for these plaques (Adams, 1977; Cuzner and Davison, 1979).

Although there is no known cause as yet for MS, the major factor responsible for the symptoms is the demyelination of the nerves. Therefore before one can interpret and understand the disease state, the normal myelin must first be discussed.

Myelin is a membrane characteristic of the vertebrate nervous system, laid down in segments along selected nerve fibers, functioning as an insulator to increase the velocity of stimuli being transmitted between a nerve cell body and its target. Structurally myelin is recognized as a lipid bimolecular leaflet, sandwiched between two layers of protein and wrapped in a spiral fashion around a segment of axon. This myelin sheath consists of up to 40 lipoprotein lamellae encircling the axon (Raine, 1977; Cuzner and Davison, 1979; Braun et al., 1980).

The composition of the myelin sheath can be broken up into

two major components: lipid and protein. The myelin sheath contains, in significant quantities, a greater variety of bipolar lipids than most membranes. Approximately 70% of the myelin dry weight is lipid (Cuzner and Davison, 1979). Even with this large amount of lipid there are only small variations in myelin composition in different species and in different areas of the central nervous system. The membrane has a lipid molar ratio of cholesterol:phospholipid:galactolipid varying between 4:3:2 and 4:4:2 (Norton, 1977). Cholesterol is the most abundant lipid in myelin. Since the lipids in myelin are all above their phase transitions at physiological temperatures, cholesterol decreases the fluidity of the membrane. Although not exclusive to myelin, the most characteristic lipid is the galactolipid, cerebroside. Its concentration is directly proportional to the amount of myelin in the brain (Norton and Poduslo, 1973). Another characteristic marker is ethanolamine plasmologen. The ethanolamine phospholipid in the vinyl form is located almost exclusively in the white matter (Cuzner et al., 1965). Small amounts of polyphosphoinositides and gangliosides are also present in myelin (Eichburg and Dawson, 1965). The monosialoganglioside $G_{M1}$ accounts for 80-90 mole % of myelin ganglioside in the rat (Suzuki et al., 1968), but an unusual ganglioside, sialosylgalactose-ceramide ($G_7$) is the major component of human myelin gangliosides (Ledeen et al., 1973). Despite the large amount of detailed comparative data on lipid composition of myelin from many sources, very little is known about the distribution of these lipids in the two halves of the bilayer, or the amounts and types of lipid that interact selectively with each of the myelin proteins. This gap in knowledge stems mainly from the way native

myelin is found in the myelin sheath. The spiral fashion in which the sheath is made makes it difficult for any extensive biochemical analysis to be carried out on intact native myelin.

Central nervous system myelin has a uniquely simple protein constitution, yet its major proteins include both an intrinsic and an extrinsic type of membrane protein. This makes it extremely useful for studying the effects of both types of proteins on membrane structure.

The extrinsic protein is known as the basic protein of myelin (Kies, 1965), the encephalitogenic protein or the $A_1$ protein (Eylar et al., 1971). This protein comprises about 30% of the myelin proteins. The myelin basic protein has been independently isolated in a number of laboratories (Roboz-Einstein et al., 1962; Lowden et al., 1966; Tomasi and Kornguth, 1967; Eylar et al., 1969; Carnegie, 1971). Isolation from spinal cord involves preliminary extraction with acetone/petroleum ether followed by isolation of the protein from the residue with 5-10% KCl (Roboz-Einstein et al., 1962). In other procedures the tissue is defatted first with chloroform/methanol (Kies et al., 1965; Wolfgram, 1965; Nakoa et al., 1966; Carnegie et al., 1967; Tomasi and Kornguth, 1967; Kibler and Shapiro, 1968).

The amino acid sequence of human myelin basic protein is known (Carnegie, 1971) and reveals some interesting features. Cysteine is completely absent and 25% of the residues are basic in nature, i.e. lysine, arginine or histidine (Lowden et al., 1966). There is an arginine residue at position 107 which is near a proline rich region, Pro-Arg-Thr-Pro-Pro-Pro. This region may induce a sharp bend in the molecule, giving it an overall hairpin shape which is in agreement with

the axial ratio of 10:1 (Brostoff and Eylar, 1971; Epand et al., 1974). The secondary and tertiary structure is not completely known but the protein seems to be without much α-helical or β structure (Eylar and Thompson, 1969; Palmer and Dawson, 1969; Choa and Einstein, 1970). X-ray scattering data show the protein to be rodlike in structure with dimensions of 15 by 150 Å (Epand et al., 1974).

The location of myelin basic protein in intact myelin has been investigated. Poduslo and Braun (1975) and Golds and Braun (1976) concluded that the protein was present on the inner cytoplasmic surface of the membrane or the major dense line. Wood et al. (1975 and 1977), using a non-penetrating reagent found that lipophilin was ten times more labelled than basic protein. Histochemical (Adams et al., 1971), immunochemical (Lennon et al., 1973) also showed that the basic protein is located at the cytoplasmic surface of the membrane while other studies suggest it may also be located at the outer surface of the sheath (Dickenson et al., 1971; Wood et al., 1974).

Myelin basic protein has been used extensively to elucidate the effects it has on synthetic bilayers. Numerous studies have been undertaken, but since this protein is not the one used in this project only the results will be presented here.

Experiments with myelin basic protein-phospholipid complexes show that it preferentially interacts with acidic phospholipids (Palmer and Dawson, 1969; Banik and Davison, 1974; Jones and Rumsby, 1977), but can also cause phase separation of lipids such as phosphatidylserine,

phosphatidylglycerol and phosphatidic acid in mixtures with phosphatidylcholine (Boggs et al., 1977). There are 10-20% 'acidic lipids' in the native myelin that provide the domains for the myelin basic protein-lipid electrostatic interactions for this lysine and arginine rich protein. However an examination of the sequence reveals 14 segments of hydrophobic and/or uncharged residues (Stollery et al., 1980). There is considerable evidence that some of these hydrophobic segments may be able to interact with the hydrocarbon region of the bilayer, either by partially penetrating or by deforming the bilayer enough to allow hydrophobic contacts at the lipid-water interface. The degree of this penetration depends on the lipid polar head group (Demel et al., 1973; London and Vossenburg, 1973; Boggs and Moscarello, 1978a; Boggs et al., 1980; Stollery et al., 1980).

The protein also increases the permeability of lipid vesicles to glucose (Goulds and London, 1972), as well as to sodium (Papahadjopoulos et al., 1975a). The fact that the protein is partially protected from enzymatic hydrolysis when interacting with lipid (London et al., 1973; London and Vossenburg, 1973) also lends credibility to the conclusion that the protein is partially embedded in the bilayer. In complexes with lipid, this protein also decreases the enthalpy and temperature of the lipid phase transition (Papahadjopoulos et al., 1975a).

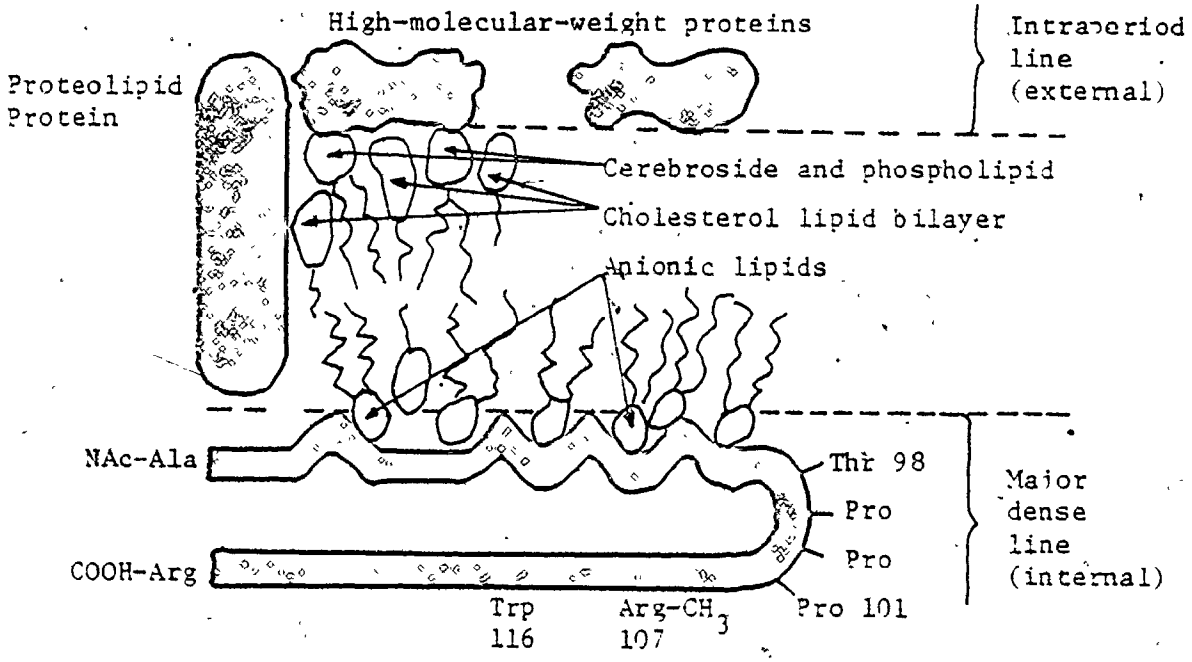
The above results describe the effects of myelin basic protein on lipids and suggest a role for the protein in natural myelin. The protein tends to stabilize whole myelin by its partitioning in regions between folded myelin regions (Maten et al., 1973) where it can organize

specific lipids into double bilayer repeating units as illustrated below (Figure 1). These features suggest a structural role in either the formation (Davison, 1970) or maintenance (Rumsby and Crang, 1977) of the compact multilamellar structure of native myelin.

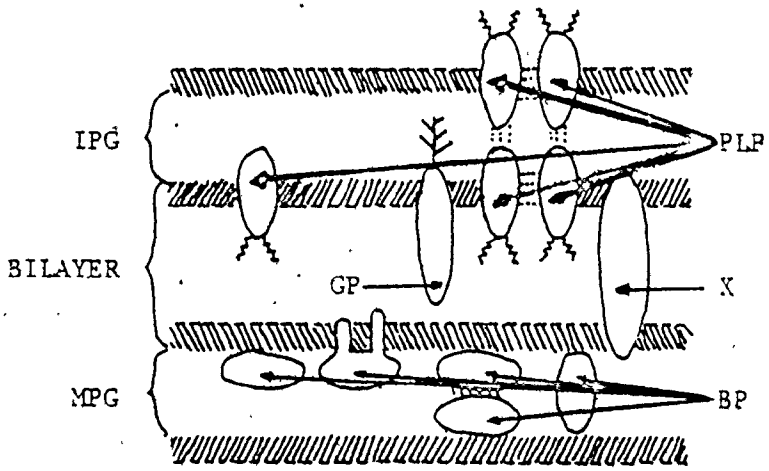
Folch and Lees (1951) described the presence in brain tissue, of a protein material insoluble in water, but soluble in chloroform-methanol mixtures. Since no other known protein was soluble in these mixtures it was assumed that the protein involved was combined with a lipid moiety that gives the complex its particular lipid-like solubility. The term proteolipid was coined to describe these complexes. There are a number of proteins in the proteolipid fraction. The proteolipid apoprotein (PLA), which is the delipidated form of the white matter proteolipid, was later shown to be the most abundant protein in the myelin sheath (Autilio et al., 1964; Conzalez-Sastre, 1970). Many references to other proteins in the proteolipid fraction can be found elsewhere (Gagnon et al., 1971; Miyamoto and Kakiuchi, 1974; Chan and Lees, 1974).

There are many different methods used to obtain the proteolipid protein fraction, including dialysis in organic solvents and Sephadex LH-20 chromatography. However the methods reviewed by Folch-Pi and Stoffyn (1972) have not yielded a single protein, so the proteolipid of different workers may vary in composition, depending on the method of preparation. Some of the properties of this protein fraction have been reported by Folch-Pi and Stoffyn (1972). By comparing the amino acid analysis of their protein fraction with the purified proteins of Gagnon et al. (1971) and Nussbaum et al. (1974), one can immediately

Figure 1. Lipid-protein interactions in unit myelin structure.




Lipid-protein interaction in unit myelin structure, (Cuzner and Davison, 1979).




A diagramatic representation of molecular organization in CNS myelin: a cross sectional view illustrating several possible arrangements of myelin proteins. Lipids which possess a configuration in the membrane other than that found in the bilayer are not illustrated in this model. The period spacings are not schematic and are not proportional to the actual periodicity. The hatched zone represents the polar head groups of the bilayer. The bilayer itself is indicated. IPG, intraperiod gap; MPG, major period gap: BP, basic protein; PLP: proteolipid protein with bound fatty acids; X, unspecified proteins, GP; glycoprotein. This diagram does not denote size and conformation, (Braun, 1977).

see several similarities. Differences in overall composition are noted in aspartic acid, serine, alanine, cysteine and histidine. However, these may be attributed to species variation.

The major purified protein from human myelin proteolipid is termed lipophilin (Boggs et al., 1976), previously being called N-2 for convenience (Gagnon et al., 1971). As the name implies, lipophilin is a hydrophobic protein with pronounced affinity for lipids and thus regarded as an intrinsic membrane protein (Moscarello, 1976). To monitor the purity of their preparation, Gagnon et al. (1971) used sedimentation velocity and equilibrium ultracentrifugation. Using the systems of Takayama et al. (1964) and Weber and Osborn (1969) they were able to demonstrate the existence of a single component on polyacrylamide gels at different pH values.

The amino acid sequence of lipophilin is not known, but the amino acid composition does afford some information. Approximately 66% of the amino acid residues are apolar (Gagnon et al., 1971). The N-terminal sequence of a performic acid-oxidized protein ($P_7$) from rat brain has been published (Nussbaum et al., 1974). Also the sequence of several small peptides from enzyme cleavages have been published (Jolles et al.,1977). The N-terminal sequence of lipophilin is similar to that of $P_7$. By automatic sequence analysis the first 23 residues of lipophilin are Gly-Leu-Leu-Glu-Cys-Cys-Ala-X-X-Leu-Val-Gly-Ala-Pro-Phe-Ala-X-Leu-Val-Ala-X-Gly-Leu (Boggs and Moscarello, 1978b).

An interesting structural feature of lipophilin is the presence of about 2% fatty acid covalently bound to the protein. In their review article, Folch-Pi and Stoffyn (1972) describe how the covalently linked

fatty acids were found by other researchers. More than 2% fatty acid is associated with the proteolipid but the attraction is mainly electrostatic and hence these fatty acids can be removed by dialysis. The first reports of non-removable fatty acids on purified lipophilin was made by Wood et al. (1971), who said that glycerol was not found in either acid or alkaline hydrolyzates of the protein. Neither phosphorus nor sphingosine was detected by careful analysis. Soxhlet extraction procedures and the charcoal method of Chen (1967) for the removal of fatty acids from serum albumin, proved unsuccessful for the fatty acids attached to lipophilin, which turned out to be $C_{16}$ and $C_{18:1}$. It was therefore concluded that the fatty acids were covalently bound to the protein. Stoffyn and Folch-Pi (1971) published a similar study to the one of Wood et al. (1971). They showed that 2.2-3.0% fatty acid, which could not be removed by extensive dialysis with chloroform-methanol, remained associated with their proteolipid apoprotein. Choline, inositol, sphingosine and hexosamine were not detected. Glycerol was less than 0.03%. Conversion of the fatty acids to their methyl esters after alkaline hydrolysis by reacting them with diazomethane followed by sodium borohydride reduction, converted them to their corresponding alcohols. This led to the conclusion that the fatty acids were esterified to the peptide chain, probably with hydroxy amino acids (Stoffyn and Folch-Pi, 1971).

The role of the bound fatty acids is not completely known, but it is assumed that they contribute to the hydrophobicity of the protein. The hydrophobicity of the protein is important for the interactions necessary with the hydrocarbon chains of the bilayer. However, several integral membrane proteins do not contain bound fatty acids. Some

authors suggest that this bound fatty acid may be involved in preventing aggregation of the protein in the bilayer. These conclusions corroborate the findings of Moscarello et al. (1973), who found two mol of fatty acid per mol of protein and stated that it contributes to the hydrophobicity of the protein.

Another interesting feature of the protein is the conformational flexibility seen in different environments. Sherman and Folch-Pi (1971) studied the ORD and CD of the brain proteolipid protein. Their water-soluble preparation showed a helical content which varied between 16 and 40%. No random coil was detected by optical rotatory dispersion (ORD), but circular dichroism (CD) showed the protein to be highly $\alpha$-helical in chloroform-methanol, but of low helicity in water.

The protein isolated by Gagnon et al. (1971) was studied by a number of physical techniques. Anthony and Moscarello (1971) prepared a water soluble form of the protein by dissolving it in phenol-acetic acid-water containing 2M urea, followed by dialysis against decreasing concentrations of acetic acid. A $\beta$ conformation or $\alpha$-helix can be obtained depending on the route of dialysis. Neither the $\beta$ conformation nor the $\alpha$-helix form are affected by performic acid oxidation or by the presence of mercaptoethanol during dialysis. Varying the pH from 1.5 - 6.0 also had no effect on the conformation.

Moscarello et al. (1973) took the above studies one step further. The $\alpha$-helical and $\beta$ conformations were confirmed by infrared analysis. They basically confirmed that the protein is highly helical in the presence of phenol. Removal of phenol and urea simultaneously results in a preparation with almost 50% helix.

Recently Cockle et al. (1978a) found lipophilin to be 100%

α-helix in 2-chloroethanol. The helicity drops to 70% when dialyzed into water from 2-chloroethanol.

To determine the molecular weight of the protein, equilibrium ultracentrifugation was carried out in water, 98% formic acid and in 0.01M sodium phosphate buffer containing 0.5% SDS (Moscarello et al., 1973). In formic acid and SDS the molecular weights are 28,000 and 24,000 respectively. The α-helical form, having a molecular weight of 86,000, is a trimer while the β form, with a molecular weight of about 500,000, is an aggregate of the protein.

Although this protein has no enzymatic activity or other dynamic function, it is a hydrophobic protein of the intrinsic type (Papahadjopoulos et al. 1975) and studies of the interaction of this protein with lipid are relevant to other membrane proteins.

Brown and Radin (1969) found that a water soluble form of the proteolipid apoprotein formed recipitable complexes only with acidic lipids. Delipidated proteolipid in a water soluble form was found to increase the surface pressure of lipid monolayers, but only of acidic lipids. This indicates that, once bound, the protein penetrates and can interact hydrophobically with bilayers (London et al., 1974). However, lipophilin can be readily incorporated into neutral lipids as well as acidic lipids, if both lipid and protein are present initially in solubilized form. A number of studies have been reported on the properties of neutral lipid vesicles containing lipophilin (Vail et al., 1974; Papahadjopoulos et al., 1975b; Boggs et al., 1976 and 1977).

The protein formed intramembranous particles in the fracture faces of the vesicles as observed by freeze fracture electron microscopy, regardless of whether the protein was present initially or added to preformed

vesicles. This indicated that lipophilin was embedded in the hydrocarbon region (Vail et al., 1974: Papahadjopoulos et al., 1975b; Boggs et al., 1976).

In an experiment designed to determine if lipophilin spanned the bilayer, single layered protein-lipid vesicles were labelled from the outside with a non-penetrating reagent, ($^{3}$H)-DIDS. A large proportion of the protein was labelled but the results did not suggest that any of the protein completely spanned the bilayer. The fact that it could be labelled indicated that the protein does protrude from the bilayer. This explained why the hydrophobic protein, which clearly penetrated into the hydrocarbon region, could be labelled in intact myelin (Wood et al., 1977).

Lipophilin-lecithin complexes have provided much information about intrinsic protein-lipid interactions using a variety of spectroscopic, x-ray diffraction and calorimetric studies (Boggs et al., 1976 and 1977; Curatolo et al., 1977 and 1978; Boggs and Moscarello, 1978c; Verma et al., 1980).

Boggs et al., (1977), using differential scanning calorimetry found that phosphatidic acid, phosphatidylglycerol and phosphatidylserine exhibit preferential binding of the hydrophobic apoprotein although no dependence upon fatty acid chain length in the C-14 to C-18 range was noted (Boggs and Moscarello, 1978c).

Boggs and Moscarello (1978c) also suggested that lipids with unsaturated trans fatty acids interact less favorably with the apoprotein. Comparison of the effects of the proteolipid on saturated and unsaturated bilayerlipids were made by Curatolo et al. (1978) using

dimyristoylphosphatidylcholine (DMPC) and egg yolk phosphatidylcholine (EYPC). With apoprotein-DMPC recombinants there is greater population of acyl chain gauche conformers in the gel state and a smaller population of gauche conformers in the liquid-crystalline state than for a DMPC bilayer alone. Lavialle and Levin (1980) obtained similar results using DMPC and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). However, by NMR, Rice et al. (1979) showed that with lipophilin-DMPC recombinants there are more gauche isomers below the transition temperature in the presence of protein, but also a more disordered lipid hydrocarbon chain above the transition temperature.

The effect of lipophilin on lipid organization in vesicles has been studied using differential scanning calorimetry, electron spin resonance spectroscopy and circular dichroism. Lipophilin has very little effect on the phase transition temperature of DMPC or DMPG. The phase transition of the lipid is broadened and the enthalpy of the transition is decreased by the protein. This indicates that the cooperativity of the transition is lessened and that less lipid is participating in the phase transition (Papahadjopoulos et al., 1975b). Boggs et al. (1976), using spin-labelled fatty acid probes, showed that lipophilin immobilizes some of the fatty acid chains surrounding itself, similar to what has been shown for other intrinsic membrane proteins (Dehlinger et al., 1971; Jost et al., 1973). This immobilized lipid does not go through the phase transition and hence the enthalpy decreases. The decrease in enthalpy is proportional to the amount of protein added, up to approximately 50% protein by weight. From the decrease in enthalpy it was estimated that approximately 15 lipid molecules are in the boundary layer of each

protein assuming a molecular weight of 25,000 for lipophilin (Boggs et al., 1976; Papahadjopoulos et al., 1978c). Assuming the same molecular weight for the protein, Boggs and Moscarello (1978c), arrived at 21-25 molecules of lipid per monomer of protein. The number surrounding the protein depends on the type of lipid. This boundary lipid has restricted motion on the electron-spin resonance (ESR) time scale but not on the nuclear magnetic resonance (NMR) time scale (Rice et al., 1979). Rice et al. (1979) also suggested that boundary lipid is more disordered than the liquid-crystalline phase lipid. Curatolo et al. (1977) found a new transition two degrees higher than that of pure lipid, but Boggs et al. (1980) were able to show that there was only one transition; that of the bulk lipid. The higher melting component was due to the presence of decomposition products; namely lysophosphatidylcholine.

In this study, the effects of lipophilin on DMPC vesicles is investigated by different techniques. A temperature scanning densimeter (Anton Paar, Graz, Austria) is employed. Results of the densimeter are verified by density gradient ultracentrifugation. Boggs et al. (1976) found that pure lipophilin, dialyzed from 2-chloroethanol, will settle between 40 and 50% sucrose, while pure lipid (DMPC) will settle between 10 and 15% sucrose. Lipid-protein complexes settle somewhere in between depending on the composition. In order to determine if the protein is embedded, other tests are employed on some lipid-protein complexes.

Temperature scanning densimetry is a relatively new technique which combines the extreme precision of density measurements by the oscillator principle with the help of continuous scanning of temperature.

The oscillator principle is based on the determination of the natural frequency of an electronically excited mechanical oscillator whose effective mass is composed of its own unknown mass plus the well defined, but also unknown, volume of the sample under investigation. The mode of vibration is the bending type oscillator, which is designed in such a way as to excite and maintain undamped oscillation of the filled vibrator as the temperature range is scanned.

In its traditional form the oscillator method yields the oscillation period of the density measuring vibrator by comparing it to a highly stabilized quartz crystal frequency. When using the densimeter with only one measuring cell, the T values, (the values expressed on the densimeter), are the absolute periods of oscillation ($^1/_f$), where f is the frequency. When using a second density measuring cell, (used as a reference cell), it becomes a time base for the period measurement of the first measuring cell (the sample cell). In this mode, the relative period T* is obtained. This represents the ratio of the periods of sample and reference cell. When the cells are coupled within the same thermostated circuit, the temperature dependance of the individual cells are almost ideally cancelled out (Kratky et al. 1973).

Possible errors in this technique are limited, but will cause large deviations in results. One such error involves keeping the temperature increase relatively slow so that the sample and reference will be in thermal equilibrium. Another error is caused by residues in the vibrator which can lead to day to day variations. This can be eliminated by careful cleaning of the cells after each experiment. Gas dissolved in the sample or the reference has only negligible effects on density

measurements, but bubbles make the measurement completely worthless. However bubbles can not be missed since they make stable readings impossible.

At present this is probably one of the best methods for density measurements, since it is easy, requires only a small amount of sample and from past studies employing it (Epand and Epand, 1980; Fodor and Epand, 1981), shows a high degree of reproducibility and precision.

## Materials and Methods

1. Materials

Dimyristoylphosphatidylcholine (DMPC) was obtained from Calbiochem. It showed a single spot in TLC using chloroform-methanol-7M ammonia (230:90:15 v/v/v) and was used without furthur purification.

Lipophilin was a kind gift from Dr. M.A. Moscarello, Sick Childrens Hospital in Toronto, Ontario. The myelin used for these preparations was isolated from normal human white matter by the method of Lowden et al. (1966). Lipophilin was isolated from this myelin and purified by chromatography on Sephadex LH-20 in chloroform:methanol (1:1 v/v) containing 5% of 0.1M HCl by the method of Gagnon et al.. (1971) and stored in lyophilized form.

The buffer system used for most experiments consisted of 100mM NaCl, 2mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.1mM EDTA (Ethylenediaminotetraacetic acid) and 0.31mM sodium azide. The buffer is adjusted to a final pH of 7.4.

2. Methods

i) Preparation of lipophilin-containing vesicles.

Pure DMPC was dissolved in a small volume of 2-chloroethanol (5 mg/ml). Pure lipophilin was also dissolved (5 mg/ml) in 2-chloroethanol and sonicated. The 2-chloroethanol was purified by redistillation over charcoal (Cockle et al. 1978c).

When both protein and lipid are solubilized, the two are mixed so as to obtain the desired protein to lipid ratio. Approximately 5 ml of the mixture is dialyzed at 25°C against two liters of buffer for

18 hours with two changes of buffer. The buffer is saturated with $N_2$ and air excluded during dialysis. The resulting suspensions are centrifuged at 6800 x g in a Sorvall SS-34 rotor at room temperature for 20 minutes. The supernatant is decanted off and the pellet resuspended by vortexing into a small amount of buffer.

Some of the experiments with pure lipophilin require a water soluble form of the protein. This was achieved by solubilizing the protein in 2-chloroethanol (5-10 mg/ml) and dialyzing against distilled water (Cockle et al. 1978a). The solubilized protein (approximately 5 ml total solution) was dialyzed for 18 hours against 2 liters of distilled water with two changes. The resulting solutions are then centrifuged for 20 minutes at 6800 x g in a Sorvall SS-34 rotor at room temperature to remove any suspended particles.

ii) Amino acid analysis to determine the protein concentration of the sample.

Approximately 0.3 mg of protein was transferred to a hydrolysis vial with norleucine as an internal standard (10 nmoles norleucine per 40 µg protein). To the hydrolysis vial was then added 1.0 ml of 6M HCl. The samples were then evacuated, sealed and hydrolyzed for 22 hours in a 110°C oven. The samples were then brought to dryness and run on a Technicon TSM amino acid analyzer at the Hospital for Sick Children in Toronto, Ontario.

iii) Phosphate determination to determine lipid concentration of the samples (Folch et al. 1957; Bartlett, 1959; Radin, 1969; Murison et al. 1976).

In this assay the sample sizes should not exceed 150 nmoles of

phosphate. The samples were dried in vacuo and 0.5 ml of 10N $H_2SO_4$ was added to each. The samples were then incubated overnight, uncovered, in a 150°C oven. The following day, 3 drops of 30% $H_2O_2$ were added to each tube and the tubes returned to the oven for another 1.5 hours. If the samples did not become clear by this time 3 more drops of 30% $H_2O_2$ were added to them and they were returned to the oven for another 1.5 hours.

Stock solutions:

a. phosphorus standard (20 µg/ml).

b. 10N $H_2SO_4$

c. 5% w/v ammonium molybdate

d. reducer: grind together and store in dark:

15 g sodium bisulfite

0.5 g sodium sulfite

0.25 g 1-amino-2-naphthol-4-sulfonic acid

A solution containing 1.6 g/10 ml distilled water was freshly prepared the day of analysis.

For the standard curve samples were prepared containing 0,1,2,3,4,6,8, and 10 µg phosphorus. The total volume of each was brought to 4.1 ml with distilled water followed by an addition of 0.5 ml of 10N $H_2SO_4$ to each standard.

For the reaction 4.4 ml of distilled water was added to each sample and 0.2 ml of 5% ammonium molybdate was added to all the tubes (standards and samples). Immediately after vortexing, 0.2 ml of reducing solution was added to each tube, again followed by vortexing. All tubes were then incubated for 10 minutes at 100°C. The tubes were then cooled and the absorbance at 830nm was determined.

iv) Densimetry measurements.

The density difference between the protein-lipid suspensions and the buffer was measured with the use of two DMA 602 external cells using a DMA 60 measuring unit in the phase lock loop mode (A. Paar K.G., Graz, Austria). The temperature of the external cells was controlled by a circulating thermostated fluid from a Neslab RTE-4 temperature bath. The temperature of the bath can be varied at a constant rate using a Neslab ETP-3 temperature programmer. Runs were generally performed at a scan rate of 0.5°C/minute. The temperature was measured by a thermistor probe placed next to the sample capillary in the thermostated fluid. The thermistor was attached to a digital thermometer (Precision Digital). The DMA 60 also provides an analog signal that is proportional to the last two digits of the densimeter reading. The DMA 60 was also attached to a digital printer (model 810, Newport Laboratories Inc.) which allows direct recording of the densimeter reading. The densimeter and the thermistor were connected to two channels of a Bascom-Turner disk-storage microprocessor unit and the data plotted in an x-y, x-t or y-t mode as desired.

Solution densities were measured by a procedure recommended by the manufacturer. All solutions were degassed before measurements were made. A calibration temperature of 20°C was used and the calibration constant k, of the instrument was calculated using the instrument periods T, and the following formula:

$$k = \frac{T^2_{H_2O} - T^2_{air}}{\rho_{H_2O} - \rho_{air}}$$ (Kratky et al. 1973)

where $\rho_{H_2O}$ and $\rho_{air}$ correspond to the densities of water and air respectively. The density of air was corrected for atmospheric pressure changes measured by a Fortin type mercury barometer. Scans were performed with buffer in the reference cell and air, water, buffer and sample suspensions in the sample cell. Densities of the solutions can be calculated relative to water at the desired temperature, according to the following formula: $\rho_{solution} = k(T^2_{solution} - T^2_{H_2O}) + \rho_{H_2O}$ (McDonald, 1977).

With T values read from the scans, the corresponding sample density at any temperature can be calculated from increments of density relative to the density obtained at the calibration temperature. The apparent partial specific volume $\bar{v}$, was obtained from:

$$\bar{v} = \frac{1}{\rho_{buffer}} \left(1 - \frac{\rho_{lipid} - \rho_{buffer}}{C}\right)$$

where $\bar{v}$ is the apparent partial specific volume at a weight concentration C, and $\rho_{lipid}$ is the density of the sample under investigation.

The density measurements must be very accurate since the density difference between sample and reference is very small. With average concentrations of about 5 mg/ml, the corresponding values for $(\rho_{lipid} - \rho_{buffer})/C$ are in the range of $10^{-1}$. The densimeter is capable of measuring densities to $\pm(1 \times 10^{-5})$ g/ml hence allowing for the accurate determination of $\bar{v}$.

The volume change ($\Delta\bar{v}$), at the transition temperature was obtained by extrapolating the variation of $\bar{v}$ with the temperature outside the phase transition region to the transition temperature and measuring the difference in volumes between the high and low temperatures respectively.

The volume coefficient of expansion $\alpha$, given by

$$\alpha = \frac{1}{\bar{v}} \frac{\delta \bar{v}}{\delta T}$$

was calculated from the slopes of the plots of partial specific volume versus temperature.

2-chloroethanol has a density quite different from the samples. Therefore if there was a substantial amount of 2-chloroethanol present after dialysis, the densities of the buffer before and after dialysis would be different. A substantial difference would indicate the presence of 2-chloroethanol in the sample. To ascertain this, the density of the buffer before and after dialysis was determined.

At high protein to lipid ratios, clumping may occur, resulting in large bits of aggregated material in the sample tube. This may give different densimetry readings on the same sample. In order to ascertain what effects this has and to check the precision of the partial specific volume at high protein to lipid ratios, repeated measurements were made on the same sample. Between measurements the sample cell was emptied and cleaned thoroughly.

It was necessary to know the partial specific volume of lipophilin accurately to measure its effects on lipid packing, so this parameter of the protein was determined in a variety of ways. Lipophilin in suspension form, as well as in a water soluble form (Cockle et al. 1978 a and b) was measured densimetrically at different protein concentrations.

Another way to check the partial specific volume of lipophilin densimetrically was to measure it in solutions of the protein in 2-chloroethanol. In addition, lipophilin can be solubilized in chloroform:methanol

(2:1 v/v). After solubilizing lipophilin in this solvent, the protein was dried down under a stream of nitrogen, finally dried in vacuo and resuspended in buffer. Protein concentration was therefore by dry weight determination (Kupke and Dorrier, 1978). Densimetry was used to measure the partial specific volume of the resulting suspensions.

To determine the accuracy of the densimeter, two well characterized proteins, BSA and lysozyme, were run at 25°C to determine their partial specific volumes and compare them to values cited in literature.

v) Density gradient ultracentrifugation.

Samples with high protein to lipid ratios (weight percent) were analyzed by density gradient ultracentrifugation. The gradients were formed by a mechanical gradient maker in cellulose nitrate tubes that fit into an SW27 swinging bucket type rotor. The gradients were usually between 20 and 65% sucrose (w/w). Samples were applied so approximately 1 mg of material was introduced to the gradient. Runs were for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected with a Gilson microfractionator. Fraction sizes were 25 drops. The linearity of the gradient was ascertained by determining the refractive index of each tube.

Protein detection was by a mini Folin-Lowry assay for insoluble proteins (Hess et al. 1978).

To assay for any free lipid that may be solubilized in the gradient or not visually detected, each fraction was extracted with chloroform and the lower phase removed and evaporated to dryness. Phosphate determinations were then performed on the residues.

It was necessary to determine the accuracy of the density gradient

results. To achieve this, a labelled protein with a well characterized partial specific volume was used ($^{125}$I-fibrinogen). The gradients were made so that the labelled fibrinogen was equally dispersed throughout the gradient. A lipophilin suspension was also run with this protein. The tubes were spun in an SW27 rotor at 76,000 x g for 24 hours at 20°C. Fractionation was as described above. $^{125}$I-fibrinogen was counted by a gamma counter. Protein determination was as noted above.

## Results

DMPC has a 3.1% increase in $\bar{v}$ over the main phase transition at 24°C (Figure 2,a). There should also be a premelt transition which is about one order of magnitude smaller than the main transition and occurs at 15°C, however, none is observed. Possible reasons for the lack of a premelt transition in this preparation will be discussed later.

Incorporation of lipophilin into DMPC bilayers leads to some interesting observations. As seen in Figure 2, (b-i) there is very little change in the transition temperature of 24°C regardless of the percent lipophilin incorporated. However, there is a broadening of the transition which is noticeable beginning at 17.8% lipophilin by weight (Figure 2,f) and the transition is almost completely abolished by 50% lipophilin (Figure 2,j). In addition, the change in $\bar{v}$ which occurs at the phase transition temperature ($\Delta\bar{v}$) decreases as the weight percent of lipophilin is increased (Table 1). The observed decrease is greater than that which would be expected on the basis of the replacement of lipid by protein (Table 1, Theoretical). This indicates that less lipid goes through the phase transition in the presence of protein.

If it is assumed that there is no volume change on the mixing of lipophilin with DMPC then:

$\bar{v}_{complex}$ = (weight fraction of lipid) ($\bar{v}_{lipid}$) + (weight fraction of protein) ($\bar{v}_{protein}$)

where for a particular temperature $\bar{v}_{lipid}$ is the partial specific volume of pure DMPC, $\bar{v}_{protein}$ is the partial specific volume of pure lipophilin and $\bar{v}_{complex}$ is the partial specific volume of the lipophilin-DMPC complex.

Figure 2. Densimetry measurements of lipophilin/DMPC complexes. The samples are dialyzed from 2-chloroethanol into the buffer system described in Materials and Methods. The scan rate for all experiments was 0.5°C/minute. The average sample concentration was 5 mg/ml. Actual experimental procedures are described in Materials and Methods. Lower case letters represent, by weight, lipophilin incorporated into the bilayer; a, 0%; b, 3.0%; c, 7.3%; d, 7.4%; e, 9.3%; f, 17.8%; g, 21.7%; h, 30.7%; i, 46.2%; j, 49.5%; k, 100%.

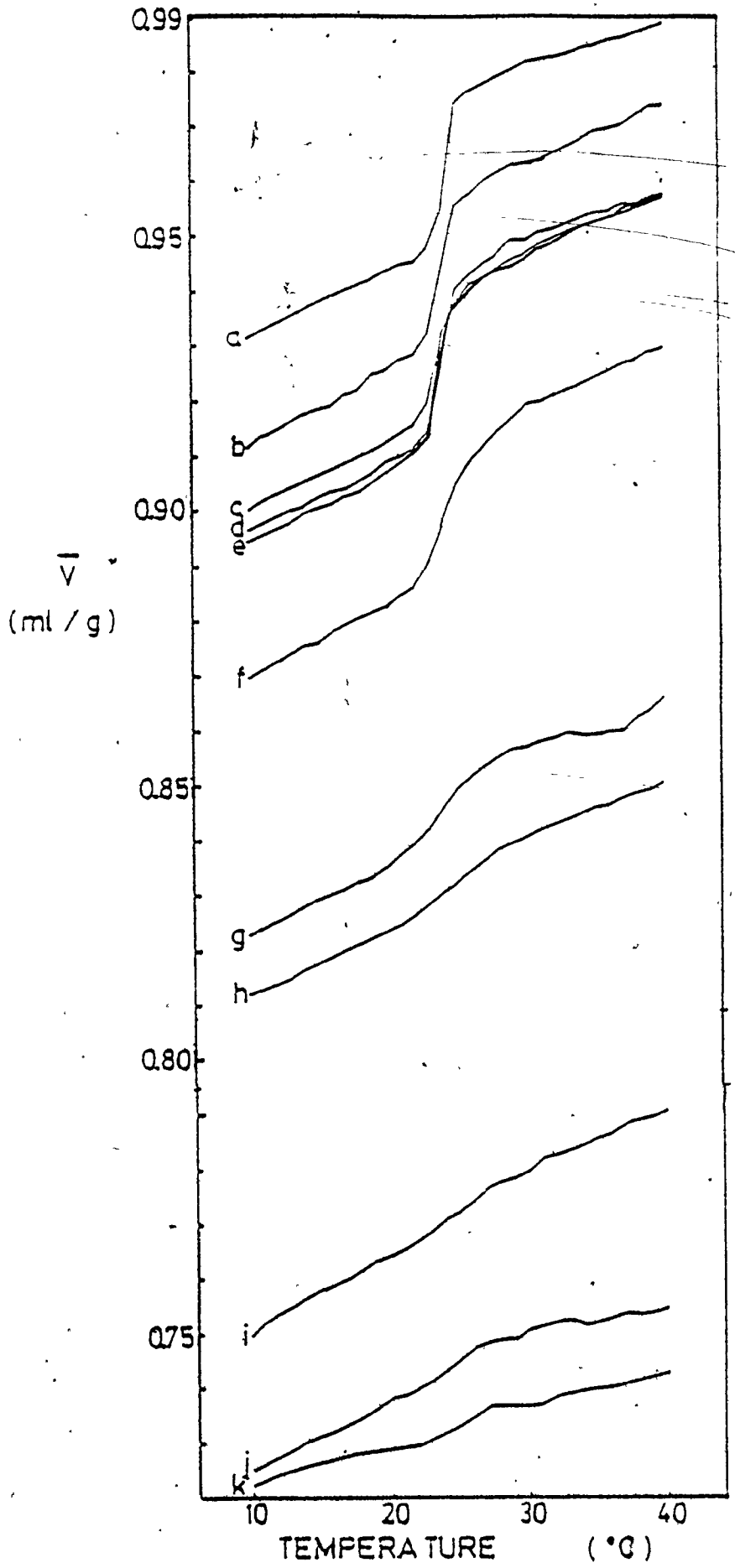

0.99
0.95
0.90
0.85
0.80
0.75
$\bar{V}$
(ml / g)
a
b
c
d
e
f
g
h
i
j
k
10
20
30
40
TEMPERATURE (°C)

Table 1. Change in partial specific volume ($\Delta\bar{v}$) for lipophilin-DMPC complexes. Materials and Methods describe the approach taken to obtain $\Delta\bar{v}$. Experimental represents densimetry results while Theoretical represents $\Delta\bar{v}$ if there was no effect of lipophilin on DMPC bilayers. Results are ml/g.

| % lipophilin by weight | Experimental | Theoretical |
|---|---|---|
| 0.0 | 0.029 | — |
| 3.0 | 0.027 | 0.028 |
| 3.2 | 0.026 | 0.028 |
| 7.3 | 0.026 | 0.027 |
| 9.3 | 0.030 | 0.027 |
| 10.7 | 0.026 | 0.026 |
| 17.8 | 0.032 | 0.024 |
| 20.9 | 0.015 | 0.024 |
| 21.7 | 0.023 | 0.022 |
| 30.7 | 0.014 | 0.019 |
| 46.2 | 0.010 | 0.018 |
| 49.5 | 0.006 | 0.016 |
| 72.8 | — | 0.011 |

A comparison of the experimental and theoretical values of $\bar{v}$ are presented in Table 2, together with a percent difference between the two. Theoretical values of $\bar{v}$ are calculated using curves a and k of Figure 2 to represent pure DMPC and lipophilin, respectively. Graphical representations of the theoretical curves are shown in Figure 3. These can be compared with the observed experimental curves of Figure 2.

The shapes of the experimental and theoretical $\bar{v}$ graphs (compare Figure 2,g with Figure 3,g) and the values of $\Delta\bar{v}$ (Table 1) are very similar up to approximately 22% lipophilin.

Large discrepancies between the experimental and the theoretical curves appear as low as 21.7% lipophilin (Figure 2 and 3,g) with respect to the absolute value of $\bar{v}$. The experimental values are consistently lower than the theoretical values.

The coefficient of expansion data ($\alpha$), can be found in Table 3 and illustrated in Figure 4. Both Table 3 and Figure 4 depict the experimental as well as the theoretical situation. Figure 4 illustrates that below the phase transition temperature, the $\alpha$ values are very similar to those above the phase transition. This indicates that the protein has the same effects below and above the transition. The $\alpha$ values observed are similar to those obtained using other perturbants in the bilayer (Fodor and Epand, 1981). The largest differences in $\alpha$ occur through the main transition. There is quite a large amount of error in these values because of the marked temperature dependence of $\alpha$ in this region. However, there appear to be some similarities between the experimental and theoretical $\alpha$s through the transition. The similarities do not rest in the absolute values, but in the shape (Figure 4). Both experimental and

Table 2. Experimental $\bar{v}$ (a), Theoretical $\bar{v}$ (b) and percent difference between the two (c), at different temperatures and weight percents of lipophilin. Densimetry experiments are described in methods. Approach to determine theoretical results are discussed in text.

| T(°C) | 3.0% Lipophilin | | | 3.2% Lipophilin | | |
|---|---|---|---|---|---|---|
| | a | b | c | a | b | c |
| | (ml/g) | (ml/g) | | (ml/g) | (ml/g) | |
| 10 | 0.9114 | 0.9250 | 1.47 | 0.8972 | 0.9246 | 2.96 |
| 11 | 0.9135 | 0.9264 | 1.39 | 0.8998 | 0.9260 | 2.83 |
| 12 | 0.9144 | 0.9275 | 1.41 | 0.9012 | 0.9271 | 2.79 |
| 13 | 0.9162 | 0.9290 | 1.38 | 0.9026 | 0.9286 | 2.80 |
| 14 | 0.9175 | 0.9302 | 1.37 | 0.9040 | 0.9298 | 2.77 |
| 15 | 0.9184 | 0.9314 | 1.40 | 0.9058 | 0.9310 | 2.71 |
| 16 | 0.9187 | 0.9324 | 1.47 | 0.9064 | 0.9319 | 2.74 |
| 17 | 0.9211 | 0.9332 | 1.30 | 0.9087 | 0.9328 | 2.58 |
| 18 | 0.9221 | 0.9345 | 1.33 | 0.9101 | 0.9341 | 2.57 |
| 19 | 0.9247 | 0.9356 | 1.17 | 0.9120 | 0.9352 | 2.48 |
| 20 | 0.9253 | 0.9374 | 1.29 | 0.9139 | 0.9369 | 2.45 |
| 21 | 0.9275 | 0.9381 | 1.13 | 0.9157 | 0.9377 | 2.35 |
| 22 | 0.9284 | 0.9391 | 1.14 | 0.9176 | 0.9387 | 2.25 |
| 23 | 0.9324 | 0.9411 | 0.92 | 0.9200 | 0.9407 | 2.20 |
| 24 | 0.9436 | 0.9487 | 0.54 | 0.9270 | 0.9483 | 2.25 |
| 25 | 0.9555 | 0.9667 | 1.16 | 0.9413 | 0.9662 | 2.58 |
| 26 | 0.9574 | 0.9692 | 1.22 | 0.9449 | 0.9688 | 2.47 |
| 27 | 0.9601 | 0.9705 | 1.07 | 0.9477 | 0.9700 | 2.30 |
| 28 | 0.9616 | 0.9714 | 1.01 | 0.9506 | 0.9710 | 2.10 |
| 29 | 0.9627 | 0.9728 | 1.04 | 0.9521 | 0.9723 | 2.08 |
| 30 | 0.9630 | 0.9744 | 1.17 | 0.9550 | 0.9739 | 1.94 |
| 31 | 0.9636 | 0.9749 | 1.16 | 0.9565 | 0.9744 | 1.84 |
| 32 | 0.9656 | 0.9752 | 0.98 | 0.9581 | 0.9747 | 1.70 |
| 33 | 0.9663 | 0.9761 | 1.00 | 0.9597 | 0.9756 | 1.63 |
| 34 | 0.9675 | 0.9770 | 0.97 | 0.9617 | 0.9765 | 1.52 |
| 35 | 0.9694 | 0.9777 | 0.84 | 0.9625 | 0.9773 | 1.50 |
| 36 | 0.9698 | 0.9785 | 0.89 | 0.9646 | 0.9780 | 1.37 |
| 37 | 0.9705 | 0.9792 | 0.89 | 0.9666 | 0.9787 | 1.24 |
| 38 | 0.9721 | 0.9798 | 0.79 | 0.9691 | 0.9793 | 1.04 |
| 39 | 0.9741 | 0.9806 | 0.66 | 0.9698 | 0.9801 | 1.05 |
| 40 | 0.9738 | 0.9816 | 0.80 | 0.9715 | 0.9811 | 0.98 |

Table 2. continued...

| T(°C) | 7.3% Lipophilin | | | 7.4% Lipophilin | | |
|---|---|---|---|---|---|---|
| | a (ml/g) | b (ml/g) | c | a (ml/g) | b (ml/g) | c |
| 10 | 0.9003 | 0.9160 | 1.71 | 0.8966 | 0.9158 | 2.10 |
| 11 | 0.9019 | 0.9174 | 1.69 | 0.8979 | 0.9172 | 2.10 |
| 12 | 0.9032 | 0.9185 | 1.67 | 0.8989 | 0.9183 | 2.11 |
| 13 | 0.9042 | 0.9200 | 1.72 | 0.9000 | 0.9198 | 2.15 |
| 14 | 0.9051 | 0.9211 | 1.74 | 0.9008 | 0.9209 | 2.18 |
| 15 | 0.9064 | 0.9223 | 1.72 | 0.9026 | 0.9221 | 2.11 |
| 16 | 0.9076 | 0.9233 | 1.70 | 0.9034 | 0.9230 | 2.12 |
| 17 | 0.9087 | 0.9241 | 1.67 | 0.9039 | 0.9239 | 2.16 |
| 18 | 0.9100 | 0.9254 | 1.66 | 0.9055 | 0.9252 | 2.13 |
| 19 | 0.9110 | 0.9264 | 1.66 | 0.9069 | 0.9262 | 2.08 |
| 20 | 0.9124 | 0.9281 | 1.69 | 0.9092 | 0.9279 | 2.01 |
| 21 | 0.9142 | 0.9289 | 1.58 | 0.9097 | 0.9287 | 2.05 |
| 22 | 0.9158 | 0.9298 | 1.51 | 0.9114 | 0.9296 | 1.96 |
| 23 | 0.9197 | 0.9318 | 1.30 | 0.9144 | 0.9316 | 1.85 |
| 24 | 0.9328 | 0.9391 | 0.67 | 0.9270 | 0.9389 | 1.27 |
| 25 | 0.9370 | 0.9564 | 2.03 | 0.9404 | 0.9561 | 1.64 |
| 26 | 0.9397 | 0.9589 | 2.00 | 0.9433 | 0.9587 | 1.61 |
| 27 | 0.9425 | 0.9601 | 1.83 | 0.9451 | 0.9599 | 1.54 |
| 28 | 0.9442 | 0.9610 | 1.75 | 0.9466 | 0.9608 | 1.48 |
| 29 | 0.9458 | 0.9623 | 1.71 | 0.9496 | 0.9621 | 1.30 |
| 30 | 0.9470 | 0.9638 | 1.74 | 0.9492 | 0.9636 | 1.49 |
| 31 | 0.9487 | 0.9643 | 1.62 | 0.9510 | 0.9641 | 1.36 |
| 32 | 0.9498 | 0.9647 | 1.54 | 0.9513 | 0.9645 | 1.37 |
| 33 | 0.9511 | 0.9656 | 1.50 | 0.9526 | 0.9653 | 1.32 |
| 34 | 0.9519 | 0.9665 | 1.51 | 0.9535 | 0.9662 | 1.31 |
| 35 | 0.9526 | 0.9671 | 1.50 | 0.9544 | 0.9669 | 1.29 |
| 36 | 0.9534 | 0.9680 | 1.51 | 0.9545 | 0.9677 | 1.36 |
| 37 | 0.9544 | 0.9686 | 1.47 | 0.9561 | 0.9684 | 1.27 |
| 38 | 0.9553 | 0.9693 | 1.44 | 0.9551 | 0.9690 | 1.43 |
| 39 | 0.9561 | 0.9701 | 1.44 | 0.9564 | 0.9698 | 1.38 |
| 40 | 0.9572 | 0.9710 | 1.41 | 0.9568 | 0.9708 | 1.44 |

Table 2. continued...

| T(°C) | 9.3% Lipophilin | | | 10.7% Lipophilin | | |
|---|---|---|---|---|---|---|
| | a (ml/g) | b (ml/g) | c | a (ml/g) | b (ml/g) | c |
| 10 | 0.8946 | 0.9119 | 1.90 | 0.8996 | 0.9089 | 1.02 |
| 11 | 0.8957 | 0.9133 | 1.93 | 0.9010 | 0.9103 | 1.02 |
| 12 | 0.8968 | 0.9143 | 1.91 | 0.9022 | 0.9114 | 1.01 |
| 13 | 0.8979 | 0.9158 | 1.95 | 0.9030 | 0.9128 | 1.07 |
| 14 | 0.8995 | 0.9169 | 1.90 | 0.9043 | 0.9140 | 1.06 |
| 15 | 0.9004 | 0.9181 | 1.93 | 0.9055 | 0.9152 | 1.06 |
| 16 | 0.9013 | 0.9190 | 1.93 | 0.9067 | 0.9161 | 1.03 |
| 17 | 0.9027 | 0.9199 | 1.87 | 0.9078 | 0.9169 | 0.99 |
| 18 | 0.9034 | 0.9211 | 1.92 | 0.9095 | 0.9182 | 0.95 |
| 19 | 0.9050 | 0.9221 | 1.85 | 0.9105 | 0.9191 | 0.94 |
| 20 | 0.9069 | 0.9238 | 1.83 | 0.9121 | 0.9208 | 0.94 |
| 21 | 0.9086 | 0.9246 | 1.73 | 0.9140 | 0.9216 | 0.82 |
| 22 | 0.9106 | 0.9255 | 1.61 | 0.9160 | 0.9225 | 0.70 |
| 23 | 0.9133 | 0.9275 | 1.53 | 0.9187 | 0.9245 | 0.63 |
| 24 | 0.9324 | 0.9347 | 0.25 | 0.9267 | 0.9315 | 0.52 |
| 25 | 0.9376 | 0.9516 | 1.47 | 0.9328 | 0.9482 | 1.62 |
| 26 | 0.9410 | 0.9541 | 1.37 | 0.9362 | 0.9507 | 1.53 |
| 27 | 0.9426 | 0.9553 | 1.33 | 0.9388 | 0.9520 | 1.39 |
| 28 | 0.9439 | 0.9562 | 1.29 | 0.9407 | 0.9528 | 1.27 |
| 29 | 0.9444 | 0.9575 | 1.37 | 0.9426 | 0.9541 | 1.21 |
| 30 | 0.9466 | 0.9589 | 1.28 | 0.9435 | 0.9555 | 1.26 |
| 31 | 0.9482 | 0.9594 | 1.17 | 0.9456 | 0.9560 | 1.09 |
| 32 | 0.9487 | 0.9598 | 1.16 | 0.9464 | 0.9564 | 1.05 |
| 33 | 0.9505 | 0.9607 | 1.06 | 0.9475 | 0.9573 | 1.02 |
| 34 | 0.9522 | 0.9616 | 0.98 | 0.9483 | 0.9581 | 1.02 |
| 35 | 0.9527 | 0.9622 | 0.99 | 0.9494 | 0.9588 | 0.98 |
| 36 | 0.9538 | 0.9631 | 1.44 | 0.9504 | 0.9596 | 0.96 |
| 37 | 0.9551 | 0.9637 | 0.89 | 0.9510 | 0.9603 | 0.97 |
| 38 | 0.9552 | 0.9644 | 0.95 | 0.9521 | 0.9609 | 1.00 |
| 39 | 0.9573 | 0.9652 | 0.79 | 0.9529 | 0.9617 | 0.92 |
| 40 | 0.9577 | 0.9661 | 0.87 | 0.9540 | 0.9627 | 0.90 |

Table 2. continued...

| T(°C) | 17.8% Lipophilin | | | 20.9% Lipophilin | | |
|---|---|---|---|---|---|---|
| | a (ml/g) | b (ml/g) | c | a (ml/g) | b (ml/g) | c |
| 10 | 0.8697 | 0.8950 | 2.83 | 0.8752 | 0.8876 | 1.40 |
| 11 | 0.8714 | 0.8964 | 2.79 | 0.8768 | 0.8890 | 1.37 |
| 12 | 0.8727 | 0.8975 | 2.76 | 0.8778 | 0.8901 | 1.38 |
| 13 | 0.8741 | 0.8988 | 2.75 | 0.8803 | 0.8914 | 1.25 |
| 14 | 0.8756 | 0.9000 | 2.71 | 0.8837 | 0.8925 | 0.99 |
| 15 | 0.8762 | 0.9012 | 2.77 | 0.8815 | 0.8937 | 1.37 |
| 16 | 0.8781 | 0.9020 | 2.65 | 0.8821 | 0.8945 | 1.39 |
| 17 | 0.8795 | 0.9028 | 2.58 | 0.8831 | 0.8953 | 1.36 |
| 18 | 0.8807 | 0.9040 | 2.58 | 0.8837 | 0.8965 | 1.43 |
| 19 | 0.8817 | 0.9049 | 2.56 | 0.8849 | 0.8973 | 1.38 |
| 20 | 0.8830 | 0.9065 | 2.59 | 0.8858 | 0.8989 | 1.46 |
| 21 | 0.8849 | 0.9072 | 2.46 | 0.8873 | 0.8996 | 1.37 |
| 22 | 0.8865 | 0.9081 | 2.38 | 0.8894 | 0.9005 | 1.23 |
| 23 | 0.8903 | 0.9101 | 2.18 | 0.8904 | 0.9025 | 1.34 |
| 24 | 0.8973 | 0.9166 | 2.11 | 0.8946 | 0.9088 | 1.56 |
| 25 | 0.9047 | 0.9321 | 2.94 | 0.8992 | 0.9237 | 2.65 |
| 26 | 0.9092 | 0.9346 | 2.72 | 0.9014 | 0.9262 | 2.68 |
| 27 | 0.9123 | 0.9359 | 2.52 | 0.9034 | 0.9247 | 2.59 |
| 28 | 0.9145 | 0.9366 | 2.36 | 0.9050 | 0.9282 | 2.50 |
| 29 | 0.9170 | 0.9378 | 2.22 | 0.9062 | 0.9292 | 2.48 |
| 30 | 0.9196 | 0.9391 | 2.08 | 0.9078 | 0.9305 | 2.44 |
| 31 | 0.9197 | 0.9396 | 2.12 | 0.9089 | 0.9310 | 2.37 |
| 32 | 0.9210 | 0.9401 | 2.03 | 0.9096 | 0.9315 | 3.35 |
| 33 | 0.9220 | 0.9409 | 2.01 | 0.9108 | 0.9323 | 2.31 |
| 34 | 0.9233 | 0.9418 | 1.96 | 0.9120 | 0.9332 | 2.27 |
| 35 | 0.9243 | 0.9418 | 1.86 | 0.9133 | 0.9338 | 2.20 |
| 36 | 0.9256 | 0.9432 | 1.87 | 0.9140 | 0.9346 | 2.20 |
| 37 | 0.9270 | 0.9539 | 1.79 | 0.9141 | 0.9353 | 2.27 |
| 38 | 0.9276 | 0.9445 | 1.79 | 0.9153 | 0.9359 | 2.20 |
| 39 | 0.9292 | 0.9453 | 1.70 | 0.9163 | 0.9367 | 2.18 |
| 40 | 0.9297 | 0.9562 | 1.74 | 0.9173 | 0.9375 | 2.15 |

Table 2. continued...

| T(°C) | 21.7% Lipophilin | | | 30.7% Lipophilin | | |
|---|---|---|---|---|---|---|
| | a (ml/g) | b (ml/g) | c | a (ml/g) | b (ml/g) | c |
| 10 | 0.8230 | 0.8859 | 7.10 | 0.8121 | 0.8671 | 6.34 |
| 11 | 0.8245 | 0.8873 | 7.08 | 0.8132 | 0.8685 | 6.37 |
| 12 | 0.8254 | 0.8884 | 7.09 | 0.8142 | 0.8696 | 6.37 |
| 13 | 0.8267 | 0.8897 | 7.08 | 0.8151 | 0.8708 | 6.40 |
| 14 | 0.8284 | 0.8908 | 7.00 | 0.8167 | 0.8719 | 6.33 |
| 15 | 0.8295 | 0.8920 | 7.01 | 0.8178 | 0.8731 | 6.33 |
| 16 | 0.8305 | 0.8928 | 6.98 | 0.8189 | 0.8737 | 6.27 |
| 17 | 0.8312 | 0.8936 | 6.98 | 0.8203 | 0.8745 | 6.20 |
| 18 | 0.8330 | 0.8948 | 6.91 | 0.8215 | 0.8756 | 6.18 |
| 19 | 0.8338 | 0.8956 | 6.90 | 0.8224 | 0.8764 | 6.17 |
| 20 | 0.8356 | 0.8971 | 6.86 | 0.8237 | 0.8778 | 6.16 |
| 21 | 0.8378 | 0.8979 | 6.69 | 0.8250 | 0.8785 | 6.09 |
| 22 | 0.8395 | 0.8988 | 6.60 | 0.8266 | 0.8793 | 6.06 |
| 23 | 0.8422 | 0.9008 | 6.50 | 0.8287 | 0.8813 | 5.97 |
| 24 | 0.8463 | 0.9070 | 6.69 | 0.8306 | 0.8870 | 6.36 |
| 25 | 0.8595 | 0.9218 | 7.84 | 0.8323 | 0.9000 | 7.52 |
| 26 | 0.8516 | 0.9242 | 7.86 | 0.8350 | 0.9025 | 7.48 |
| 27 | 0.8540 | 0.9255 | 7.73 | 0.8368 | 0.9039 | 7.42 |
| 28 | 0.8555 | 0.9262 | 7.63 | 0.8386 | 0.9045 | 7.28 |
| 29 | 0.8570 | 0.9273 | 7.58 | 0.8398 | 0.9054 | 7.25 |
| 30 | 0.8570 | 0.9286 | 7.71 | 0.8410 | 0.9065 | 7.23 |
| 31 | 0.8583 | 0.9291 | 7.63 | 0.8420 | 0.9070 | 7.17 |
| 32 | 0.8590 | 0.9296 | 7.59 | 0.8433 | 0.9076 | 7.08 |
| 33 | 0.8597 | 0.9304 | 7.60 | 0.8441 | 0.9084 | 7.08 |
| 34 | 0.8591 | 0.9312 | 7.74 | 0.8452 | 0.9092 | 7.04 |
| 35 | 0.8592 | 0.9319 | 7.80 | 0.8466 | 0.9098 | 6.95 |
| 36 | 0.8601 | 0.9326 | 7.77 | 0.8471 | 0.9105 | 6.96 |
| 37 | 0.8604 | 0.9333 | 7.81 | 0.8482 | 0.9113 | 6.92 |
| 38 | 0.8627 | 0.9339 | 7.62 | 0.8490 | 0.9118 | 6.89 |
| 39 | 0.8641 | 0.9347 | 7.55 | 0.8496 | 0.9127 | 6.91 |
| 40 | 0.8664 | 0.9356 | 7.40 | 0.8512 | 0.9134 | 6.81 |

Table 2. continued...

| T(°C) | 46.2% Lipophilin | | | 49.5% Lipophilin | | |
|---|---|---|---|---|---|---|
| | a (ml/g) | b (ml/g) | c | a (ml/g) | b (ml/g) | c |
| 10 | 0.7497 | 0.8347 | 10.2 | 0.7249 | 0.8278 | 12.4 |
| 11 | 0.7526 | 0.8361 | 9.99 | 0.7266 | 0.8292 | 12.4 |
| 12 | 0.7542 | 0.8371 | 9.90 | 0.7279 | 0.8302 | 12.3 |
| 13 | 0.7553 | 0.8382 | 9.89 | 0.7293 | 0.8313 | 12.3 |
| 14 | 0.7570 | 0.8392 | 9.80 | 0.7306 | 0.8323 | 12.2 |
| 15 | 0.7583 | 0.8404 | 9.77 | 0.7313 | 0.8335 | 12.3 |
| 16 | 0.7594 | 0.8409 | 9.69 | 0.7324 | 0.8340 | 12.2 |
| 17 | 0.7603 | 0.8517 | 9.67 | 0.7338 | 0.8347 | 12.1 |
| 18 | 0.7620 | 0.3427 | 9.58 | 0.7352 | 0.8357 | 12.0 |
| 19 | 0.7637 | 0.8432 | 9.43 | 0.7366 | 0.8362 | 11.9 |
| 20 | 0.7645 | 0.8445 | 9.47 | 0.7383 | 0.8374 | 11.8 |
| 21 | 0.7657 | 0.8452 | 9.41 | 0.7388 | 0.8381 | 11.9 |
| 22 | 0.7674 | 0.8459 | 9.28 | 0.7405 | 0.8388 | 11.7 |
| 23 | 0.7691 | 0.8479 | 9.29 | 0.7417 | 0.8408 | 11.8 |
| 24 | 0.7713 | 0.8524 | 9.51 | 0.7436 | 0.8451 | 12.0 |
| 25 | 0.7725 | 0.8629 | 10.5 | 0.7455 | 0.8550 | 12.8 |
| 26 | 0.7748 | 0.8652 | 10.5 | 0.7476 | 0.8573 | 12.8 |
| 27 | 0.7770 | 0.8666 | 10.3 | 0.7485 | 0.8587 | 12.8 |
| 28 | 0.7780 | 0.8670 | 10.3 | 0.7490 | 0.8590 | 12.8 |
| 29 | 0.7787 | 0.8676 | 10.3 | 0.7492 | 0.8596 | 12.8 |
| 30 | 0.7800 | 0.8686 | 10.2 | 0.7513 | 0.8605 | 12.7 |
| 31 | 0.7823 | 0.8671 | 9.99 | 0.7518 | 0.8610 | 12.7 |
| 32 | 0.7830 | 0.8698 | 9.98 | 0.7421 | 0.8618 | 12.7 |
| 33 | 0.7838 | 0.8705 | 9.96 | 0.7526 | 0.8624 | 12.7 |
| 34 | 0.7848 | 0.8713 | 9.93 | 0.7516 | 0.8633 | 12.9 |
| 35 | 0.7861 | 0.8719 | 9.84 | 0.7522 | 0.8638 | 12.9 |
| 36 | 0.7869 | 0.8724 | 9.80 | 0.7528 | 0.8643 | 12.9 |
| 37 | 0.7887 | 0.8733 | 9.69 | 0.7540 | 0.8652 | 12.9 |
| 38 | 0.7893 | 0.8738 | 9.67 | 0.7533 | 0.8657 | 13.0 |
| 39 | 0.7898 | 0.8746 | 9.70 | 0.7543 | 0.8665 | 13.0 |
| 40 | 0.7911 | 0.8753 | 9.62 | 0.7549 | 0.8671 | 12.9 |

Table 2. continued...

| T(°C) | 72.8% Lipophilin a (ml/g) | b (ml/g) | c |
|---|---|---|---|
| 10 | 0.6723 | 0.7790 | 13.7 |
| 11 | 0.6722 | 0.7804 | 13.9 |
| 12 | 0.6735 | 0.7815 | 13.8 |
| 13 | 0.6741 | 0.7823 | 13.8 |
| 14 | 0.6755 | 0.7833 | 13.8 |
| 15 | 0.6767 | 0.7845 | 13.7 |
| 16 | 0.6780 | 0.7847 | 13.6 |
| 17 | 0.6764 | 0.7853 | 13.9 |
| 18 | 0.6775 | 0.7861 | 13.8 |
| 19 | 0.6771 | 0.7864 | 13.9 |
| 20 | 0.6775 | 0.7873 | 14.0 |
| 21 | 0.6782 | 0.7879 | 13.9 |
| 22 | 0.6782 | 0.7885 | 13.9 |
| 23 | 0.6790 | 0.7905 | 14.1 |
| 24 | 0.6794 | 0.7931 | 14.3 |
| 25 | 0.6801 | 0.7990 | 14.8 |
| 26 | 0.6895 | 0.8011 | 13.9 |
| 27 | 0.6807 | 0.8026 | 15.2 |
| 28 | 0.6811 | 0.8026 | 15.2 |
| 29 | 0.6819 | 0.8029 | 15.1 |
| 30 | 0.6834 | 0.8035 | 15.0 |
| 31 | 0.6844 | 0.8040 | 14.9 |
| 32 | 0.6849 | 0.8049 | 14.9 |
| 33 | 0.6864 | 0.8055 | 14.8 |
| 34 | 0.6871 | 0.8063 | 14.8 |
| 35 | 0.6889 | 0.8067 | 14.6 |
| 36 | 0.6902 | 0.8071 | 14.5 |
| 37 | 0.6915 | 0.8081 | 14.4 |
| 38 | 0.6933 | 0.8085 | 14.3 |
| 39 | 0.6946 | 0.8043 | 14.2 |
| 40 | 0.6969 | 0.8098 | 14.0 |

Figure 3. Theoretical partial specific volumes ($\bar{v}$) for the complexes versus temperature. The $\bar{v}$ was calculated under the assumption that there was no volume change upon mixing. Therefore $\bar{v}$=(wt. %)($\bar{v}_L$)+(wt. %)($\bar{v}_p$) where the $\bar{v}$s of lipid and protein are those of pure lipid and protein respectively. Weight percent lipid weight percent protein are taken from the composition of the samples. Lower case letters represent percent by weight, lipophilin; a, 0%; b, 3.0%; c, 7.3%; d, 7.4%; e, 9.3%; f, 17.8%; g, 21.7%; h, 30.7%; i, 46.2%; j, 49.5%.

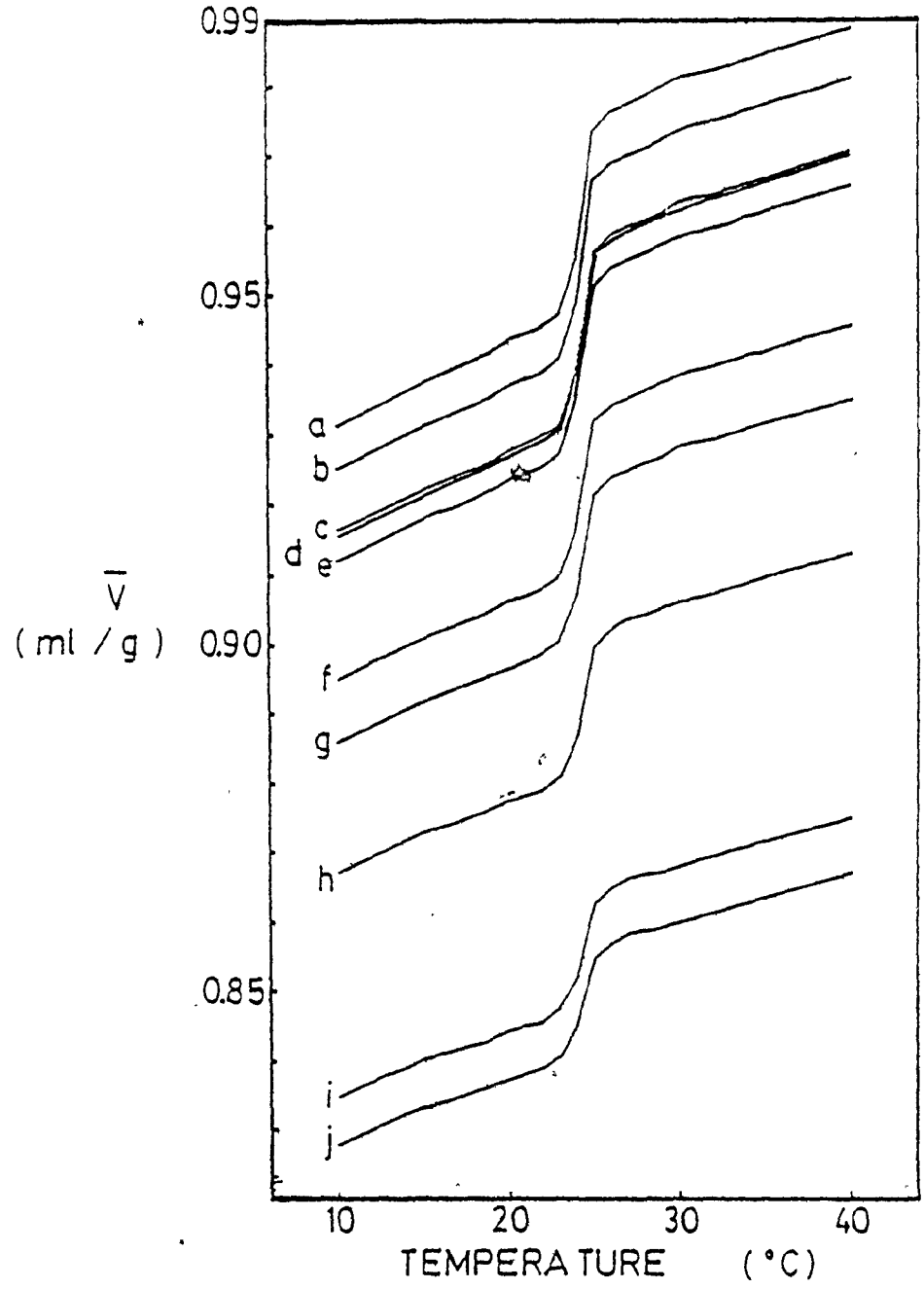
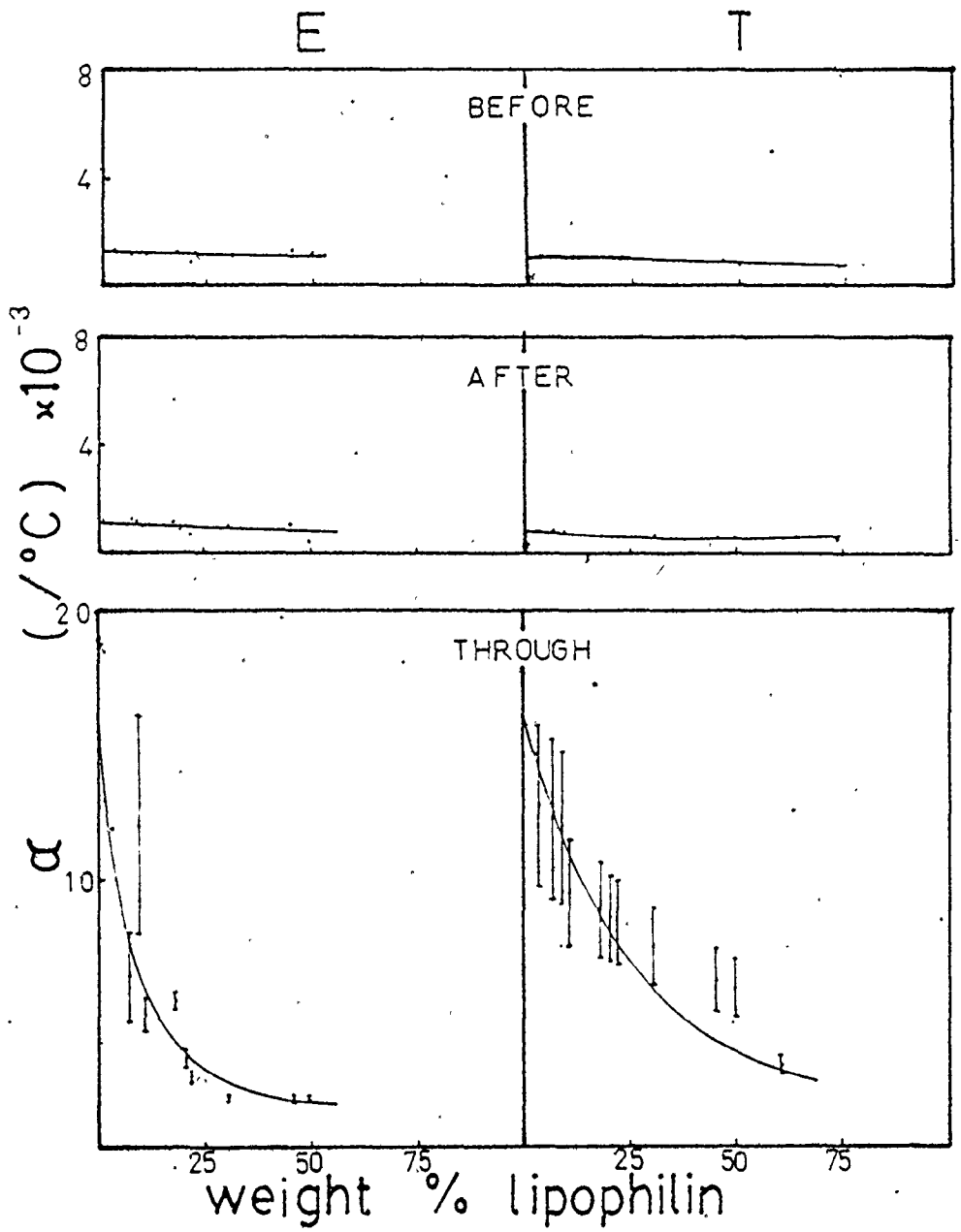

0.99
0.95
0.90
0.85
V̄
( ml / g )
a
b
c
d
e
f
g
h
i
j
10
20
30
40
TEMPERATURE ( °C )

Table 3. Coefficients of expansion of the experimental (E), and theoretical (T) systems as they relate to the phase transition, ie. before, through or after the phase transition of the lipid. Units are per °C and all values are $10^{-3}$.

| Wt. % lipophilin | before | | through | | after | |
|---|---|---|---|---|---|---|
| | E | T | E | T | E | T |
| 0 | 1.205±0.017 | | 18.50 | | 0.848±0.028 | |
| 3.0 | 1.468±0.057 | 1.170±0.018 | 11.90 | 12.80±3.002 | 1.128±0.037 | 0.838±0.028 |
| 3.2 | 1.626±0.033 | 1.168±0.017 | 8.900±1.382 | 12.75±2.973 | 1.781±0.038 | 0.834±0.026 |
| 7.3 | 1.229±0.250 | 1.145±0.017 | 6.420±1.725 | 12.30±2.887 | 1.084±0.041 | 0.826±0.024 |
| 7.4 | 1.211±0.035 | 1.145±0.017 | 10.01±1.729 | 12.25±2.868 | 0.848±0.063 | 0.821±0.024 |
| 9.3 | 1.276±0.042 | 1.127±0.016 | 12.15±4.013 | 12.05±2.800 | 1.209±0.033 | 0.819±0.023 |
| 10.7 | 1.261±0.029 | 1.125±0.018 | 4.931±0.436 | 9.530±1.834 | 1.054±0.037 | 0.786±0.021 |
| 17.8 | 1.333±0.022 | 1.080±0.019 | 5.517±0.358 | 8.900±1.679 | 1.196±0.047 | 0.764±0.020 |
| 20.9 | 0.977±0.103 | 1.060±0.018 | 3.280±0.351 | 8.600±1.602 | 1.030±0.034 | 0.757±0.015 |
| 21.7 | 1.219±0.027 | 1.058±0.018 | 2.712±0.129 | 8.500±1.602 | 0.722±0.097 | 0.752±0.016 |
| 30.7 | 1.166±0.018 | 0.998±0.021 | 1.923±0.047 | 7.660±1.357 | 1.002±0.022 | 0.725±0.009 |
| 46.2 | 1.402±0.051 | 0.910±0.022 | 1.861±0.054 | 6.240±1.063 | 1.102±0.033 | 0.679±0.007 |
| 49.5 | 1.263±0.022 | 0.890±0.024 | 1.960±0.034 | 5.940±0.985 | 0.443±0.045 | 0.665±0.009 |
| 72.8 | —— | 0.760±0.040 | —— | 3.091±0.293 | —— | 0.618±0.012 |

Figure 4. Coefficient of expansion ($\alpha$) versus weight percent lipophilin for densimetry results (E), and theoretical results (T). The method to obtain $\alpha$ is described in Materials and Methods. Theoretical $\alpha$s are from the plots of theoretical $\bar{v}$s (Figure 3). The headings Before, After and Through are with respect to the phase transition.


E
T
BEFORE
AFTER
THROUGH
8
4
8
4
20
10
α (/°C) ×10⁻³
25
50
75
25
50
75
weight % lipophilin

theoretical plots show a sharp decrease in $\alpha$ up to about 20-25% lipophilin followed by a gradual levelling off. The relevance of these findings will be discussed later.

The method for incorporation of lipophilin into DMPC involves dialysis from 2-chloroethanol into buffer. Even though there is an initial volume of approximately 5 ml of 2-chloroethanol which is dialyzed against 6 liters of buffer, in 2 liter aliquots, theoretically leaving only $1.6 \times 10^{-6}$ % of the original 2-chloroethanol in the retentate. However, there may be some additional 2-chloroethanol remaining. If any is remaining it would probably be trapped within the hydrocarbon region of the vesicle formed during dialysis. The density of 2-chloroethanol at 20°C is 1.197 g/ml, so it would change the density of the complex noticeably if not completely dialyzed out.

In order to determine if there was much remaining 2-chloroethanol in the buffer after dialysis, the density of the buffer before and after dialysis was determined. The density of the buffer before dialysis was 1.002686 g/ml while it was 1.002675 g/ml after dialysis. The difference between the two is not significant indicating that all of the 2-chloroethanol was dialyzed out. However, some 2-chloroethanol may be trapped inside the vesicles, but as will be shown later, this was not the case.

By visual inspection, lipophilin/DMPC complexes tend to aggregate and clump at high lipophilin percentages. The fact that this occurs may alter the densimetry measurements since the aggregated material may clump in one area or another of the sample cell. However, it was found that this had no substantial effect on the precision of the measurements. By repeatedly filling and emptying the cell with a complex containing

76% lipophilin, the $\bar{v}$ was found to be 0.718 ± 0.006 ml/g, of similar precision to measurements of other lipid samples.

However, this still leaves the question of why the $\bar{v}$ at high protein to lipid ratios are so low compared to pure protein alone. The results obtained for these high protein to lipid complexes may be influenced by the rate of temperature scanning. Since the sample is so highly aggregated it may not have sufficient time to reach thermal equilibrium. Therefore, the temperature indicated on the thermometer may not be the actual temperature at which the sample is being measured. Hence, most later measurements were done isothermally at 20°C. By doing isothermal measurements, the samples are allowed to come to equilibrium. In this respect the results should be more accurate. Although no other scan rates were used, this seems a logical explanation.

The measuring capabilities of the densimeter also had to be evaluated. To determine the accuracy of the instrument, two well-characterized, water soluble proteins were used; BSA and lysozyme. At 25°C and at a protein concentration of 2.2 mg/ml we measured $\bar{v}$s of 0.7395 and 0.7459 ml/g for BSA and lysozyme respectively. The reported literature values are 0.7376 ml/g for BSA (Bull and Breese, 1968) and 0.7477 ml/g for lysozyme (Blazyk et al. 1975) at infinite dilution. The literature values and experimental values are very similar indicating that the densimeter gives very accurate values for soluble proteins.

Another reason why the complexes with high protein to lipid ratios may give seemingly erroneous results may be the possibility that some of the complex may adhere to the sample tube and hence be taken out of measurements. To try to eliminate this, a water soluble form of the

protein was prepared by dialyzing the protein, solubilized in 2-chloroethanol, against distilled water. The partial specific volume was found to be: $\bar{v}$ = 0.4947 ± 0.0004 ml/g at C = 0.0580 mg/ml; $\bar{v}$ = 0.7186 ± 0.0004 ml/g at C = 0.933 mg/ml and $\bar{v}$ = 0.7105 ± 0.0005 ml/g at C = 1.874 mg/ml. Although these values are at high precision, we believe that the value for C = 0.0580 mg/ml is inaccurate due to undetermined effects such as the adsorption of protein to glass surfaces in the presence of impurities. The other two values are much closer to what would be expected based on the amino acid composition.

Pure lipophilin dialyzed from 2-chloroethanol into buffer had a somewhat higher partial specific volume at 20°C ($\bar{v}$ = 0.7288 ml/g at C = 1.083 mg/ml and $\bar{v}$ = 0.7353 ml/g at C = 2.828 mg/ml). The differences between the $\bar{v}$ of the water soluble form and the buffer suspended form of lipophilin seems to be due to the solvation of the protein as will be discussed later.

The partial specific volume of lipophilin was measured in different solvents in an attempt to determine what effects they have on the protein. The $\bar{v}$ of lipophilin solubilized in 2-chloroethanol was 0.7891 ml/g at 20°C. When lipophilin was solubilized in chloroform:methanol (2:1 v/v), the solvent evaporated and the residue resuspended in buffer, the $\bar{v}$ of lipophilin at 20°C was 0.7973 ml/g. The implications of this and possible explanations will be discussed later.

Sucrose density gradient ultracentrifugation was performed on some of the complexes in an attempt to substantiate the densimetry results. The results are tabulated in Table 4 and graphically presented in Figures 5 through 14. The results from density gradient ultracentrifugation do

Table 4. Information obtained from density gradient ultracentrifugation. The procedures for centrifugation and fractionation are described in methods.

| % lipophilin (by weight) | Density (g/ml) | $\bar{v}$ (ml/g) |
|---|---|---|
| 30.7 | 1.1076±0.0030 | 0.9029±0.0077 |
| 46.2 | 1.1168±0.0081 | 0.8954±0.0065 |
| 53.6 | 1.1271±0.0124 | 0.8872±0.0098 |
| 66.5 | 1.1703±0.0091 | 0.8545±0.0066 |
| 68.3 | 1.1744±0.0076 | 0.8515±0.0059 |
| 75.5 | 1.1909±0.0083 | 0.8397±0.0050 |
| 84.0 | 1.1892±0.0071 | 0.8406±0.0050 |
| 84.0 (after sonication) | 1.1896±0.0085 | 0.8409±0.0060 |
| 100 | 1.2431±0.0083 | 0.8044±0.0020 |

Figure 5. Sucrose density ultracentrifugation results on a sample comprised of 30.7% (by weight) lipophilin in DMPC. The sample is dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient is checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).

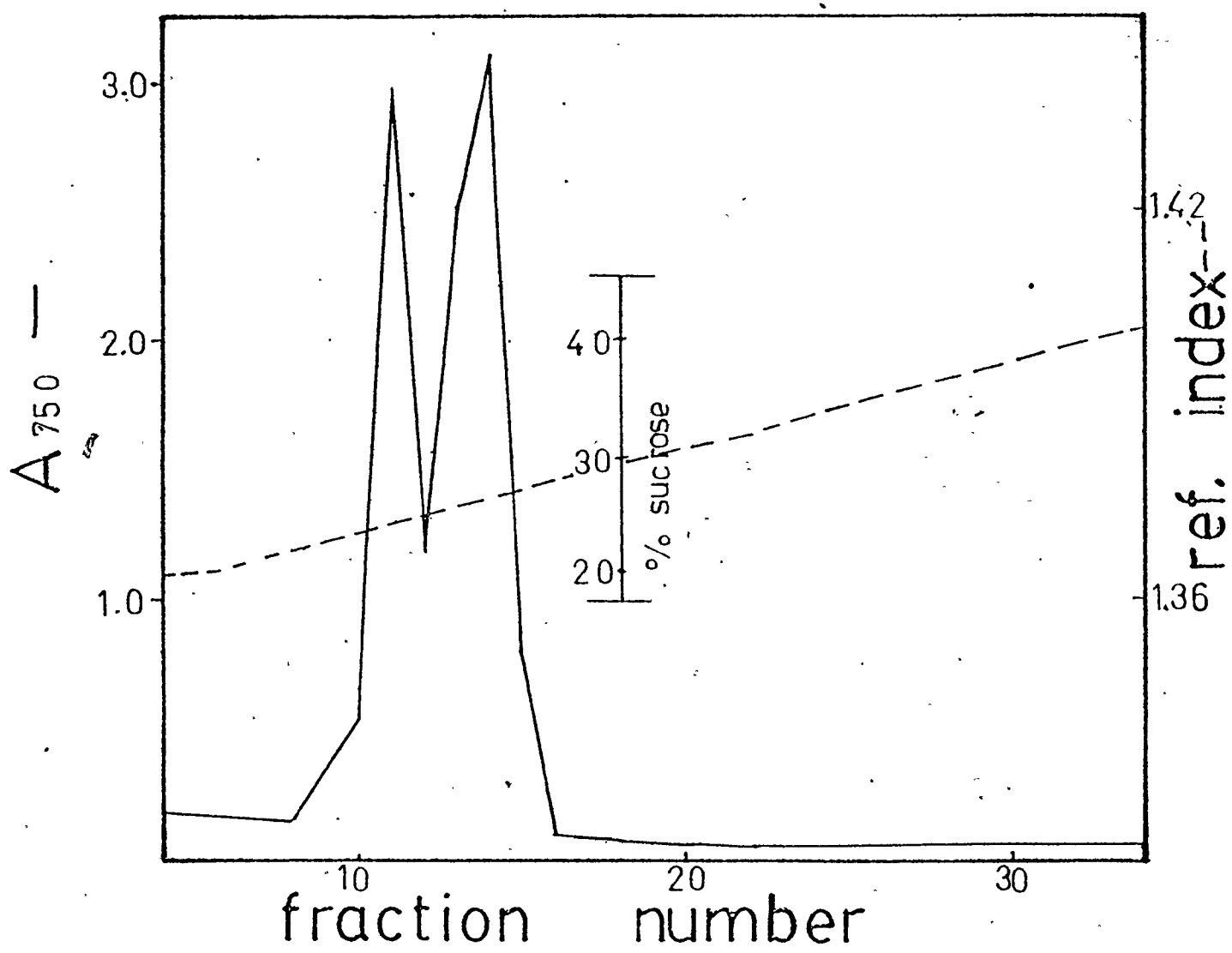

3.0
2.0
1.0
$A_{750}$
40
30
20
% sucrose
1.42
1.36
ref. index
10
20
30
fraction number

Figure 6. Sucrose density ultracentrifugation results on a sample comprised of 46.2% (by weight) lipophilin in DMPC. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).

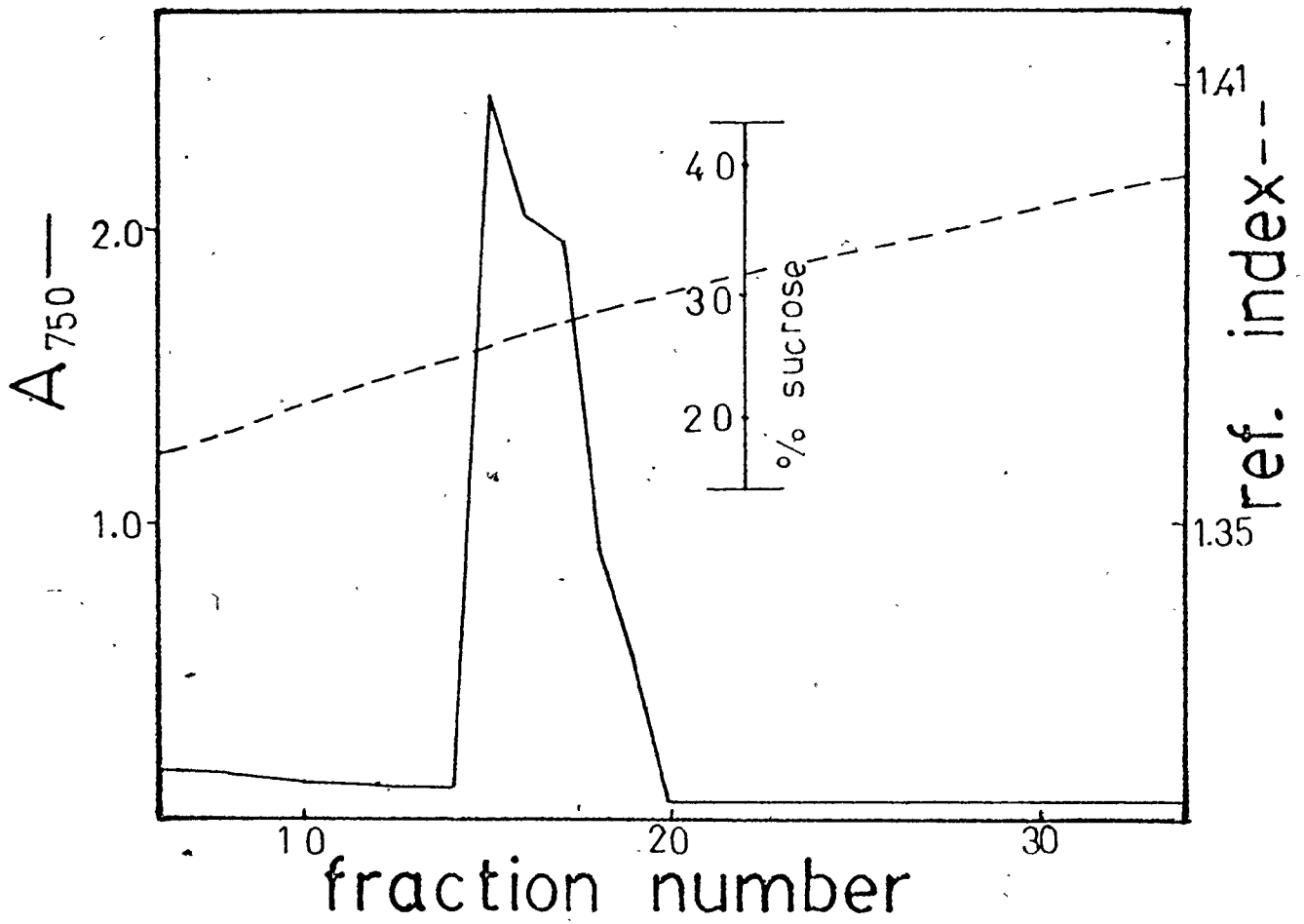

A750
2.0
1.0
40
30
20
% sucrose
1.41
1.35
ref. index
10
20
30
fraction number

Figure 7. Sucrose density ultracentrifugation results on a sample comprised of 53.6% (by weight) lipophilin in DMPC. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$(——).

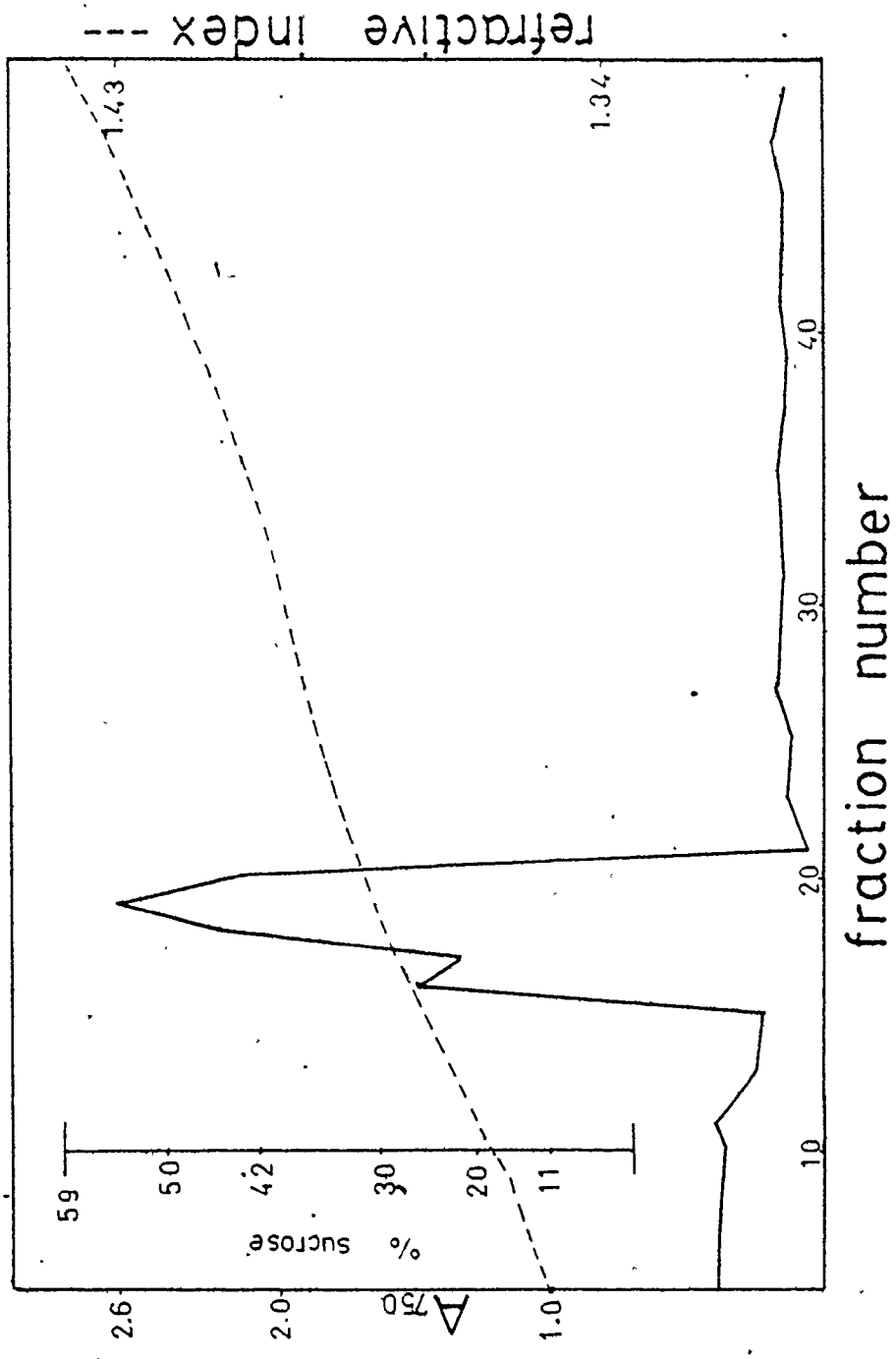

refractive index ---
1.43
1.34
fraction number
10
20
30
40
A750
2.6
2.0
1.0
% sucrose
59
50
42
30
20
11

Figure 8. Sucrose density ultracentrifugation results on a sample comprised of 66.5% (by weight) lipophilin in DMPC. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).

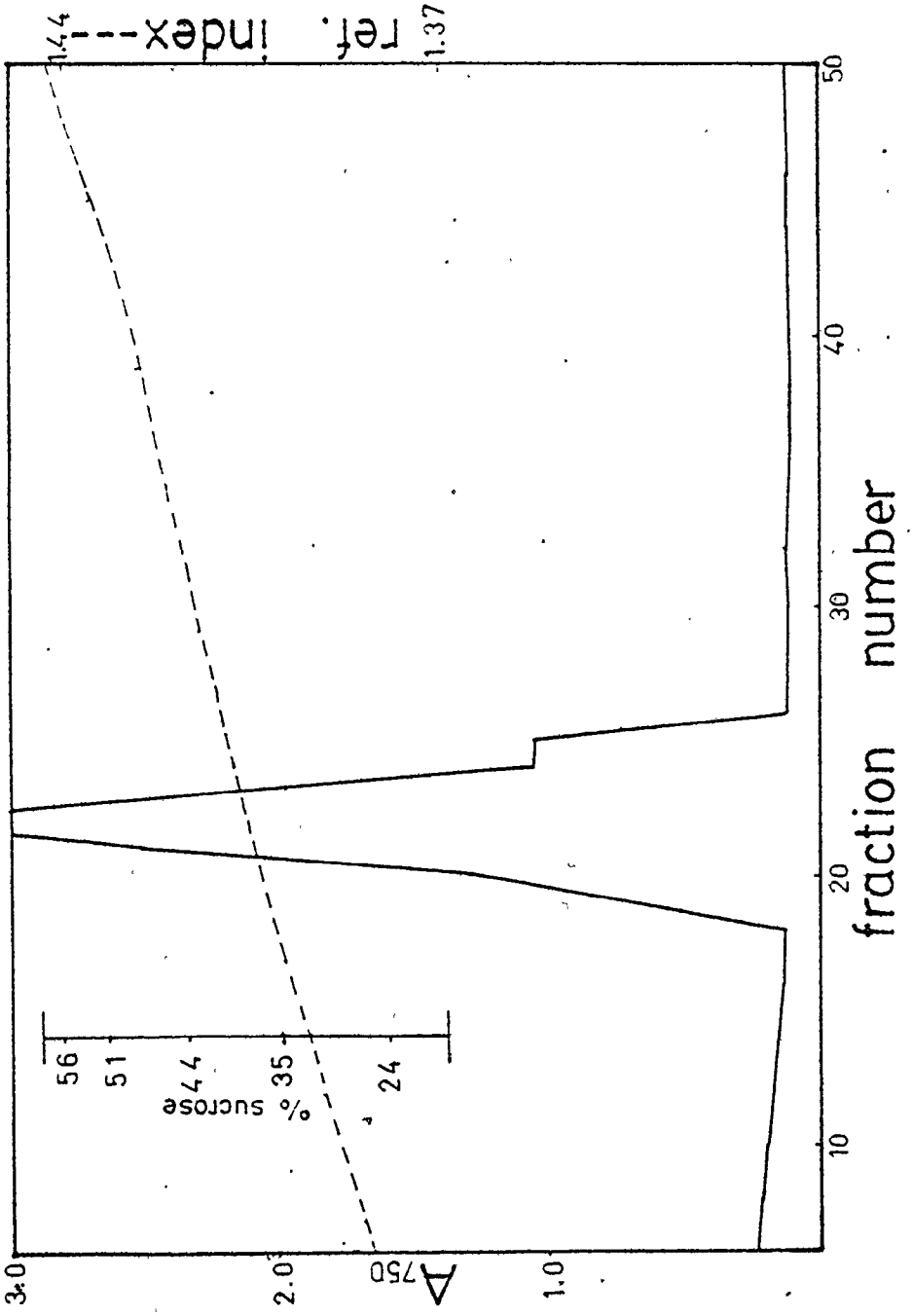

ref. index---
1.44
1.37
A750
3.0
2.0
1.0
10
20
30
40
50
fraction number
% sucrose
56
51
44
35
24

Figure 9. Sucrose density ultracentrifugation results on a sample comprised of 68.3% (by weight) lipophilin in DMPC. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).

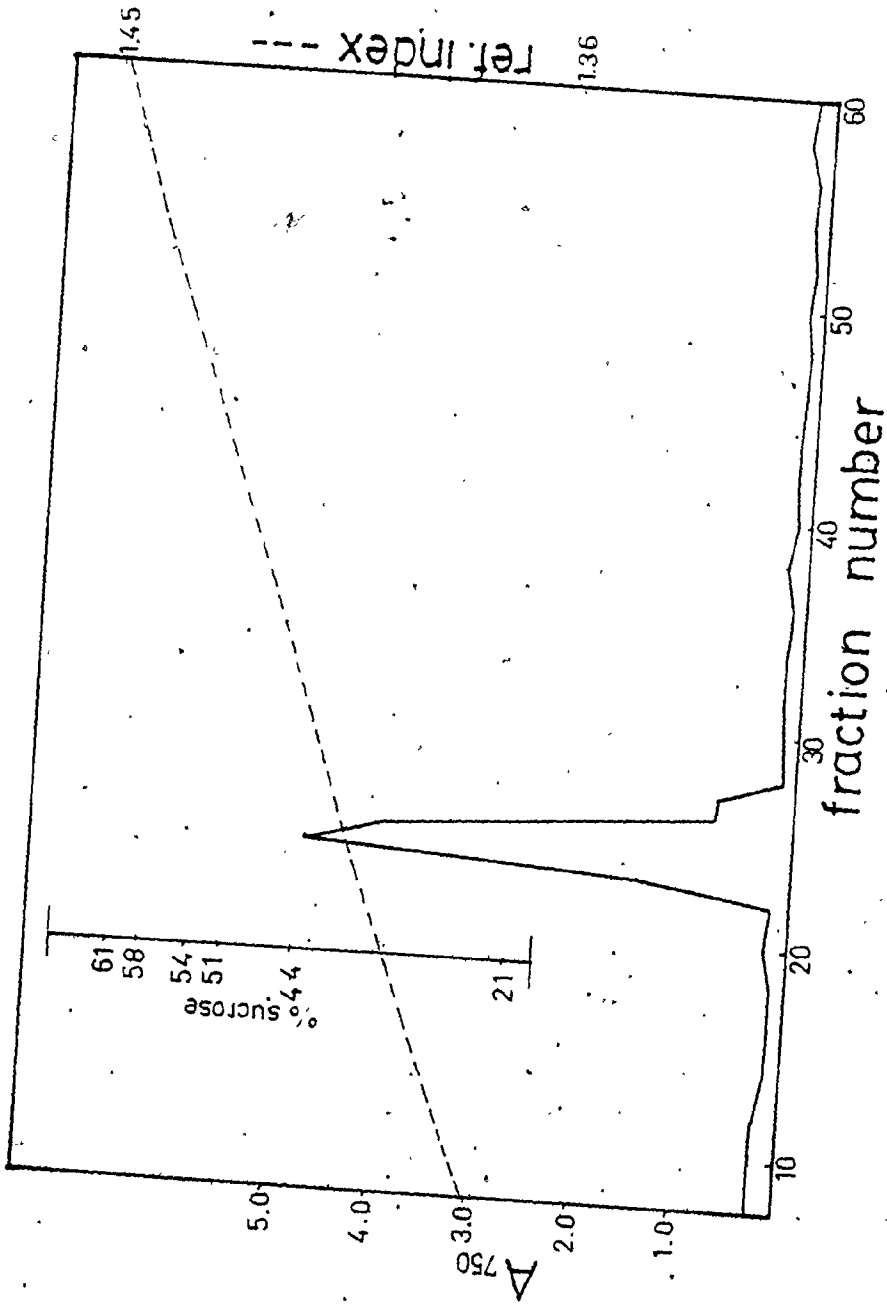

ref. index ---
1.45
1.36
A 750
5.0
4.0
3.0
2.0
1.0
% sucrose
61
58
54
51
44
21
fraction number
10
20
30
40
50
60

Figure 10. Sucrose density ultracentrifugation results on a sample comprised of 75.5% (by weight) lipophilin in DMPC. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——). Lipid determination was also performed. The fractions were extracted with chloroform, the lower phase evaporated and phosphate determination performed on the residues. These results are illustrated as $A_{830}$ (...).

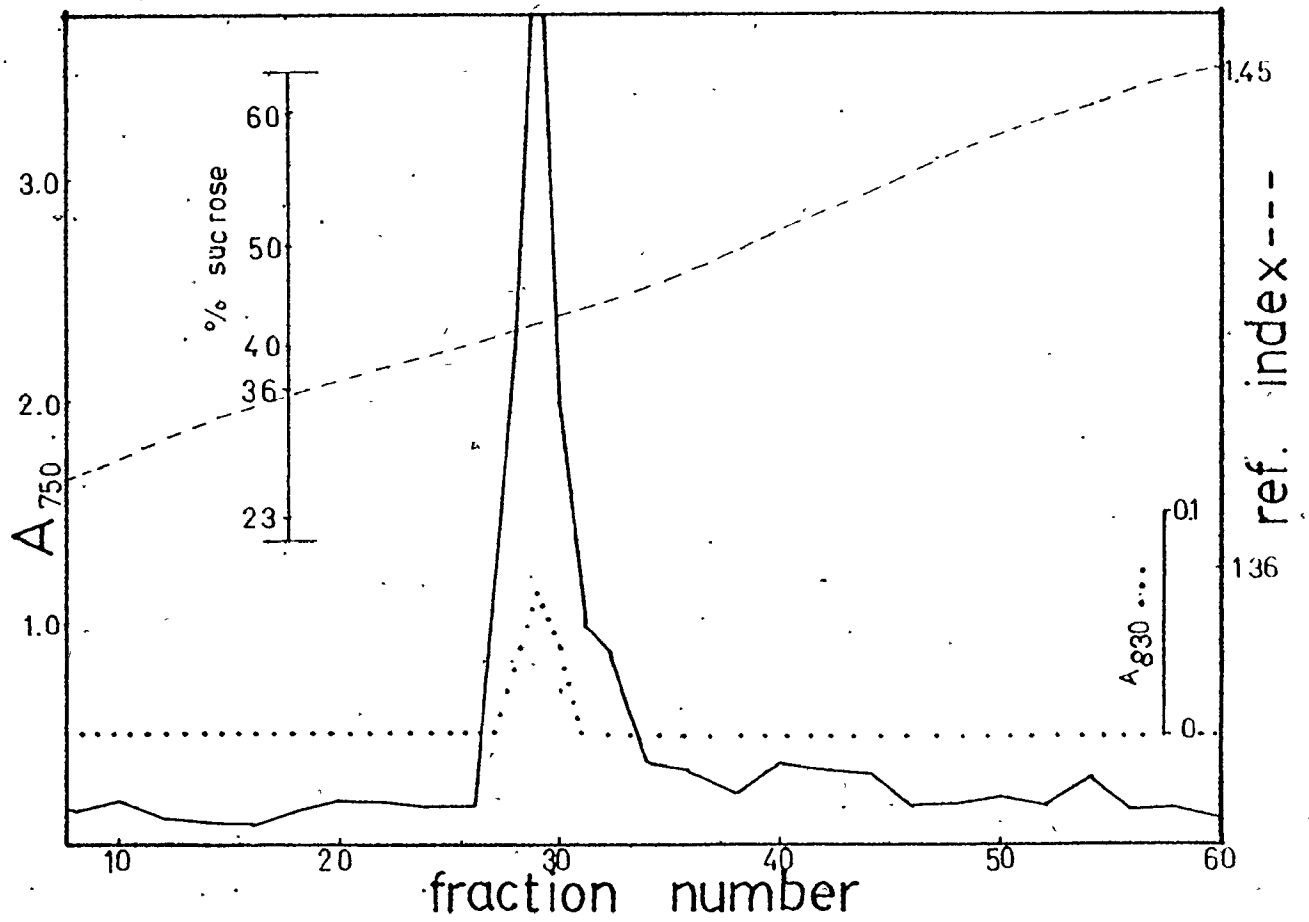

3.0
2.0
1.0
A750
60
50
40
36
23
% sucrose
1.45
1.36
ref. index---
0.1
0
A830 ....
10
20
30
40
50
60
fraction number

Figure 11. Sucrose density ultracentrifugation on a sample comprised of 84.0% (by weight) lipophilin in DMPC. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).

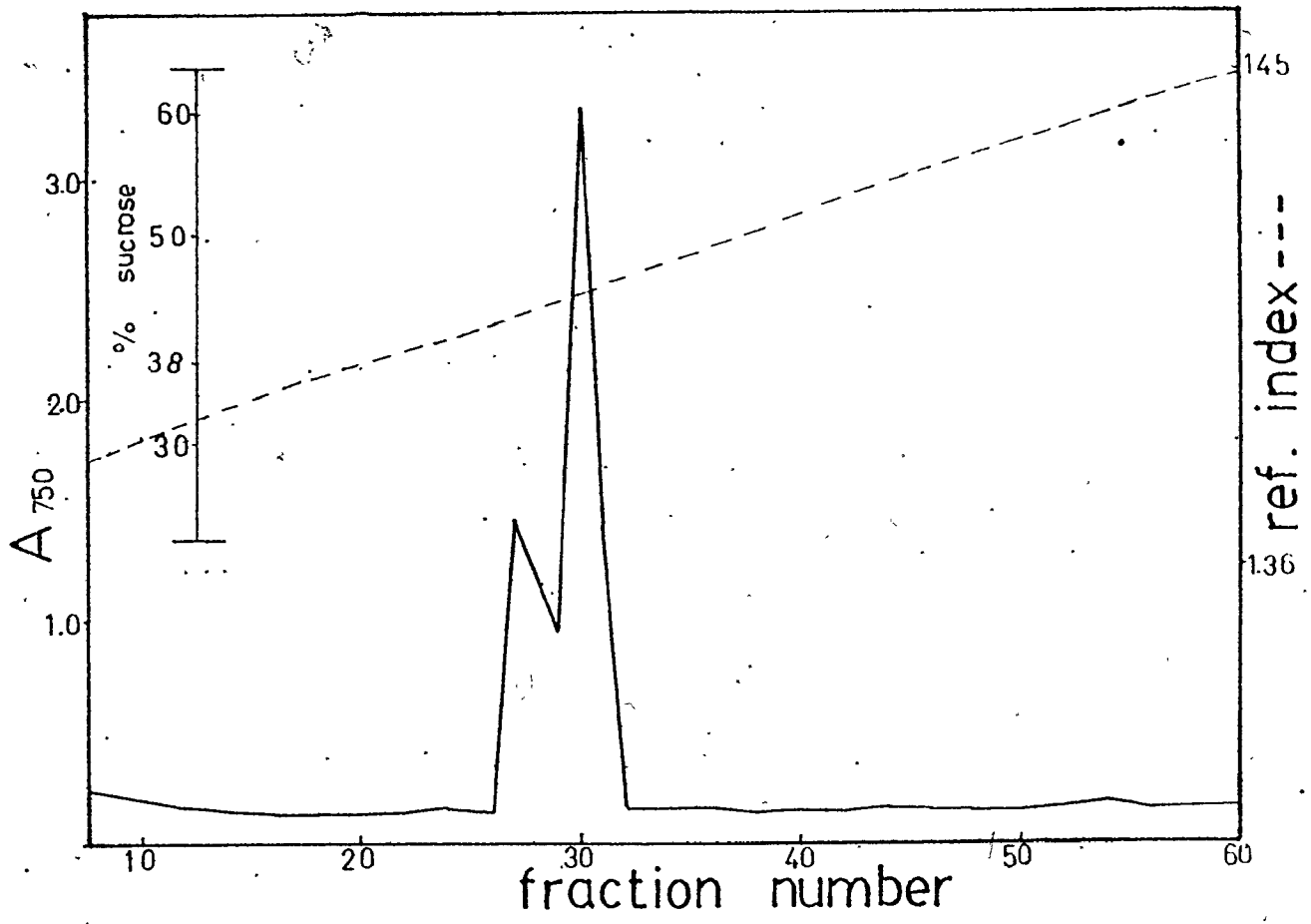

A 750
3.0
2.0
1.0
% sucrose
60
50
38
30
1.45
1.36
ref. index---
10
20
30
40
50
60
fraction number

Figure 12. Sucrose density ultracentrifugation results on a sample comprised of 84.0% (by weight) lipophilin in DMPC. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods). The sample in buffer was sonicated extensively before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).

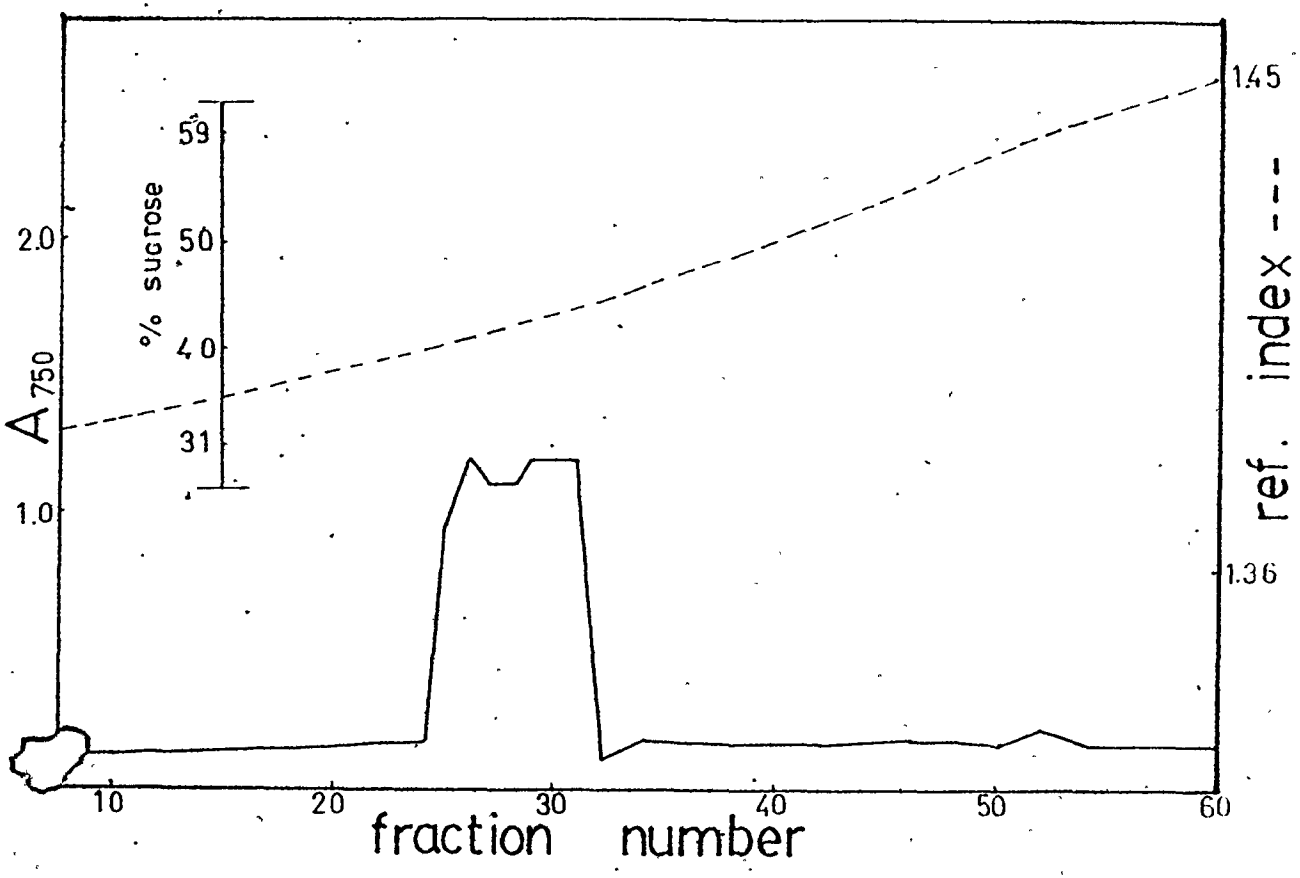

59
50
40
31
% sucrose
2.0
1.0
A750
1.45
1.36
ref. index ---
10
20
30
40
50
60
fraction number

Figure 13. Sucrose density ultracentrifugation results on a sample comprised of pure lipophilin. The sample was dialyzed from 2-chloroethanol into buffer (see Materials and Methods) before application to the gradient. The linearity of the gradient was checked by refractometer (---). Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. Protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).

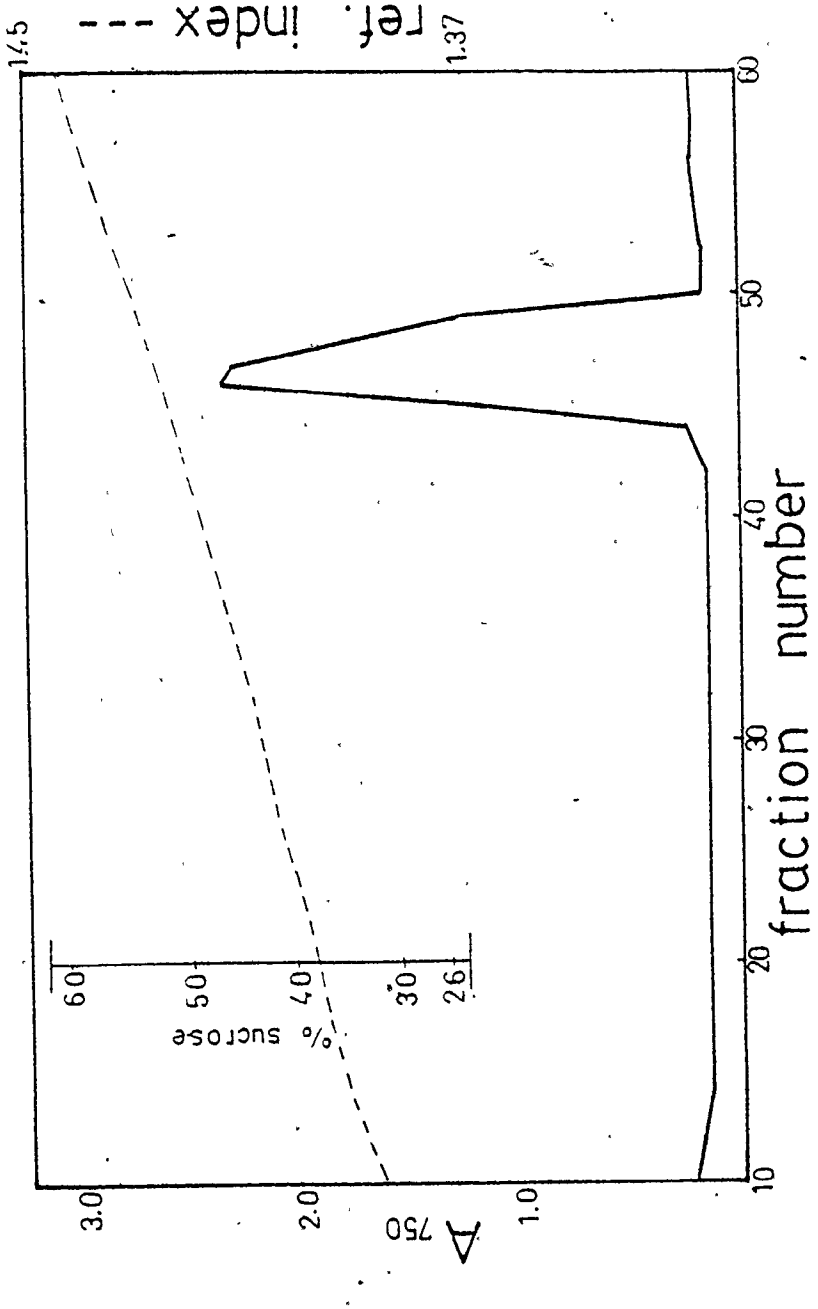
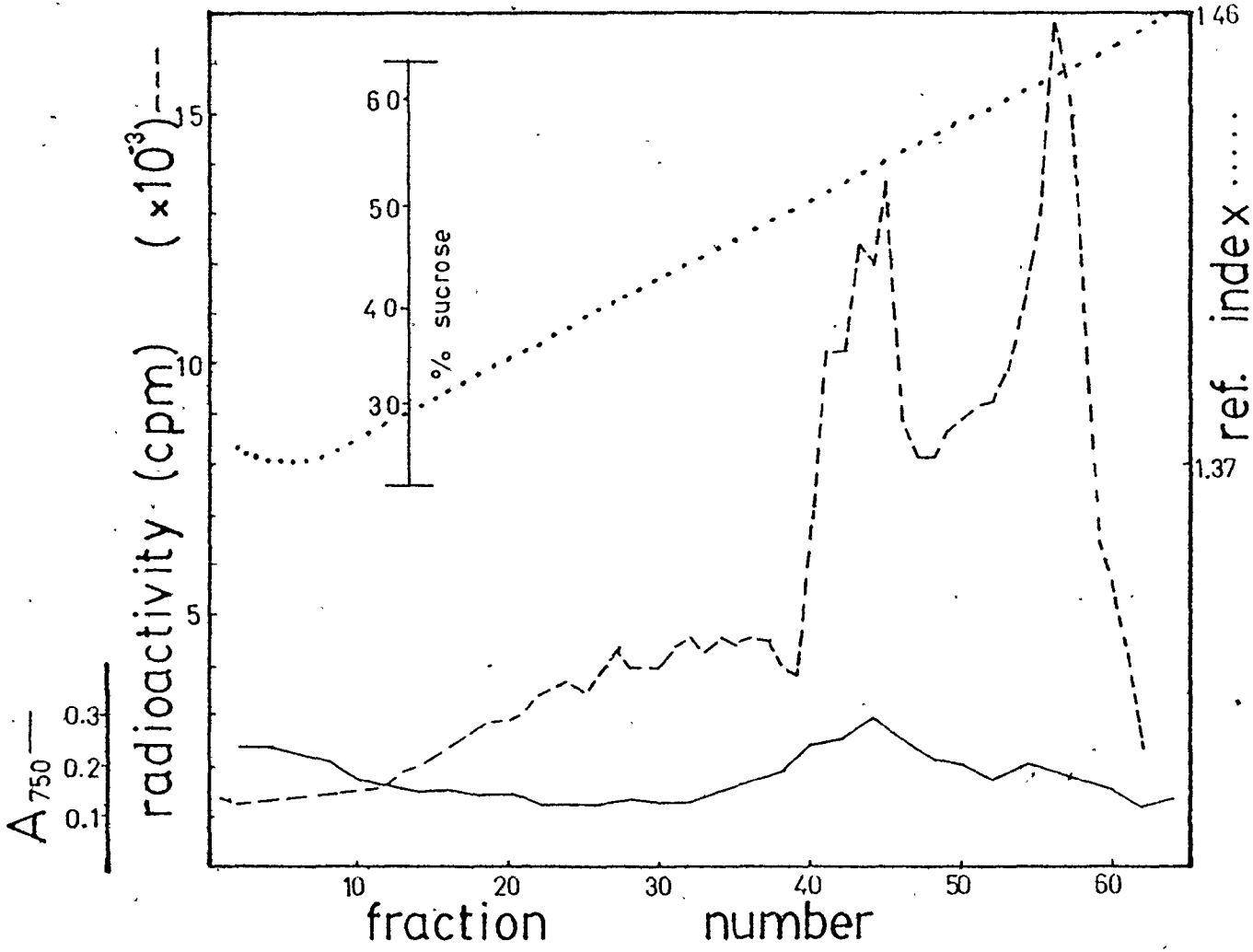
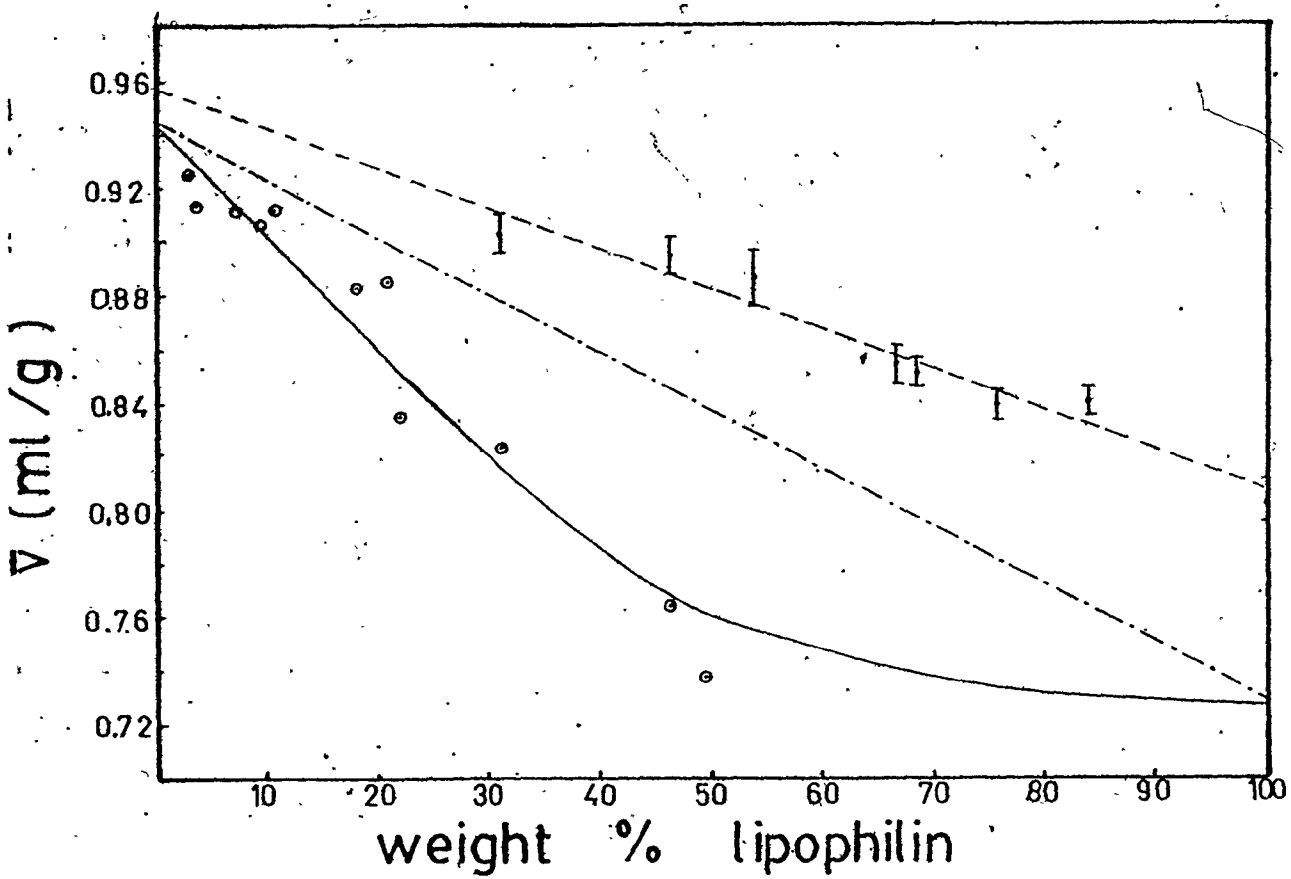

ref. index ---
1.45
1.37
A 750
3.0
2.0
1.0
% sucrose
60
50
40
30
26
10
20
30
40
50
60
fraction number

Figure 14. Sucrose density ultracentrifugation results on pure lipophilin and $^{125}$I-fibrinogen. The lipophilin was dialyzed from 2-chloroethanol into buffer (see Materials and Methods). The water soluble $^{125}$I-fibrinogen was dispersed evenly throughout the gradient while the lipophilin was applied to the top of the gradient. Centrifugation was in an SW27 rotor for 24 hours at 76,000 x g at 20°C. The linearity of the gradient was checked by refractometer (···). Fractionation was by an ISCO Density Gradient Fractionator and collected by a Gilson microfractionator. Fraction sizes were 25 drops. The labelled protein was counted by a gamma counter (---) while the protein determination was by a mini Folin-Lowry assay for insoluble proteins (see Materials and Methods) and illustrated as $A_{750}$ (——).


1.46
1.37
ref. index .....
60
50
40
30
% sucrose
15
10
5
radioactivity (cpm) (×10⁻³)---
A750 —
0.3
0.2
0.1
10
20
30
40
50
60
fraction number

not agree well with the densimetry results. Figure 15 illustrates this graphically. The $\bar{v}$ of lipophilin by ultracentrifugation was 0.8044 ± 0.0020 ml/g at 20°C (Figure 13), while by densimetry it was 0.7146 ± 0.0041 ml/g in the water soluble form and 0.7321 ± 0.0033 ml/g suspended in buffer at 20°C. Since the samples used in density gradient ultracentrifugation were in the form suspended in buffer, this method would be expected to give the same results as densimetry. To ascertain the cause of the discrepancy, lipophilin was run on a density gradient together with a well-characterized protein, fibrinogen, as described in Materials and Methods. Since within the time of the experiment the fibrinogen can not reach equilibrium in the gradient, it was initially dispersed evenly throughout the gradient. During the time of the experiment, the protein will begin to accumulate at its equilibrium position, which will show up as a peak of radioactivity. Figure 14 illustrates the results. The partial specific volume of fibrinogen at 20°C is 0.725 ml/g (Handbook of Biochemistry, 2nd ed., 1970) while, according to the results presented in Figure 14, it was 0.7698 ± 0.0105 ml/g. Superficially it seems evident that the density gradients do not accurately measure the partial specific volume. The second peak of radioactivity centered around 50% sucrose in Figure 14 is the lipophilin peak. The reason for this is that as lipophilin travels down the gradient, it picks up fibrinogen and traps it since the lipophilin is highly aggregated. Fibrinogen, run alone, did not band at all in the gradient (results not shown). The reason for this is the time and the speed of the run. For a protein the size of fibrinogen it would take days to reach equilibrium at these speeds. Even higher speeds do not significantly

Figure 15. Partial specific volume ($\bar{v}$) results from three sources at 20°C. Density gradient ultracentrifugation (---), theoretical results assuming that there is no volume change upon mixing of lipophilin and DMPC (-·-·-) and experimental densimetry results (——).


0.96
0.92
0.88
0.84
0.80
0.76
0.72
V (ml/g)
10
20
30
40
50
60
70
80
90
100
weight % lipophilin

alter the time to reach equilibrium. Without the fibrinogen control run, the density gradient results are still somewhat ambiguous. Part of the difficulty may arise from effects of the high concentrations of sucrose on the $\bar{v}$ of proteins.

The gradients are still useful to isolate complexes of varying composition. Figure 15 shows that at 20°C the complexes are easily distinguishable. The density gradients also show that lipophilin incorporates into the bilayer. By doing protein determinations on all gradients (Figures 5-13, $A_{750}$), it is clearly evident that all the protein is located in the band. There is no free DMPC in the gradient as seen in Figure 10 ($A_{830}$). The lipid determination on the fractions are not quantitative. It does not add up to the amount of lipid originally added. However, this is mainly due to the extraction procedures for DMPC from the density gradient fractions (see Materials and Methods).

The density gradients also clear up the possibility that 2-chloroethanol may be trapped in the vesicles. Figure 12 shows that the partial specific volume of the complex does not significantly change after sonication, before application to the gradient ($\bar{v}$ = 0.8409 ± 0.0050 ml/g at 20°C before sonication and $\bar{v}$ = 0.8406 ± 0.0060 ml/g at 20°C after sonication. Sample was 84.0% lipophilin by weight). Through sonication the vesicles would be disrupted to free any trapped 2-chloroethanol. Also evident from the profile of the density gradient in Figure 12 is that sonication does not disrupt the complex. This could be added evidence that lipophilin is embedded in the bilayer. To substantiate these findings a sample comprised of 75.5% (by weight) lipophilin was measured densimetrically before and after sonication. The partial specific volume

before sonication was 0.718 ml/g while it was 0.723 ml/g after sonication. This is also evidence that there is no 2-chloroethanol remaining in the complex.

## Discussion

This work can be included with the many that describe lipophilin/lipid interactions, (refer to references cited in Introduction). However this is the first investigation of this nature to employ the vibrating reed densimeter to determine the properties of the complexes. The vibrating reed densimeter has the advantage over other methods for the determination of densities, that it can be performed with about one tenth the sample and measurements can be obtained easily on commercially available instruments. Changes in volume, which can be easily measured by this method, occur upon reaction of proteins with phospholipids, and yield a specific fundamental physical parameter with which to characterize these systems.

The temperature for the phase transition of pure DMPC, namely 24°C, is in good agreement with that found by other methods (Mabrey and Sturtevant, 1976; Albon and Sturtevant, 1978; Verma et al. 1980; Lavialle and Levin, 1980; Fodor and Epand, 1981). This indicates that the inhomogeneous nature of the sample does not introduce sizable errors in the results obtained by densimetry.

However the scans for pure DMPC do not show the expected behavior of the lipid. Not evident in Figure 2,a is the premelt transition. This is a transition which is about one order of magnitude smaller (Laggner and Stabinger, 1976), occurs at a lower temperature and is much broader than the main transition (Melchoir and Steim, 1976; Lee, 1977 a,b,c). A premelt transition occurs in pure lecithins and phosphatidic acid

at high pH, but is absent in any other synthetic lipids (Melchoir and Steim, 1976; Mabrey and Sturtevant, 1976; Laggner and Stabinger, 1976; Mabrey and Sturtevant, 1977). This transition is thought to reflect a change in the state of the polar head groups in lecithins (Ladbrooke and Chapman, 1969; Janiak et al. 1976). The lack of a premelt is difficult to explain. In another study employing this method (Fodor and Epand, 1981) there is a distinct premelt transition. The lack of one in this study could probably be explained by some impurity in the lipid. Even a small amount of impurity is known to abolish the premelt phase transition (Lee, 1976; Eliasz et al. 1976; Papahadjopoulos et al. 1976; Laggner and Stabinger, 1976; Mabrey and Sturtevant, 1976 and 1977; Lee, 1977; Podo and Blasie, 1976; Usher et al. 1978; Wilkinson and Nagle, 1979).

There is considerable evidence that lipophilin is incorporated into the bilayer by dialysis from 2-chloroethanol (Boggs et al. 1976). By using 100mM NaCl instead of 10mM NaCl, Boggs et al. (1976) found better incorporation of the lipophilin, but this gave a more diffuse band in sucrose gradient centrifugation. Freeze-fracture showed that the vesicles prepared in this way were multilayered, (using 100mM NaCl), while they were single layered using 10mM NaCl (Boggs et al. 1976). In this study no free lipid or free protein was found outside the band in sucrose density centrifugation (refer to Figures 5-13).

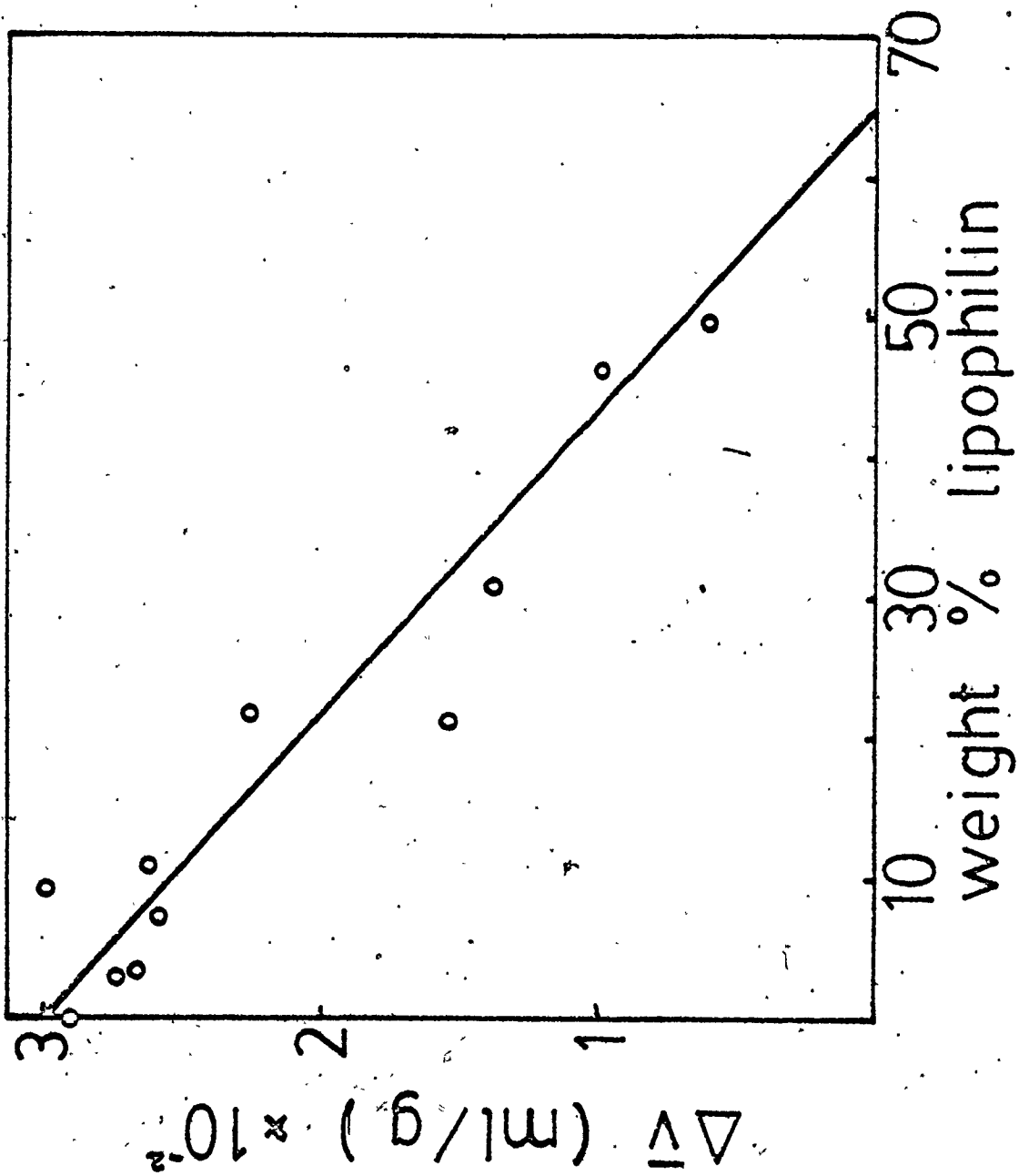
It has been suggested that the volume changes observed in phospholipid bilayers are caused predominantly by trans to gauche isomerization of the hydrocarbon chains (Nagle and Wilkinson, 1978). Therefore the volume coefficient of expansion $\alpha$, is probably closely related to

the rate of increase in gauche bonds with increasing temperature. Therefore at a maximal number of gauche bonds one would expect a low value of $\alpha$. This would explain why DMPC has an $\alpha$ value of $5 \times 10^{-4}$ deg.$^{-1}$ at 50°C (Nagle and Wilkinson, 1978). Systems which are particularly stable to changes in temperature, such as crystalline forms, may also have low values of $\alpha$. Figure 4 and Table 3 show that the volume coefficient of expansion remains constant both below and above the main phase transition as the percent lipophilin is increased in both experimental and theoretical cases. The decrease in the baseline with higher temperatures could be attributed to a maximal number of gauche bonds being reached. The greatest change in $\alpha$ occurs in the region of the phase transition. Even though there are large errors in $\alpha$ some conclusions can be reached. Increasing the percentages of lipophilin decreases $\alpha$ through the phase transition. This is evident in both the experimental and theoretical cases. As the lipophilin concentration increases, the curve levels out between 25 and 50% lipophilin. This can be correlated with the cooperativity unit since a broader transition means a smaller cooperativity unit as well as a smaller $\alpha$.

A recent calorimetric study of proteolipid apoprotein/DMPC complexes has shown that, in the presence of PLA, a population of DMPC molecules undergoes a perturbed order-disorder transition (Curatolo et al. 1977). Thermodynamics indicated that this population, referred to as "boundary" lipid (Jost et al. 1973), must possess partially disordered acyl chains below the transition or partially ordered ("bound") acyl chains above the transition, or both. However thermodynamic measurements were insufficient to distinguish between the possibilities.

In this study the presence of this so called boundary lipid is evident. Using spin-labels, Boggs et al. (1976) concluded that there were two distinct components of the lipid in lipophilin-lipid recombinants. One was immobilized, presumably that which was bound to the protein. The second component was similar to that of phosphatidylcholine vesicles and thought to be the lamellar phase. In this study the phase transition temperature of 24°C is altered very little no matter how much lipophilin is incorporated. Even though the phase transition temperature is altered very little, the protein does broaden the phase transition as the percent lipophilin is increased. The broadening effect is due to a reduction in the cooperativity of the transition. The protein seemingly takes a layer of lipid surrounding itself out of the normal transition. This bound lipid does not go through a phase transition. The change in state that is seen in the densimetry scans is due to the bulk lipid. Figure 16 demonstrates this point. Figure 16 illustrates the change in partial specific volume $\Delta\bar{v}$, plotted against weight percent lipophilin. It would seem logical that as more lipid is prevented from participating in the phase transition, this $\Delta\bar{v}$ would decrease. However in Table 1 there is one value of $\Delta\bar{v}$ that is somewhat high even though the general trend is a decrease in $\Delta\bar{v}$ as the weight percent of lipophilin is increased. This high value can be attributed to experimental error. In some of the scans it was somewhat arbitrary where the tangent to the lines are drawn (refer to Figure 2, g). Since $\Delta\bar{v}$ is an indication of the amount of lipid participating in the phase transition one can estimate the amount of lipid that the protein has in its boundary layer. DMPC has a molecular weight of 695.9 while the

Figure 16. Change in partial specific volume ($\Delta\bar{v}$) versus weight percent lipophilin. Procedures used to obtain $\Delta\bar{v}$ are described in Materials and Methods. The line was obtained through linear regression.


$\Delta\bar{v}$ (ml/g) $\times 10^{-2}$
3
2
1
10
30
50
70
weight % lipophilin

monomer form of lipophilin has a molecular weight of 25,000. Therefore if one plots $\Delta\bar{v}$ versus the weight percent lipophilin, the number of lipid molecules surrounding a monomer of lipophilin can be estimated (Figure 16). By doing a linear regression analysis on the points in Table 1 one arrives at an x-intercept of 65% lipophilin by weight. This is the point where the change in partial specific volume is zero, so theoretically there is no free lipid. Expressed as amolar ratio there are approximately 19 lipid molecules per molecule of lipophilin. This presumably represents the boundary layer. This value is in agreement with that found by Boggs and Moscarello (1978c). They arrived at their value of 21-25 lipid molecules per molecule of lipophilin by plotting the enthalpy of the transition against the protein to lipid mole ratio and extrapolating to $\Delta H=0$. The difference between the value obtained in this study and those found by Boggs and Moscarello (1978c) can be attributed to the respective methods of investigation. In calorimetry one is dealing with the first derivative of the curves obtained by densimetry. The first derivative is more sensitive to baseline shifts and hence small changes are more evident. Therefore one can expect a somewhat poorer detection of broad transitions by densimetry. However the values for $\Delta\bar{v}$ at 46.2 and 49.5% lipophilin can be questioned. There seems to be very little change in $\bar{v}$ at the phase transition. However the original scans from the densimeter for these complexes definitly do show a transition. Therefore the values obtained for $\Delta\bar{v}$ may be inaccurate, but are definitely not zero. Even by placing less emphasis on these points when obtaining the line for Figure 16, the intercept on the x-axis is still close to what is found when these points are

included.

The amount of boundary lipid found for lipophilin is comparable to that found for other proteins, approximately 30 for the sarcoplasmic reticulum $Ca^{2+}$, $Mg^{2+}$- ATPase (Warren et al. 1975) and 47 for cytochrome oxidase (Jost et al. 1973). However these two proteins have a much higher molecular weight than lipophilin. The radius of lipophilin in vesicles is 54Å as determined by x-ray diffraction (Brady et al. 1979). That means that lipophilin in vesicles has a circumference of 339Å which allows about 70 phospholipids to interact in its boundary layer. That implies 23 lipids/monomer of protein (Boggs and Moscarello, 1978c) which is in good agreement with the results of this study. However it is not known what state of association of lipophilin is in the bilayer. It would be anticipated that if the protein were monomeric more lipid could bind per molecule of protein, but this may not be the case if there is some lipid entrapped in an aggregated form of the protein.

Figures 17-19 show what effect lipophilin has on DMPC bilayers. Figure 15 shows what would be expected if there was no effect on the bilayer due to the incorporation of lipophilin at 20°C (labelled as theoretical results). The solid line represents the experimental results. The deviation from the theoretical is significant, indicating that the protein does affect the bilayer. At low protein to lipid ratios the differences are not great between the theoretical and experimental results. This is probably due to the fact that when little protein is incorporated, most of the lipid is bulk lipid. As the amount of protein is increased its effects become more evident. Figure 17 is a plot of the partial specific volume of the lipid calculated from the partial

Figure 17. Calculated partial specific volume ($\bar{v}$) of DMPC from varying protein to lipid complexes at 20°C. The $\bar{v}_{DMPC}$ was calculated from the experimentally obtained partial specific volumes of the complexes assuming that the partial specific volume of the lipophilin does not change. Curve was obtained through linear regression.

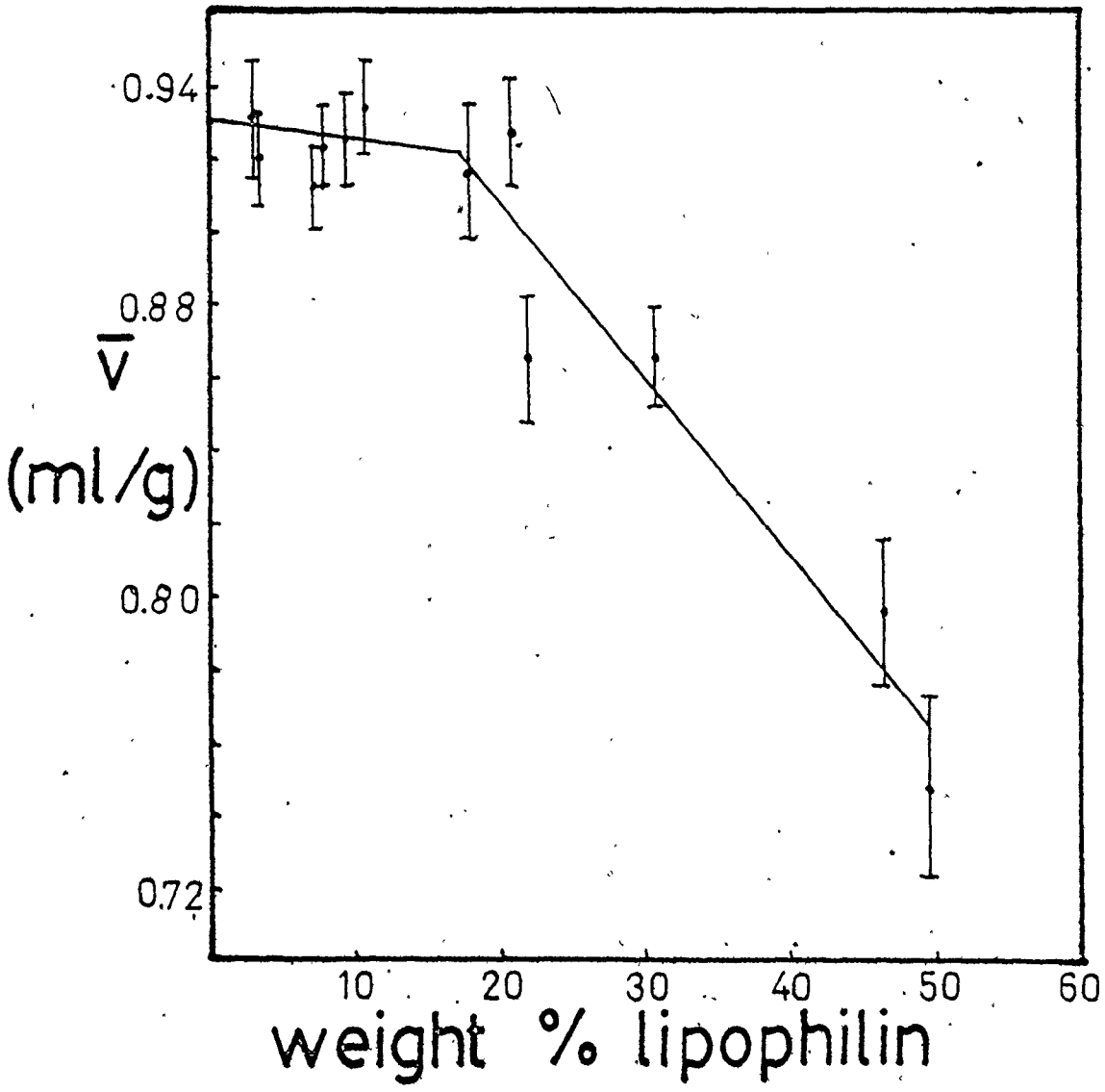

0.94
0.88
0.80
0.72
$\bar{V}$
(ml/g)
10
20
30
40
50
60
weight % lipophilin

Figure 18. Calculated partial specific volume of DMPC from varying protein to lipid complexes at 10°C. The $\bar{v}_{DMPC}$ is calculated from the experimentally obtained partial specific volumes of the complexes assuming that the partial specific volume of the lipophilin does not change. The curve is obtained through linear regression.

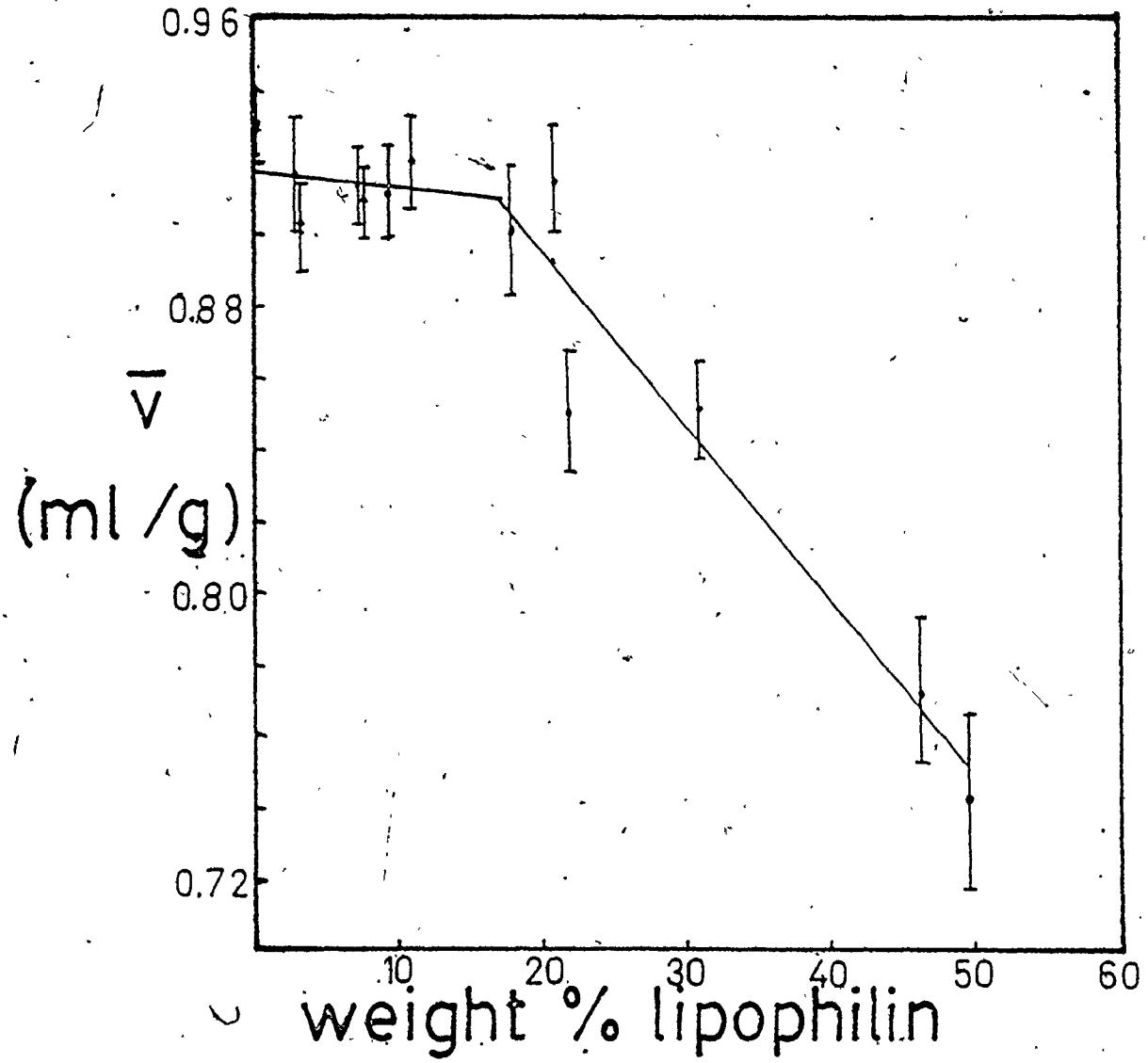
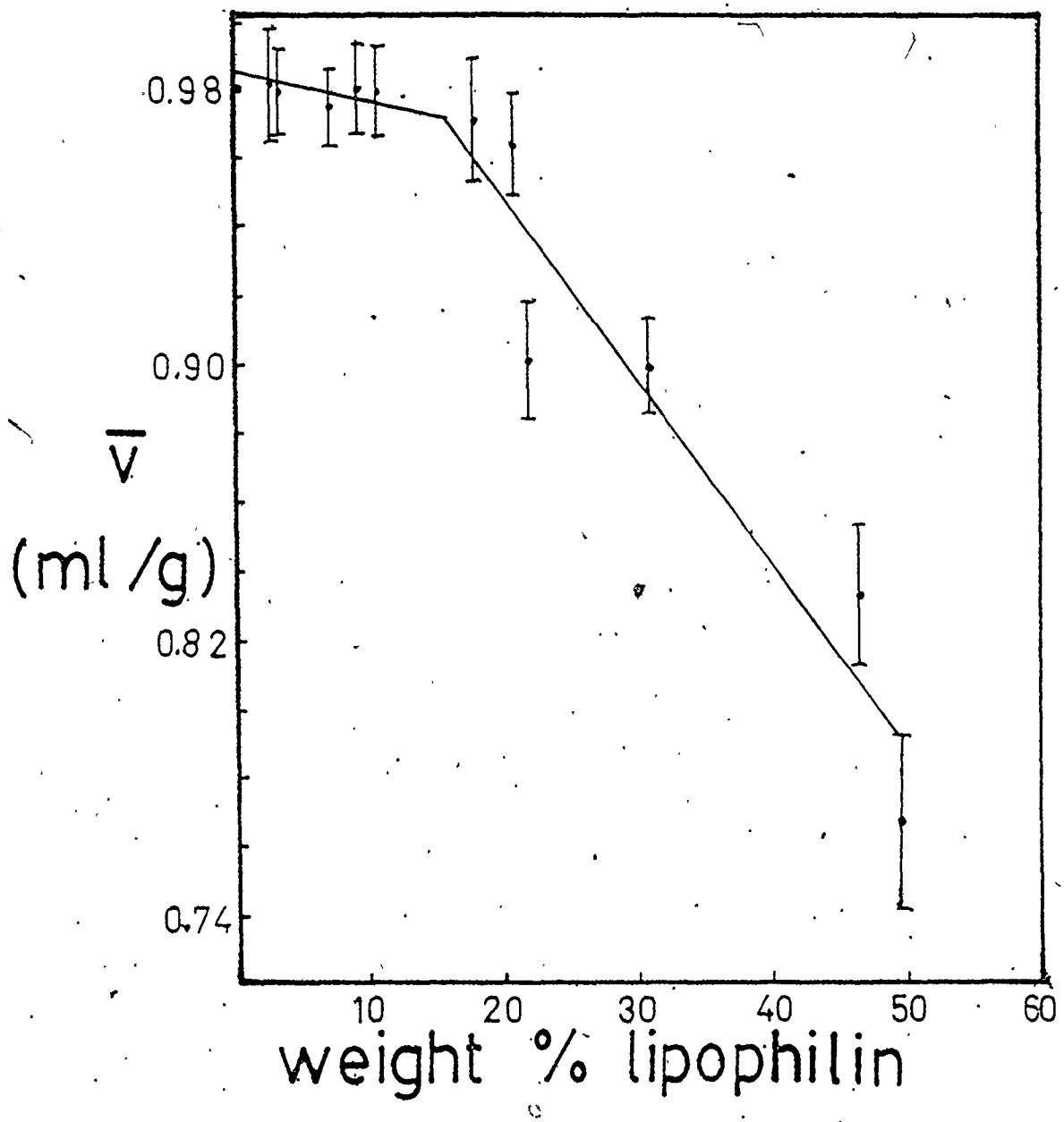

0.96
0.88
0.80
0.72
$\bar{v}$
(ml/g)
10
20
30
40
50
60
weight % lipophilin

Figure 19. Calculated partial specific volume ($\bar{v}$) of DMPC from varying protein to lipid complexes at 40°C. The $\bar{v}_{DMPC}$ is calculated from the experimentally obtained partial specific volumes of the complexes assuming that the partial specific volume of the lipophilin does not change. The curve is obtained through linear regression.


0.98
0.90
0.82
0.74
$\bar{v}$
(ml/g)
10
20
30
40
50
60
weight % lipophilin

specific volume of the complexes, assuming that the partial specific volume of the protein does not change when incorporated into the bilayer. Thus the figure shows that as the amount of the protein is increased there is a decrease in the partial specific volume of the lipid. What this means is that the protein imparts a condensing effect on the bilayer. Figures 18 and 19 illustrate what is occuring at 10 and 40°C respectively. The effects throughout the temperature range appear to be the same. However the two step shapes of Figures 17, 18 and 19 can not be taken as proven. One can, by linear regression, obtain a single line with a regression coefficient between 0.85 and 0.87, while for the two component system the regression coefficient is about 0.90. The points do have a large amount of error, but do indicate a condensing effect. At low amounts of protein incorporated, the changes are similar, since not much lipid is taken out of the bulk lamellar phase. The changes are much more evident at higher amounts of incorporated protein. However these points may be in somewhat of error since at higher amounts of incorporated protein, the densimeter may not give accurate results. The precision of the densimeter is about $5.5 \times 10^{-3}$ ml/g at high lipophilin concentrations in the bilayer (see Results).

It is not known in what state of aggregation lipophilin is found in the bilayer. It has been suggested that it is in a trimeric state (Boggs and Moscarello, 1978c). If it is in this state and lipophilin is somewhat spherical then it is possible that there may be some lipid that is not in the boundary layer, but could be trapped within the pocket made by the three lipophilin monomers. Due to its proximity to boundary lipids, its conformation may not be like that of the bulk lipid.

This would give this type of lipid a lower partial specific volume. Therefore at low protein to lipid ratios there would not be much of this sort of lipid because the protein would be monomeric. However as the protein to lipid ratio increases the protein would begin to aggregate and hence there would be more of this "trapped" form of lipid. The effects are seen in Figures 17-19.

Curatolo et al. (1977), using proteolipid apoprotein incorporated into DMPC attempted to explain what is occuring as the amount of PLA is increased. At low PLA concentrations there is a lot of free lipid compared to bound lipid. They represent bound lipid as concentric circles around the protein. As the amount of protein is increased these concentric circles converge, decreasing the amount of free lipid. At high protein concentrations all the lipid could quite likely be bound lipid. However it is difficult to visualize a discrete boundary between lipid that is in contact with the protein and that which is not. Two recent theoretical studies predicted that membrane proteins should perturb several layers of lipid beyond the boundary layer (Marcelja, 1976; Owicki et al. 1978). Therefore this perturbed lipid adjacent to the boundary lipid may contribute to the low partial specific volume of DMPC as the lipophilin percentage is increased.

The decrease in partial specific volume of the lipid assumes that there is no change in the partial specific volume of the protein at the given temperature. Between 10 and 40°C there is a 2.9% increase in the partial specific volume of the protein (Figure 2, k). The lipophilin for these experiments is in a highly aggregated form. The partial specific volume of this form of lipophilin is 0.732±0.003 ml/g at 20°C

while the water soluble form of the protein has a partial specific volume of 0.715±0.004 ml/g at 20°C. The differences seen seem to be the result of the hydration states in different solvents. The differences are also emphasized when one notes that the partial specific volume of lipophilin in 2-chloroethanol is 0.789 ml/g. It is known that the more hydrated a protein is, the lower the volume becomes (Haurowitz, 1965). The water content in 2-chloroethanol is less than in buffer which may be the cause of the difference in partial specific volumes. The differences in the partial specific volume of lipophilin in buffer and water can be explained if one takes into account the charged species of the buffer and the aggregation state of lipophilin in buffer. In the buffer system, the charged species of the buffer would be in competition with water molecules, thus decreasing the amount of hydration and consequently the partial specific volume of the protein. Since the protein aggregates in buffer this would also change the solvation of the protein which would increase the partial specific volume.

By the method of Zamyatnin (1972), the partial specific volume of lipophilin at 25°C is 0.7433 ml/g. This method uses only the amino acid composition of the protein. At 25°C the experimental partial specific volume of lipophilin is 0.7336 ml/g. However this method does not take into account hydration or any amino acid interactions resulting from tertiary structure. However partial specific volume is relatively insensitive to these factors. Therefore the partial specific volume calculated by the method of Zamyatnin (1972) can be considered reliable.

It seems that the partial specific volume of the protein is dependent on the environment it is in. Since the protein is embedded

in the bilayer, it may have a different partial specific volume than it has in buffer since it may interact selectively with charged head groups and hydration would be minimal within the bilayer.

However the results obtained from the densimeter can be considered both accurate and precise. The precision of the partial specific volume is about $5.5 \times 10^{-3}$ ml/g while the accuracy is almost as good. The accuracy was determined by measuring the partial specific volume of BSA and lysozyme and comparing then to literature values (see Results).

The actual method to determine the partial specific volume has limited error. The calibration constant, k, which is $T^2_{H_2O} - T^2_{air} / \rho_{H_2O} - \rho_{air}$ has minimal error since $\rho_{H_2O}$ and $\rho_{air}$ are international values having little or no error. The T values are again in error of about $1 \times 10^{-5}$ as shown by the precision measurements. To calculate the density of the sample or buffer, the equation used is:
$\rho_{sample} = [(T^2_{sample} - T^2_{H_2O})/k] + \rho_{H_2O}$. The T values have a $1 \times 10^{-5}$ error inherent in them while k has about $2 \times 10^{-5}$ error. Most error comes when partial specific volume, $\bar{v}$, is calculated.
Since $\bar{v} = (1/\rho_{buffer})[1 - (\rho_{sample} - \rho_{buffer}/C)]$ we can see by the error analysis above, that the densities have very little, if any, error. So we can with good confidence subtract $\rho_{buffer}$ from $\rho_{sample}$. The main error comes from the concentration. The error in protein determination is about 5% as it is in the phosphate determination. This gives us an overall error of about 5% in C, thus giving an overall error of 5% in $\bar{v}$. Therefore one can see that this method for determining partial specific volume has little error. By looking at the error in the partial specific

volume determination of BSA and lysozyme we find it to be as low as 2.5%. So an overall error estimate of 5% in partial specific volume seems quite reasonable.

Overall this study has shown a number of things. Lipophilin has a condensing effect on DMPC bilayers throughout the lipophilin percentages tested, below, near and above the phase transition of the lipid. The partial specific volume of lipophilin has never been reported. In this study, this parameter of the protein is evaluated using a relatively new technique; namely densimetry. In the course of this study the technique itself has been shown to be accurate as well as precise.

## References


Adams, C.W.M. (1977) Brit. Med. Bull. 33, 15-20.

Adams, C.W.M., Bayliss, O.B., Hallpike, J.F. and Turner, D.R. (1971) J. Neurochem. 18, 389-394.

Albon, N, and Sturtevant, J.M. (1978) Proc. Nat. Acad. Sci. 75, 2258-2260.

Anthony, J. and Moscarello, M.A. (1971) FEBS Letts. 15, 335-339.

Autilio, L.A. and Norton, W.T. (1964) J. Neurochem. 11, 17.

Banik, N.L. and Davison, A.N. (1974) Biochem. J. 143, 39-45.

Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.

Blazyk, J.F., Melchior, D.L. and Steim, J.M. (1975) Anal. Biochem. 68, 586-599.

Boggs, J.M., Clement, I.R. and Moscarello, M.A. (1980) Biochim. Biophys. Acta 601, 134-151.

Boggs, J.M. and Moscarello, M.A. (1978a) J. Mem. Biol. 39, 75-96.

Boggs, J.M. and Moscarello, M.A. (1978b) Biochim. Biophys. Acta 515, 1-21.

Boggs, J.M. and Moscarello, M.A. (1978c) Biochemistry 17, 5734-5739.

Boggs, J.M., Moscarello, M.A. and Papahadjopoulos, D. (1977) Biochemistry 16, 5420-5426.

Boggs, J.M., Stollery, J.G. and Moscarello, M.A. (1980) Biochemistry 19, 1226-1234.

Boggs, J.M., Vail, W.J. and Moscarello, M.A. (1976) Biochim. Biophys. Acta 448, 517-530.

Boggs, J.M., Wood, D.D., Moscarello, M.A. and Papahadjopoulos, D. (1977) Biochemistry 16, 2325-2329.

Brady, G.W., Birnbaum, P.S., Moscarello, M.A. and Papahadjopoulos, D. (1979) Biophys. J. 26, 23-42.

Braun, P.E. (1977) in: Myelin, Pierre Morell (ed.), Plenum Press, New York and London, pp. 91-115.

Braun, P.E., Pereyra, P.M. and Greenfield, S. (1980) in: Myelin: Chemistry and Biology, George A. Hashim (ed.), Alan R. Liss Inc., New York.

Braun, P.E. and Radin, N.S. (1969) Biochemistry 8, 4310-4318.

Brostoff, S.W. and Eylar, E.H. (1971) Proc. Nat. Acad. Sci. 68, 765-769.

Bull, H.B. and Breese, K. (1968) J. Phys. Chem. 72, 1817.

Carnegie, P.R. (1971) Nature 229, 25-28.

Carnegie, P.R., Bencina, B. and Lamoureux, G. (1967) Biochem. J. 105, 559-568.

Chan, D.S. and Lees, M.B. (1974) Biochem. J. 13, 2704-2712.

Chen, R.F. (1967) J. Biol. Chem. 242, 173-181.

Choa, L.P. and Einstein, E.R. (1970) J. Neurochem. 17, 1121-1132.

Cockle, S.A., Epand, R.M., Boggs, J.M. and Moscarello, M.A. (1978a) Biochemistry 17, 624-629.

Cockle, S.A., Epand, R.M. and Moscarello, M.A. (1978b) Biochemistry 17, 630-637.

Cockle, S.A., Epand, R.M. and Moscarello, M.A. (1978c) J. Biol. Chem. 253, 8019-8026.

Curatolo, W., Sakura, J.D., Small, D.M. and Shipley, G.G. (1977) Biochemistry 16, 2313-2319.

Curatolo, W., Verma, S.P., Sakura, J.D., Small, D.M., Shipley, G.G. and Wallach, D.F.H. (1978) Biochemistry 17, 1802-1807.

Cuzner, M.L. and Davison, A.N. (1979) in: Molecular Aspects of Medicine, Baum, H. and Gergely, J. (eds.), Pergamon Press, Oxford, New York and London.

Cuzner, M.L., Davison, A.N. and Gregson, N.A. (1965) J. Neurochem. 12, 469-481.

Davison, A.N. (1970) in: Myelination, Davison, A.N. and Peters, A. (eds.), C.C. Thomas, IL.

Dehliger, P.J., Jost, P.C. and Griffith, O.H. (1974) Proc. Nat. Acad. Sci. 71, 2280-2284.

Demel, R.A., London, Y., Geurt von Kessel, W.S.M. and Vossenberg, F.G.A. (1973) Biochim. Biophys. Acta 311, 507-519.

Dickenson, J.P., Jones, K.M., Aparicio, S.R. and Lumsden, C.E. (1971) Nature 227, 1133-1134.

Eichburg, J. and Davison, R.M.C. (1965) Biochem. J. 96, 644-650.

Eliasz, A.W., Chapman, D. and Ewing, D.F. (1976) Biochim. Biophys. Acta 448, 220-230.

Epand, R.M. and Epand, R.F. (1980) Chem. Phys. Lipids 27, 139-150.

Epand, R.M., Moscarello, M.A., Zirenburg, B. and Vail, W.J. (1974) Biochemistry 13, 1264-1267.

Eylar, E.H., Brostoff, S., Hashim, G., Caccam, J. and Burnet, P. (1971) J. Biol. Chem. 246, 5770-5784.

Eylar, E.H., Salk, J., Beverage, G. and Braun, L. (1969) Arch. Biochem. Biophys. 132, 34-48.

Fodor, D. and Epand, R.M. (1981) Chem. Phys. Lipids 28, 159-164.

Folch, J. and Lees, M. (1951) J. Biol. Chem. 191, 807-817.

Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497.

Folch-Pi, J. and Stoffyn, P.J. (1972) Ann. N.Y. Acad. Sci. pp. 86-107.

Gagnon, J., Finch, P.R., Wood, D.D. and Moscarello, M.A. (1971) Biochemistry 10, 4756-4763.

Golds, E.E. and Braun, P. (1976) J. Biol. Chem. 251, 4729-4735.

Gonzalez-Sastre, F. (1970) J. Neurochem. 17, 1049.

Goulds, R.M. and London, Y. (1972) Biochim. Biophys. Acta 290, 200-218.

Haurowitz, F. (1965) in: Chemistry and Functions of Proteins, Academic Press, New York, NY.

Herndon, R.M., Raunch, H.C. and Einstein, E.R. (1973) Immunol. Commun. 2, 163-172.

Hess, H.H., Lees, M.B. and Derr, J.E. (1978) Anal. Biochem. 85, 295-300.

Janiak, M.J., Small, D.M. and Shipley, G.G. (1976) Biochemistry 15, 4575-4580.

Jolles, J., Nussbaum, J.L., Schoentgen, F., Mandel, P. and Jolles, P. (1977) FEBS Letts. 74, 190-194.

Jost, P.C., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973) Proc. Nat. Acad. Sci. 70, 480-484.

Kibler, R.F. and Shapiro, R. (1968) J. Biol. Chem. 243, 281-286.

Kies, M. (1965) Ann. N.Y. Acad. Sci. 122, 161-170.

Kies, M., Thompson, B.E. and Alvord, E.C. (1965) Ann. N.Y. Acad. Sci. 122, 148-160.

Kratky, O., Leopold, H. and Stabinger, H. (1973) Methods in Enzymology 27, 98-110.

Kupke, D.W. and Dorrier, T.E. (1978) Methods in Enzymology 48, 155-162.

Ladbrooke, B.D. and Chapman, D. (1969) Chem. Phys. Lipids 2, 304-367.

Laggner, P. and Stabinger, H. (1976) in: Colloid and Interface Science, Vol. 5.

Lavialle, F. and Levin, I.W. (1980) Biochemistry 19, 6044-6050.

Ledeen, R.W., Yu, R.K. and Eng, L.F. (1973) J. Neurochem. 21, 829-840.

Lee, A.G. (1976) Biochim. Biophys. Acta 448, 34-44.

Lee, A.G. (1977a) Biochim. Biophys. Acta 472, 237-281.

Lee, A.G. (1977b) Biochim. Biophys. Acta 472, 285-344.

Lee, A.G. (1977c) Biochemistry 16, 835.

Lennon, V.A., Whittingham, S., Carnegie, P.R., McPherson, T.A. and MacKay, I.R. (1971) J. Immunol. 107, 56-62.

London, Y., Demel, R.A., Geurts von Kessel, W.S.M., Vossenburg, F.G.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 311, 520-530.

London, Y., Demel, R.A., Geurts von Kessel, W.S.M., Zahler, P. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 332, 69-84.

London, Y. and Vossenburg, F.G.A. (1973) Biochim. Biophys. Acta 307, 478-490.

Lowden, J.A., Moscarello, M.A. and Morecki, R. (1966) Can. J. Biochem. Physiol. 44, 567-577.

Mabrey, S. and Sturtevant, J.M. (1976) Proc. Nat. Acad. Sci. 73, 3862-3866.

Mabrey, S. and Sturtevant, J.M. (1977) Biochim. Biophys. Acta 486, 444.

Marcelja, S. (1976) Biochim. Biophys. Acta 455, 1-7.

Maten, L., Luzzati, V., London, Y., Gould, R.M., Vossenburg, F.G.A. and Olive, J. (1973) J. Mol. Biol. 75, 697-709.

Melchior, D.L. and Steim, J.M. (1976) in: Thermotropic Transitions in Biomembranes.

Miyamoto, E. and Kakiuchi, I. (1974) J. Biol. Chem. 249, 2769-2777.

Moscarello, M.A. (1976) Curr. Top. Memb. Trans. 8, 1-28.

Moscarello, M.A., Gagnon, J., Wood, D.D., Anthony, J. and Epand, R.M. (1973) Biochemistry 12, 3402-3406.

Murison, J., Festi, D., Ross, P.E. and Bouchier, I.A.D. (1976) Clin. Chim. Acta 68, 159-166.

Nagle, J.F. and Wilkinson, D.A. (1978) Biophys. J. 23, 159-175.

Nakoa, A., Davis, W.J. and Einstein, E.R. (1966) Biochim. Biophys. Acta 130, 163-170.

Norton, W.T. (1977) in: Myelin, Morell, P. (ed.), Plenum Press, New York and London.

Norton, W.T. and Poduslo, S.E. (1973) J. Neurochem. 21, 759-773.

Nussbaum, J.L., Rouayreng, J.F., Mandel, P., Jolles, J. and Jolles, P. (1974) Biochem. Biophys. Res. Commun. 57, 1240-1247.

Owicki, J.C., Springgate, M.W. and McConnell, H.M. (1978) Proc. Nat. Acad. Sci. 75, 1616-1619.

Palmer, F.B. and Dawson, R.M.C. (1969a) Biochem. J. 111, 629-636.

Palmer, F.B. and Dawson, R.M.C. (1969b) Biochem. J. 111, 637-646.

Papahadjopoulos, D., Hui, S., Vail, W.J. and Poste, G. (1976) Biochim. Biophys. Acta 448, 245-264.

Papahadjopoulos, D., Moscarello, M.A., Eylar, E.H. and Isac, T. (1975a) Biochim. Biophys. Acta 401, 317-335.

Papahadjopoulos, D., Vail, W.J. and Moscarello, M.A. (1975b) J. Mem. Biol. 22, 143-164.

Podo, F. and Blasie, J.K. (1976) Biochim. Biophys. Acta 419, 1-18.

Poduslo, J.F. and Braun, P. (1975) J. Biol. Chem. 250, 1099-1105.

Radin, N.S. (1969) Methods in Enzymology 14, 245-254.

Raine, C.S. (1977) in: Myelin, Pierre Morell (ed.), Plenum Press, New York.

Rice, D.M., Meadows, M.D., Scheinman, A.O., Goni, F.M., Gomez-Fernandez, J.C., Moscarello, M.A., Chapman, D. and Oldfield, E. (1979) Biochemistry 18, 5893-5903.

Roboz-Einstein, E., Robertson, D., Diaprio, J. and Moore, W. (1962) J. Neurochem. 9, 353-361.

Rumsby, M.G. and Crang, A.G. (1977) Cell Surf. Rev. 4, 247-362.

Sherman, G. and Folch-Pi, J. (1971) J. Neurochem. 17, 597-605.

Stoffyn, P. and Folch-Pi, J. (1971) Biochem. Biophys. Res. Commun. 44, 157-161.

Stollery, J.G., Boggs, J.M. and Moscarello, M.A. (1980) Biochemistry 19, 1219-1226.

Stollery, J.G., Boggs, J.M., Moscarello, M.A. and Deber, C.M. (1980) Biochemistry 19, 2391-2396.

Suzuki, K., Poduslo, J.F. and Poduslo, S.E.(1968) Biochim. Biophys. Acta 152, 576-586.

Takayama, K., MacLennan, D.H., Tzagloff, A. and Stoner, C.D. (1964) Arch. Biochem. Biophys. 114, 223-230.

Tomasi, L.G. and Kornguth, S.E. (1967) J. Biol. Chem. 242, 4933-4938.

Usher, J.R., Epand, R.M. and Papahadjopoulos, D. (1978) Chem. Phys. Lipids 22, 245-253.

Vail, W.J., Papahadjopoulos, D. and Moscarello, M.A. (1974) Biochim. Biophys. Acta 345, 463-467.

Verma, S.P., Wallach, D.F.H. and Sakura, J.D. (1980) Biochemistry 19, 574-579.

Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Nature 255, 684-687.

Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.

Wilkinson, D.A. and Nagle, J.F. (1979) Biochemistry 18, 4244-4249.

Wolfgram, F. (1965) Ann. N.Y. Acad. Sci. 122, 104-115.

Wood, D.D., Epand, R.M. and Moscarello, M.A. (1977) Biochim. Biophys. Acta 467, 120-129.

Wood, D.D., Gagnon, J., Finch, P.R. and Moscarello, M.A. (1971) Am. Soc. Neurochem. Trans. 2, 117.

Wood, D.D., Vail, W.J. and Moscarello, M.A. (1975) Brain Res. 93, 463-471.

Wood, J.G., Dawson, R.M.C. and Hauser, H. (1974) J. Neurochem. 22, 637-643.

Zamyatnin, A.A. (1972) Prog.Biophys. Mol. Bio. 24, 107-123.