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VOLUME PROPERTIES OF MIXTURES OF LIPOPHILIN AND DIMYRISTOYLPHOSPHATIDYLCHOLINE *

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The hydrophobic myelin protein, lipophilin, has been incorporated into bilayers of dimyristoylphosphatidylcholine by dialysis from 2-chloroethanol. The protein was shown to be incorporated into a protein-lipid complex of uniform density by density gradient sedimentation equilibrium. The volume properties of the resulting complexes were studied by densitometry. It was found that a molecule of the protein could prevent an increase in volume at the phase transition temperature of 19 lipid molecules. The remaining lipid underwent its phase transition over a broader temperature range, resulting in a decrease in the volume coefficient of expansion in the region of the phase transition. The protein has little effect on this parameter at higher or lower temperatures. The partial specific volume of the lipid alone was similar to what has been previously determined using freshly prepared suspensions. The partial specific volume of the protein alone was similar to the value calculated based on the amino acid composition. The partial specific volume of the lipid-protein complex, however, was less than the weighted average of the components, indicating that lipophilin could induce an increase in the density of the lipid. This condensing effect of lipophilin was observed both above and below the phase transition and may be a general property of proteins incorporated into lipid bilayers.

Introduction

The myelin membrane has relatively few major protein components. One of these, the protein lipophilin, has been purified from the human myelin proteolipid fraction [1]. Dialysis of a solution of lipophilin in chloroethanol against water results in the formation of a water-soluble form of the protein containing a high content of α-helix [2] which is particularly resistant to denaturation [3]. This procedure can also be used to incorporate the protein into phospholid bilayers. The protein has a high content of α-helix when incorporated into membranes in this manner [2] and its tryptophan residues are not readily accessible to the aqueous environment [4]. X-ray diffraction studies indicate that the protein is self-associated when bound to a membrane [5,6] and freeze-fracture studies suggest that the protein is more extensively clustered when the lipid is in the $P_{\beta'}$ phase [7].

The behaviour of phospholipid molecules in the presence of this protein has been studied with particular intensity because of interest in describing the nature of the lipid molecules surrounding integral membrane proteins. Differential scanning

Abbreviations: DMPC, dimyristoylphosphatidylcholine; TLC, thin-layer chromatography; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminotetraacetic acid.

calorimetry has demonstrated that with dimyristoylphosphatidylcholine (DMPC), lipophilin broadens the phase transition and lowers its enthalpy, indicative of the presence of boundary lipid [7,8]. Calorimetric studies have also shown that lipophilin preferentially interacts with acidic phospholipids [9] but that it does not distinguish between saturated lecithins containing fatty acids of between 14 and 18 carbon atoms although its interaction with dielaidoylphosphatidylcholine is less favourable [10]. Perturbation of the lipid structure by lipophilin has also been demonstrated by Raman spectroscopy [11–13]. There is a particularly large increase in the acyl chain gauche conformers in the gel state of DMPC in the presence of lipophilin [13]. It was shown by Boggs et al. [14] using spin-labelled fatty acid probes, that lipophilin immobilizes some surrounding lipid, at least on the time scale of electron spin relaxation. However, on the longer time scale of nuclear relaxation no immobilized boundary lipid was detected [15,16].

A fundamental property of a phospholipid bilayer is its partial specific volume. This volume can be perturbed by changes in lipid packing arrangements caused by a phase transition [17–20] or by the presence of added substances such as cholesterol [21] or peptides [22]. We have used a vibrating reed densitometer [23] to measure the effect of the incorporation of lipophilin on the packing of DMPC bilayers, their thermal coefficient of expansion and the volume change which occurs at the phase transition temperature. This is the first study to measure volume changes accompanying the incorporation of integral membrane proteins into phospholipid bilayers.

Materials and Methods

1. Materials

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

Myelin was isolated from normal human white matter by the method of Lowden et al. [24] and lipophilin extracted from it and purified by chromatography on Sephadex LH-20 and stored in lyophilized form [25].

The buffer system used for most experiments consisted of 100 mM NaCl, 2 mM Hepes, 0.1 mM EDTA and 0.02 mg/ml sodium azide, adjusted to pH 7.40 with NaOH.

2-Chloroethanol (BDH) was purified by vacuum distillation over Tris, Girard's reagent T, and Norit charcoal.

2. Methods

(i) Preparation of lipophilin-containing vesicles. DMPC and lipophilin are each individually dissolved in 2-chloroethanol at a concentration of 5 mg/ml. Brief sonication was used occasionally to affect the dissolution of lipophilin. The chloroethanol solutions of protein and lipid were mixed so as to obtain the desired protein to lipid ratio. This chloroethanol solution is then dialyzed at 25°C against 2 liters of buffer for 18 h with two changes of buffer. The buffer is saturated with N_2 and air is excluded during dialysis. The resulting suspensions are centrifuged at 7500 rpm in a Sorvall SS-34 rotor at room temperature. The supernatant is discarded and the pellet resuspended, by vortexing, into buffer.

Some of the experiments with pure lipophilin required a water soluble form of the protein. This was achieved by solubilizing the protein in 2-chloroethanol (5–10 mg/ml) and dialyzing as above but into doubly distilled water instead of buffer [2]. The resulting solutions are then centrifuged at 7500 rpm in a Sorvall SS-34 rotor at room temperature to remove suspended particles.

(ii) Amino acid analysis to determine protein concentration of the sample. Approx. 0.3 mg of protein was transferred to a hydrolysis vial with norleucine as an internal standard (10 nmol norleucine per 40 μg protein). The samples are hydrolyzed in sealed ampoules, under vacuum, with 1 ml of 6 M HCl at 110°C for 22 h. The samples are then brought to dryness, dissolved in 0.01 M HCl and run on a Durrum D-500 amino acid analyzer.

(iii) Determination of lipid concentration. The concentration of phospholipid was determined from total phosphate analysis [26] after overnight digestion of the sample in 5 M H_2SO_4 at 150°C and followed by oxidation with hydrogen peroxide [27].

(iv) Densitometry measurements. All solutions used for densitometry measurements were degassed under reduced pressure. Densitometry measurements were made with the use of two DMA 602 external cells using a DMA 60 measure unit in the phase lock loop mode (A. Paar K.G., Graz, Austria). The lipid-protein suspension was placed in one of the cells and buffer or dialyzate placed in the other. In the use of dialyzed samples, the dialyzate contained only traces of protein or phosphate. The details of the measuring system and the methods used to calculate apparent partial specific volumes are described elsewhere [20,23]. In the present work, the apparent partial specific volume has been measured at only a single concentration of solute. It is assumed that these values closely approximate the true partial specific volume, although the results were not extrapolated to infinite dilution. This is so because solute concentrations were below 1% and lipid systems, being in a separate phase, show no concentration dependence of the apparent partial specific volume in the presence of excess water (Wilkinson and Nagle, personal communication).

The volume change at the transition temperature was obtained by extrapolating the variation of $\bar{v}$ with temperature outside the phase transition region to the transition temperature and measuring the difference in volumes between the values extrapolated from high and low temperatures, respectively. The volume coefficient of expansion, α, given by

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

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

2-Chloroethanol has a density of 1.20 g/cm^3, quite different from that of water. Therefore, if there was substantial amount of 2-chloroethanol present after dialysis, the density of the dialyzate would be different from that of buffer. However, no differences in density could be detected indicating the virtual complete removal of 2-chloroethanol from the aqueous phase.

(v) Density gradient ultracentriguation. The extent of incorporation of lipophilin into DMPC bilayers and the degree of homogeneity of the product was evaluated by density gradient ultracentrifugation. The gradients were formed by a mechanical gradient maker in cellulose nitrate tubes, usually between 20 and 65% sucrose (w/w) with approx. 1 mg of sample introduced into the gradient. Runs were done with an SW 27 swinging bucket rotor, for 24 hours at 76000 xg at 20°C. Fractionation was made by an ISCO Density Gradient Fractionator and 25-drop samples collected. The linearity of the gradient was determined from the refractive index of each tube. Protein was detected by a modified Folin-Lowry assay for insoluble proteins [28]. Lipid concentration was determined for each fraction by extracting the lipid with chloroform, removing the lower phase, evaporating the solvent and finally performing phosphate determinations on the residues.

Results

The observed temperature dependence of the partial specific volume of lipophilin-DMPC mixtures (Fig. 1) can be compared to that calculated assuming no volume change on mixing lipophilin and DMPC (Fig. 2). The curves in Fig. 2 are calculated according to the formula

$$\bar{v}_{\text{complex}} = W_l \bar{v}_l + W_p \bar{v}_p$$

where W_l and W_p are the weight fractions of lipid and protein, respectively, and $\bar{v}_l$ and $\bar{v}_p$ are their partial specific volumes. The values of $\bar{v}_l$ and $\bar{v}_p$ are obtained from curves a and k, respectively, of Fig. 1. Fig. 2 shows a progressive decrease in the magnitude of the volume change at the phase transition in going from curve a to curve j, as more protein is added to the complex. This is because the $\bar{v}$ is expressed per gram of material and as lipid is replaced by protein there is less lipid remaining to undergo a transition. However, the results of Fig. 1 demonstrate that the observed change in volume decreases even more rapidly than what would be expected on the basis of the change in composition of the sample. This is particularly true for samples containing more than 22% lipophilin. The densitometer scan for the pure lipid (Fig. 1a) appears similar to published results for this lipid [17–20] demonstrating the efficacy of the dialysis procedures in eliminating the 2-chloro-

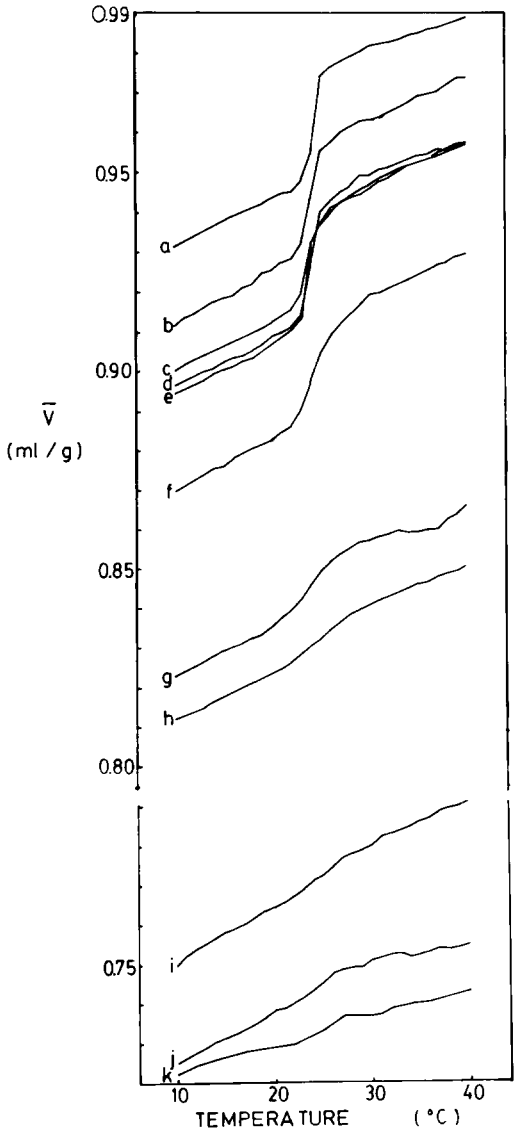

Fig. 1. Densitometry measurements of lipophilin-DMPC complexes. The heating scan rate for all experiments was 0.5 K/min. The average sample concentration was 5 mg/ml. Scans correspond to those from samples containing the following weight % of lipophilin: a, 0%; b, 3.0%; c, 7.3%; d, 7.4%; e, 9.3%; f, 17.8%; g, 21.7%; h, 30.7%; i, 46.2%; j, 49.5%; k, 100%.

ethanol. However, the premelt transition observed with freshly prepared suspensions of DMPC is not detectable in scans of lipid dialyzed from 2-chloroethanol. This could be due to trace contamination with 2-chloroethanol or with products of lipid degradation formed during the long dialysis at 25°C. It may also be caused by a change in the

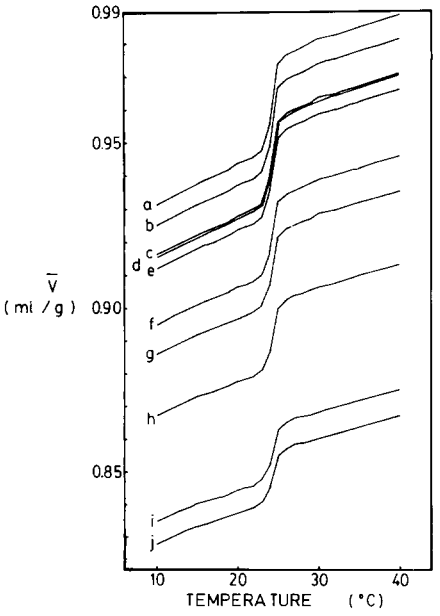

Fig. 2. Theoretical partial specific volumes ($\bar{v}$) for the lipophilin-DMPC complexes. The $\bar{v}$ was calculated assuming that there is no volume change upon mixing of the components. Curves for various weight % lipophilin labelled as in Fig. 1.

lipid organization, such as the number of lamelli or size of the liposomes formed. Thus, there are several factors which could lead to a loss of the premelt transition. If it were due to trace contamination by chloroethanol, this contamination is

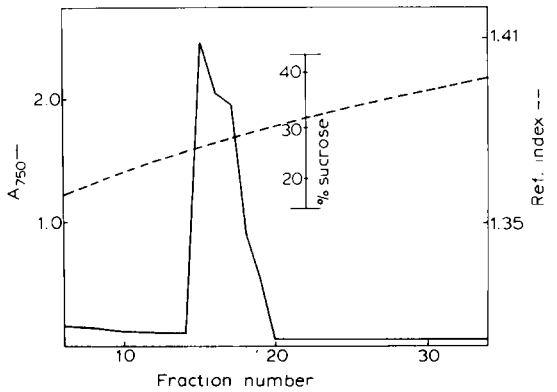

Fig. 3. Sucrose density ultracentrifugation results on a sample comprising 46.2% (by weight) lipophilin in DMPC. The gradient was monitored by refractometry (— — —). Centrifugation was in an SW27 rotor for 24 h at 76000 × g at 20°C. The gradient was divided into fractions of 25 drops. Protein determination was by a modified Folin-Lowry assay for insoluble proteins (see Materials and Methods) measuring A_{750} (———).

not sufficient to significantly effect the measured density of the lipid. The scan for the pure protein was performed on a suspension formed by dialysis of a chloroethanol solution of the protein against buffer. Dialysis against buffer gives rise to precipitated samples. These have the advantage that they can be readily concentrated by centrifugation for the densitometry measurements. However, they have the disadvantage that they are inhomogeneous and the density of a suspension, particularly the protein suspension, can not be obtained as accurately as that of a solution. In order to verify the accuracy of the measurement of the protein density, aqueous forms of the protein were prepared by dialysis of the protein from chloroethanol into distilled water, rather than buffer, resulting in the formation of a clear solution. Lipophilin at a concentration of 1–2 mg/ml in distilled water yielded a $\bar{v}$ of 0.714 ml/g at 20°C. The comparative value for lipophilin dialyzed into buffer is 0.732 ml/g which is also close to the value of 0.743 ml/g calculated from the amino acid composition [29].

Density gradient centrifugation demonstrates that lipophilin is incorporated into DMPC in a complex of relatively uniform density. For example, at 46% lipophilin the protein bands over a narrow density range in a position between that observed with pure lipid and that observed with the pure protein (Fig. 3). No bands of free lipid or free protein were observed in these gradients. In a separate experiment, the free protein was found to band anomalously at a position corresponding to a substance of partial specific volume 0.80 ml/g. In general, proteins are too small to band in a density gradient under the centrifugation conditions used here and it is not known why lipophilin bands in this manner.

Density gradient centrifugation was also used to demonstrate the absence of 2-chloroethanol trapped inside of the vesicles. A sample containing 84% lipophilin by weight in DMPC was isolated by density gradient centriguation both before and after sonciation. Both sonicated and unsonicated sample banded in the same position in the gradient. This indicates that no entrapped chloroethanol was liberated by the sonication treatment.

Discussion

The volume change per gram of solute, observed over the temperature region of the phase transition, would be expected to decrease with increasing amounts of protein since the protein does not undergo a transition over this temperature range (Fig. 2). However, the observed decrease in the change in volume at the phase transition (Fig. 1) is greater than this. This suggests that lipophilin is preventing some lipid from undergoing a phase transition while only broadening the phase transition of the remaining lipid (Fig. 1). The dependence of the volume change at the phase transition on the weight fraction of lipophilin (Fig. 4) indicates that by linear extrapolation there would be no volume change at approx. 65% lipophilin. This composition represents a molar ratio of 19 lipid molecules per molecule of lipophilin and corresponds to the layer of boundary lipid around the protein which does not undergo a phase transition. The value is close to the 21–25 boundary lipid molecules per lipophilin estimated by Boggs and Moscarello from the effect of the protein on the transition enthalpy [10].

We can also calculate how lipophilin effects the packing of phospholipid bilayers at various temperatures. As a first approximation we can assume that the $\bar{v}$ of the protein will not be altered when it is incorporated into a bilayer since proteins un-

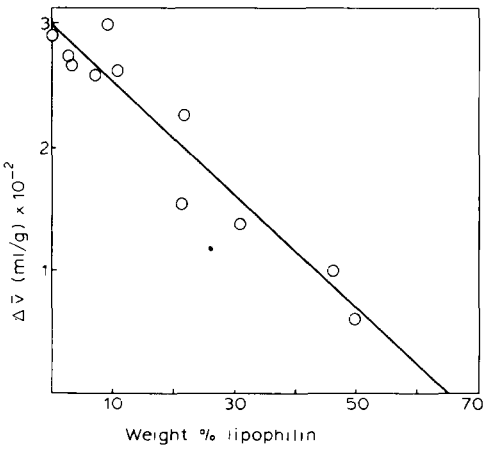

Fig. 4. Change in partial specific volume ($\Delta\bar{v}$) versus weight percent lipophilin. Procedures used to obtain $\Delta\bar{v}$ is descrived in Materials and Methods. The line is obtained by linear regression.

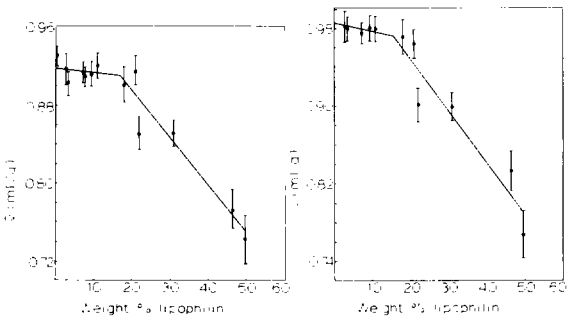

Fig. 5. Calculated partial specific volume ($\bar{v}$) of DMPC in the presence of varying amounts of lipophilin at 10°C (left) and 40°C (right). The $\bar{v}$ of the lipid component is calculated from the experimentally determined partial specific volumes of the complexes and assuming that the partial specific volume of lipophilin in the lipid is the same as it is in free solution.

dergo volume changes of only about 10^{-2} ml/g even as a result of extensive conformation change [29] and there is little effect of protein concentration on $\bar{v}$ [30]. The only known example of a large change in protein volume is a result of dehydration which leads to an increase in volume [31]. It would be expected that if there is any change in hydration in going from the water soluble form of the protein to the lipid embedded form there would be a decrease in hydration thus causing an increase in volume. This is opposite, however, to the direction of the observed volume change and therefore this could no contribute greatly to the experimentally observed change. The lipid which is of much lower molecular weight is held in a bilayer arrangement only by non-covalent bonding forces and can more readily undergo changes in volume. From the observed $\bar{v}$ of the complex at a known weight fraction of lipid and protein, and assuming the $\bar{v}$ of the protein in the presence of lipid to be the same as the pure protein, we can calculate the $\bar{v}$ of the lipid in this complex. These values are given in Fig. 5 for data taken at 10 and at 40°C, i.e. below and above the phase transition temperature of the phospholipid. The effect of the protein on the $\bar{v}$ of the lipid is similar for the two temperatures and indicates that the protein promotes an increase in the packing density of the lipid. A similar situation has been observed for the effect of glucagon on the $\bar{v}$ of DMPC [22] and may be a general effect of proteins on lipid packing. It may arise from the preferential interaction of proteins and peptides with defects in the bilayer. In this manner proteins can be incorporated into the bilayer without displacing any lipid molecules. As a consequence there is an increased density of the complex compared to the sum of its components. This is different from the incorporation of cholesterol into a bilayer which results in an increase in volume at temperatures below the phase transition temperature [21]. Thus, although the protein appears to increase the number of *gauche* bonds in the acyl side chains of the gel state lipid [13], a phenomenon that is usually accompanied by decreased membrane order and increased membrane volume, in this case the volume of the membrane actually decrease. This suggests that the acyl chains of the lipid become bent in a specific manner, perhaps to accommodate themselves to the contours of the protein. The shapes of the curves in Fig. 5 suggests little change in the $\bar{v}$ of the lipid below about 20% lipophilin. However, we cannot state this conclusion with a great degree of certainty since the deviation of these curves from a single linear relationship is not very great. It is possible that a high concentration of lipophilin is required to observe the effect because the bilayer structure of the lipid must be disrupted in order for the density to be perturbed. This could arise, for example, if phospholipids are trapped inside the protein.

Another property which can be calculated from the curves of Fig. 1 is the volume coefficient of expansion, α. It has been suggested that the compressibility of the bilayer is indicative of its permeability [20,32]. We find no large effect of lipophilin on the value of α either above or below the phase transition region. In the region of the phase transition, however, the protein causes α to decrease but this is simply a result of the broadening of the phase transition. Thus, lipophilin has no marked effect on the volume coefficient of expansion. Its main effects on the volume properties of DMPC are to lower the volume change which occurs at the phase transition and to increase the packing density of the bilayer.

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References

1 Moscarello, M.A. (1976) Curr. Top. Trans. 8, 1–28
2 Cockle, S.A., Epand, R.M., Boggs, J.M. and Moscarello, M.A. (1978) Biochemistry 17, 624–629
3 Cockle, S.A., Epand, R.M. and Moscarello, M.A. (1978) J. Biol. Chem. 253, 8019–8026
4 Cockle, S.A., Epand, R.M. and Moscarello, M.A. (1978) Biochemistry 17, 630–637
5 Brady, G.W., Birnbaum, P.S., Moscarello, M.A. and Papahadjopoulos, D. (1979) Biophys. J. 26, 23–42
6 Brady, G.W., Birnbaum, P.S. and Moscarello, M.A. (1979) Biophys. J. 26, 49–60
7 Boggs, J.M., Clement, I.R., Moscarello, M.A. (1980) Biochim. Biophys. Acta 601, 134–151
8 Curatolo, W., Sakura, J.D., Small, D.M. and Shipley, G.G. (1977) Biochemistry 16, 2313–2319
9 Boggs, J.M., Wood, D.D., Moscarello, M.A. and Papahadjopoulos, D. (1977) Biochemistry 16, 2325–2329
10 Boggs, J.M. and Moscarello, M.A. (1978) Biochemistry 17, 5734–5739
11 Curatolo, W., Verma, S.P., Sakura, J.D., Small, D.M., Shipley, G.G. and Wallach, D.F.H. (1978) Biochemistry 17, 1802–1807
12 Verma, S.P., Wallach, D.F.H. and Sakura, J.D. (1980) Biochemistry 19, 574–579
13 Lavialle, F. and Levin, I.W. (1980) Biochemistry 19, 6044–6050
14 Boggs, J.M., Vail, W.J. and Moscarello, M.A. (1976) Biochim. Biophys. Acta 448, 517–530
15 Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, H.S., Hshung, J.C., Kang, S.Y., King, T.E., Meadows, M. and Rice, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4657–4660
16 Rice, D.M., Meadows, M.D., Scheinman, A.O., Goñi, F.M., Gómez-Fernández, J.C., Moscarello, M.A., Chapman, D. and Oldfield, E. (1979) Biochemistry 18, 5893–5903
17 Blazyk, J.F., Melchior, D.L. and Steim, J.M. (1975) Anal. Biochem. 68, 586–599
18 Nagle, J.F. and Wilkinson, D.A. (1978) Biophys. J. 23, 159–175
19 Laggner, P. and Stabinger, H. (1976) Colloid Interface Sci. 5, 91–96
20 Epand, R.M. and Epand, R.F. (1980) Chem. Phys. Lipids 27, 139–150
21 Melchior, D.L. Scavitto, F.J. and Steim, J.M. (1980) Biochemistry 19, 4828–4834
22 Epand, R.M., Epand, R.F., Stewart, T.P. and Hui, S.W. (1981) Biochim. Biophys. Acta 649, 608–615
23 Kratky, O., Leopold, H. and Stabinger, H. (1973) Methods in Enzymology, Vol. XXVII, Part D (Hirs, C.H.W. and Timasheff, S.M., eds.), pp. 98–110 Academic Press, New York
24 Lowden, J.A., Moscarello, M.A. and Morecki, R. (1966) Can. J. Biochem. Physiol. 44, 567–577
25 Gagnon, J., Finch, P.R., Wood, D.D. and Moscarello, M.A. (1971) Biochemistry 10, 4756–4763
26 Bartlett, G.R. (1959) J. Biol. Chem. 234. 466–468
27 Murison, J., Festi, D., Ross, P.E. and Bouchier, I.A.D. (1976) Clin. Chim. Acta 68, 159–166
28 Hess, H.H., Lees, M.B. and Derr, J.E. (1978) Anal. Biochem. 85, 295–300
29 Zamyatnin, A.A. (1972) in Progress in Biophysics and Molecular Biology, Vol. 24 (Butler, J.A.V. and Noble, D., eds.) pp. 107–123, Pergamon Press, Oxford
30 Adair, G.S. and Adair, M.E. (1947) Proc. R. Soc., Ser. A 190, 341–356
31 Haurowitz, F. (1965) in Chemistry and Functions of Proteins, Academic Press, New York
32 Nagle, J.F. and Scott, H.L., Jr. (1978) Biochim. Biophys. Acta 513, 236–243