Biochimica et Biophysica Acta, 455 (1976) 322–331

BBA 77517

SINGLE BILAYER VESICLES PREPARED WITHOUT SONICATION

PHYSICO-CHEMICAL PROPERTIES

J. BRUNNER[a], P. SKRABAL[b] and H. HAUSER[a]

Eidgenössiche Technische Hochschule, [a]Laboratorium für Biochemie II and [b]Technisch-Chemisches Laboratorium, ETH-Zentrum, 8H-8092 Zürich (Switzerland)

(Received May 28th, 1976)

SUMMARY

Single shelled lecithin vesicles of uniform size (diameter = 300 Å) are prepared without sonication by solubilizing unsonicated lecithin dispersions with sodium cholate and removing the detergent from the mixed lecithin – cholate micelles by gel filtration on Sephadex G-50. A homogeneous population of pure lecithin single – bilayer vesicles free of multilamellar structures is obtained. The vesicle diameter is somewhat larger than the average diameter of sonicated vesicles. The curvature of the bilayer seems to be sufficiently large to allow for similar packing densities (areas/molecule) on the outer and inner layer of the bilayer. The morphology and some physico-chemical properties of these vesicles are described and compared with those of sonicated vesicles.

INTRODUCTION

Phospholipid bilayers as an integral part of biological membranes are considered to play an important role in the structure and function of these membranes. Hence their physico-chemical properties have been the subject of intensive investigation in the past. The bilayer systems used extensively comprise the "black lipid" bilayer, unsonicated and sonicated aqueous phospholipid dispersions, the advantages and disadvantages of which have been discussed in some detail [1–3]. One of the objections of using single-bilayer vesicles produced by sonication is that ultrasonic treatment may lead to chemical degradation and irregularities in the order and packing of the phospholipid molecules outweighing the many advantages of that bilayer system.

Batzri and Korn [4] were the first to report on a technique of preparing single bilayer vesicles without sonication. Here we describe another method of preparation, which also avoids ultrasonication, and discuss some of the physico-chemical properties and distinct advantages of the vesicles thus obtained.

METHOD OF PREPARATION

Egg lecithin dispersions (1–2 % (w/v) = 0.013–0.027 M) in 0.1 M NaCl containing 0.01 M Tris · HCl pH 7.3 and 0.02 % sodium azide were prepared according to standard procedures [5]. To the milky dispersion sodium cholate recrystallized as the acid from acetone/water 4 : 1 (v/v) was added to a final concentration of at least ≈ 0.03 M (≈ 1.3 %) required to disintegrate the multilamellar structures present in unsonicated lecithin dispersions. For the reaction product, small mixed micelles were formed as described by Small and coworkers [6, 7]. 2–3 ml of ≈ 1–2 % micellar solutions of egg lecithin were applied to a Sephadex G-50 (of medium particle size) column (20 × 1.5 cm) which was equilibrated and eluted with the same buffer at 4 °C (flow rate 7–8 ml/h). The sample volumes and column dimensions were such that a complete separation of lecithin vesicles eluted at the void volume V_0 and cholate micelles was achieved. The retention of cholate was tested with ^{3}H-labelled cholate (from NEN Chemicals). After the first Sephadex G-50 chromatography less than 1 % of cholate was retained giving a lecithin/cholate molar ratio of > 50.* A second chromatography or a 12-h dialysis at 4 °C reduced the cholate amount below the limit of detection giving lecithin/cholate ratios > 200–300 (i.e. less than 20 cholate molecules/vesicle). During the course of preparation the oxidative index [8] and the thin-layer chromatography pattern of lecithin remained unchanged indicating that neither autooxidation nor chemical degradation occurred.

SOME PHYSICO-CHEMICAL PROPERTIES OF THE SINGLE BILAYER VESICLES

In Fig. 1 A–C the gel filtration patterns of lecithin vesicles prepared by sonication, by injection into water of a lecithin solution in ethanol according to Batzri and Korn [4] and by the cholate treatment described above are compared. The calibration of the Sepharose 4B column is given in Fig. 1D. The "cholate" vesicles** (Fig. 1C) are more homogeneous with respect to size as evident from the symmetric, narrow peak and do not contain large multilamellar structures or aggregated vesicles eluted in the void volume of the column. When the peak fractions (Fig. 1C) were rechromatographed on Sepharose 4B after pooling and concentrating by vacuum dialysis at 4 °C, the same elution pattern as in Fig. 1C was obtained. The differences in homogeneity and particle size distribution are further illustrated in Fig. 2A and B showing ultracentrifugal Schlieren patterns of sonicated and cholate vesicles, respectively. Consistent with the gel filtration results sonicated vesicles show a wider spread of sizes and the presence of larger structures as evident from the advancing edge of the peak [9].

Some physico-chemical properties of the cholate vesicles are summarized and compared with those of sonicated vesicles in Tables I and II. The cholate vesicles are somewhat larger than the average size of sonicated vesicles. Apart from gel filtration and electron microscopy the different methods used gave consistent results

* The exact amount of cholate left in the lecithin bilayer depended on the column dimensions; e.g. on a 25 × 2.5 cm column 99.9 % of the cholate was removed.

** The term "cholate" vesicles is used to refer to lecithin vesicles prepared without sonication by the cholate treatment as described in Method of Preparation; it does not imply that the lecithin bilayer contains residual cholate.

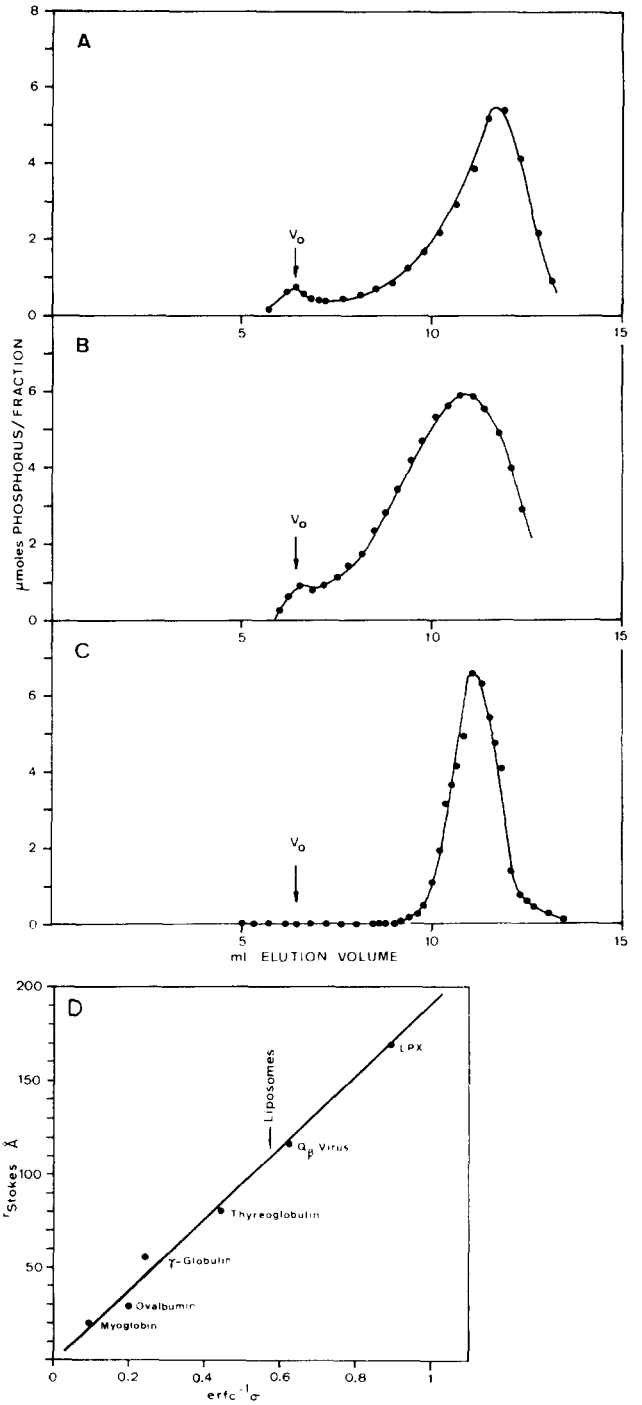


Fig. 1. Gel filtration on Sepharose 4B. (A) Egg lecithin vesicles sonicated as described before [14]; (B) Egg lecithin vesicles produced according to the method of ref. 4; (C) Egg lecithin vesicles produced by the method described in this work; (D) Calibration curve of the Sepharose 4B column relating

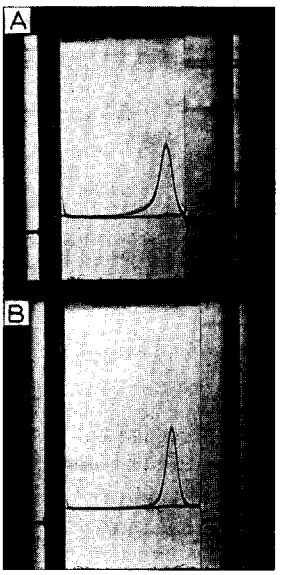


Fig. 2. Ultracentrifugal Schlieren pattern of the sedimentation of sonicated egg lecithin vesicles (A) and of vesicles prepared as described in Method of Preparation (B). Concentration, 20 mg/ml; time, 103.5 min after the start of the experiment; temperature, 19.8 °C; phase plate angle, 75°. Sedimentation is from right to left. Ultracentrifugation was carried out in a Beckman model E analytical ultracentrifuge using an RTIC temperature control unit.

for the vesicle radius of $\approx$ 150 Å (Table I) indicating that this value represents an average over a rather narrow range. The determination of the Stokes radius by gel filtration on Sepharose 4B seems to yield a minimum value. The vesicles were probably retarded due to adsorption on Sepharose 4B. The size of the cholate vesicles was found to be independent of the flow rate of the Sephadex G-50 column chromatography. However, if the cholate was removed very slowly by dialysis, large multilamellar structures were obtained.

The larger vesicle radius of about 150 Å (Table I) agrees well with the particle weight determined in the analytical ultracentrifuge (Table II). The differences in the hydrodynamic properties (sedimentation, diffusion) of the two types of vesicles (Table II) probably reflect the differences between the two samples in the particle size distribution and the state of aggregation.

Molecular details of the packing in the bilayer

The chemical shifts of all clearly discernible signals in the 360 MHz ^{1}H-NMR spectra of cholate vesicles and sonicated vesicles (Fig. 3A and B) are consistent within the error of the measurement with data reported before [15]. That the packing of the

---

the Stokes radius in Å to erfc^{-1} σ according to ref. 24; $\sigma = (V_e - V_o)/(V_t - V_o)$ where V_e = elution volume; V_o = void volume (5.65 ml); $V_t - V_o$ = internal volume (16.1 ml). As marker compounds myoglobin (V_e = 14.8 ml) from Fluka, ovalbumin (V_e = 13.72 ml), γ-globulin (V_e = 13.3 ml) both from Schwarz/Mann, thyreoglobulin (V_e = 11.25 ml) from Sigma, Q_β virus (V_e = 9.6 ml) from Miles and LPX lipoprotein (V_e = 7.85 ml) of density 1.019–1.063 g/cm^3 were used. The LPX-sample was a gift from Dr. G. Kostner.

TABLE I

DIMENSION OF EGG LECITHIN VESICLES PREPARED WITH AND WITHOUT SONICATION

Vesicle dimension Å (±S.D.)	Vesicle preparation			
	Sonication[a] (cf. refs. 12–14)	The method of Huang [11][b]	The method described in this work	
Stokes radius	100±10	140	105±5	gel filtration
Apparent vesicle radius	125±5[c]	150[c] 125[d]	135±5[c]	electron microscopy
Stokes radius	120	114	150	calculated from the diffusion coefficient using the Stokes-Einstein equation (9)
Vesicle radius	133	—	156	from the internal volume determined by incorporation of $^{22}Na^+$ [14]; a bilayer thickness of 50 Å and an area/molecule of 70 Å^2 (25) was used.
Vesicle radius	125	—	150	obtained from the ratio of molecules on the outer and inner layer of the bilayer determined from ^{31}P NMR measurements in the presence of shift probes (cf 15). The packing density was assumed to be equal on either side of the bilayer (= 70 Å^2/ molecule).

[a] The multilamellar structures were removed either by centrifugation at 100 000 × g or gel filtration on Sepharose 4B; alternatively, the sonication conditions were chosen such that all multilamellar structures were broken up and 100 % of the phospholipid was present as single bilayer vesicles [9].

[b] The preparation differs from that of column 1 in as much as the vesicles were fractionated on Sepharose 4B and filtered through a 0.1 μm Sartorius filter prior to the physical measurements [11].

[c] Negatively stained preparation.

[d] Freeze-etched preparation.

molecules is similar in both types of bilayers is shown by ESR spin label experiments (Fig. 4). The hyperfine splittings $T_{\parallel}$ and $T_{\perp}$ and thus the order parameters S_3 (using the nomenclature of ref. 16)

$$S_3 = \tfrac{1}{2}(3\langle\cos^2\theta_3\rangle - 1) = \frac{T_{\parallel} - T_{\perp}}{T_{zz} - T_{xx}}$$

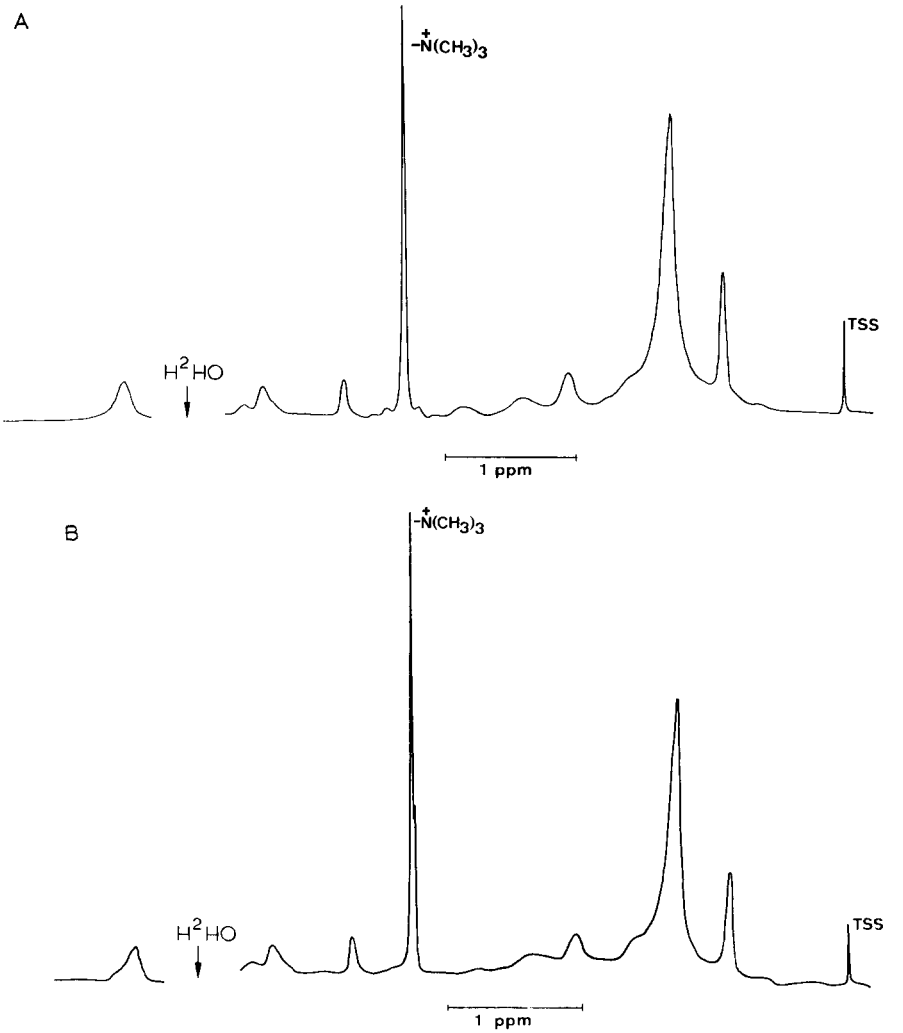
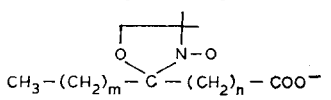


Fig. 3. 360 MHz ^{1}H-NMR spectra of vesicles prepared without sonication as described in Method of Preparation (A) and of sonicated egg lecithin vesicles (B). The assignment of the signals relative to 3-(trimethyl-silyl)-propane sulphonate (TSS) as internal reference was consistent with data published previously [15]. NMR spectra were recorded on a Bruker HXS-360 MHz instrument operating in the Fourier transform mode.

of the fatty acid spin labels $\{(m, n) = 12, 3; 1, 14\}$ incorporated in both bilayer



systems are identical within the error of the measurement; $S_3 = 0.64 \pm 0.03$ and 0.12 ± 0.02, respectively.

That the packing in the two bilayer systems is similar is also consistent with the observation of practically identical Na^+ permeabilities (Table II). The main

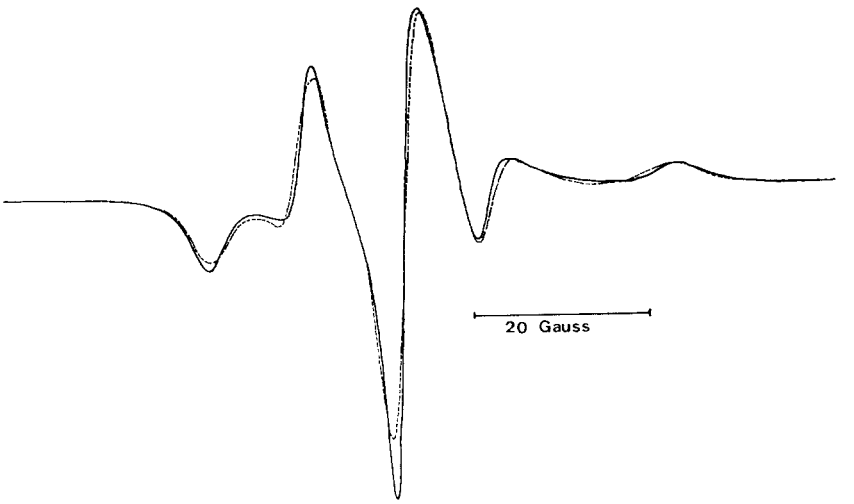
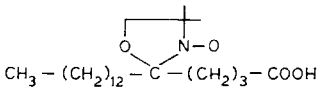


Fig. 4. ESR spectra of the spin labelled fatty acid incorporated in 0.026 M sonicated egg lecithin



vesicles (solid line) and 0.026 M vesicles prepared without sonication as described in Method of Preparation. ESR spectra were taken at ambient temperature on a Varian E-104A spectrometer using an aqueous sample cell.

feature of the two ^1H spectra (Fig. 3A and B) are in good agreement except for the shoulder (splitting) of the $-N^+(CH_3)_3$ signal observed with sonicated vesicles. This upfield shoulder has been assigned to the $-N^+(CH_3)_3$ groups on the inner layer of the bilayer [17, 18] and has been suggested to be due to slight differences in the packing between the inner and outer layer of the bilayer. Such a shoulder is missing in the ^1H spectrum of cholate vesicles indicating that the packing of the molecules is similar on the two halves of the bilayer. Using resolution enhancement techniques the $-N^+(CH_3)_3$ resonance of both preparations can be resolved into two peaks. The separation was 0.03 ppm with sonicated lecithin vesicles, and only about half (0.016 ppm) with the vesicles described in this work.

The differences in shape of the $-N^+(CH_3)_3$ resonances (Fig. 3A and B) suggests that there are small differences in the molecular packing of the two bilayer systems, and these differences are now being discussed in the light of the proposed mechanism of sonication. Finer et al. [19] suggested that the mechanism of sonication involves the break-down of the planar bilayers to small fragments which are unstable and reseal to single bilayer vesicles when the field is switched off. With our method the planar bilayers are disintegrated to small lecithin – cholate micelles by the detergent action of cholate. The micelles begin to aggregate as soon as the cholate is removed by gel filtration and eventually single bilayer vesicles are formed. Under the mild conditions of the gel filtration it may be assumed that the curved bilayers of the resulting vesicles are formed in the absence of any severe stress. This may not be so under the conditions of an ultrasonic field. Hence the packing of the bilayers of cholate vesicles should resemble more closely that of planar bilayers with approx-

TABLE II

PHYSICO-CHEMICAL PROPERTIES OF EGG LECITHIN VESICLES PREPARED WITH AND WITHOUT SONICATION

Property	Vesicles prepared by the cholate method	Vesicles prepared by sonication (cf. refs. 9, 10, 12–14)	Physical technique
Internal volume	0.47 μl/μmol lecithin or $4.99 \cdot 10^{-18}$ cm^3 per vesicle	0.33 ± 0.01 μl/μmol lecithin or $2.2 \cdot 10^{-18}$ cm^3 per vesicle	Tracer technique using ^{22}Na as described previously [9, 14]
Sedimentation coefficient	2.35 s*	3.20 s*	Analytical ultracentrifugation
Diffusion coefficient	$0.7 \cdot 10^{-7}$ cm^2/s*	$1.78 \cdot 10^{-7}$ cm^2/s	Diffusion measured in the analytical ultracentrifuge
Particle weight	$4.6 \cdot 10^6$ Dalton	$2.5 \cdot 10^6$ Dalton	Calculated from sedimentation and diffusion coefficients using the Svedberg equation; the partial specific volume $\nu_{20} = 0.984$ cm^3/g [9]
Ratio of molecules on the outer and inner layer of the bilayer	1.98	2.45	^{31}P and ^{1}H-NMR** in the presence of $Eu(NO_3)_3$ or $Pr(NO_3)_3$ (cf. refs. 20 and 21)
Permeability coefficient for Na^+	$3.4 \cdot 10^{-14}$ cm/s (at 20 °C)***	$1.2 \cdot 10^{-14}$ cm/s (at 4 °C)	Radiotracer technique using ^{22}Na as described before [14]

* Extrapolated to zero dilution.

** ^{31}P and ^{1}H-NMR measurements were carried out on a Bruker HX-E 90 spectrometer operating at 36.44 and 90 MHz, respectively.

*** The difference between the two permeability coefficients at 4 and 20 °C is accounted for by assuming that the process of the passive cation diffusion through lecithin bilayers has an activation energy of about 15 kcal/mol (cf. ref. 23).

mately equal areas per molecule being occupied on the inner and outer layers of the bilayer; in support of this is the good agreement of the vesicle radius (Table I) which is calculated from the inside/outside molar ratio under the assumption of equal packing densities (areas/molecule) on both layers of the bilayer.

The differences between the two preparations in the homogeneity and particle size distribution may also be explained in terms of the mechanisms of vesicle formation suggested above. In the case of sonication the units from which the bilayer vesicles are formed through aggregation are bilayer fragments with a wide size distribution while the building units of the cholate vesicles are very small micelles of uniform size. In the former case the vesicles, as the product of random aggregation

of the bilayer fragments, are expected to show a much wider spread of sizes than the cholate vesicles resulting from the aggregation of small micelles of uniform size.

Advantages of the cholate vesicles

(1) Ultrasonication with its undesirable side effects [8, 13, 22] is avoided. The cholate method is particularly useful for the reconstitution of biological membranes (model membranes) or lipoprotein systems (Brunner, J., Hauser, H. and Semenza, G., to be published) where the use of ultrasonication is often detrimental to the protein components.

(2) A homogeneous population of pure lecithin vesicles free of multilamellar structures is obtained reproducibly.

(3) The vesicle size is sufficiently large so that the bilayer is essentially free of stress resembling more closely the planar bilayer with equal packing densities on both sides of the bilayer.

(4) The geometry is better defined, i.e. the values for size, internal volume and bilayer area are the averages of a narrow distribution (cf. consistency of the data for cholate vesicles in Tables I and II). This should allow more accurate measurements of bilayer permeabilities and other physico-chemical parameters.

ACKNOWLEDGEMENT

We acknowledge the expert technical assistence of Mr. Kevin Howell.

REFERENCES

1 Bangham, A. D. (1970) in Permeability and Function of Biological Membranes (Bolis, L., Katchalsky, A., Keynes, R. D., Loewenstein, W. R. and Pethica, B. A., eds.) pp. 195–206, North-Holland, Amsterdam
2 Johnson, S. M. and Bangham, A. D. (1969) Biochim. Biophys. Acta 193, 82–91
3 Hauser, H., Phillips, M. C. and Stubbs, M. (1972) Nature 239, 342–344
4 Batzri, S. and Korn, E. D. (1973) Biochim. Biophys. Acta 298, 1015–1019
5 Bangham, A. D. (1968) Progr. Biophys. Mol. Biol. 18, 29–95
6 Small, D. M., Penkett, S. A. and Chapman, D. (1969) Biochim. Biophys. Acta 176, 178–189
7 Carey, M. C. and Small, D. M. (1970) Am. J. Med. 49, 590–608
8 Klein, R. A. (1970) Biochim. Biophys. Acta 210, 486–489
9 Hauser, H. and Irons, L. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1579–1590
10 Chapman, D., Fluck, D. J., Penkett, S. A. and Shipley, G. G. (1968) Biochim. Biophys. Acta 163, 255–261
11 Huang, C. H. (1969) Biochemistry 8, 344–352
12 Johnson, S. M., Bangham, A. D., Hill, M. W. and Korn, E. D. (1971) Biochim. Biophys. Acta 233, 820–826
13 Hauser, H. O. (1971) Biochem. Biophys. Res. Commun. 45, 1049–1055
14 Hauser, H., Oldani, D. and Phillips, M. C. (1973) Biochemistry 12, 4507–4517
15 Hauser, H., Phillips, M. C., Levine, B. A. and Williams, R. J. P. (1975) Eur. J. Biochem. 58, 133–144
16 Hubbell, W. L. and McConnell, H. M. (1971) J. Am. Chem. Soc. 93, 314–326
17 Jendrasiak, G. L. (1972) Chem. Phys. Lipids 9, 133–146
18 Kostelnik, R. J. and Castellano, S. M. (1973) J. Magn. Reson. 9, 291–295
19 Finer, E. G., Flook, A. G. and Hauser, H. (1972) Biochim. Biophys. Acta 260, 49–69
20 Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I. and Bergelson, L. D. (1971) Chem. Phys. Lipids 6, 343–350

21 Bystrov, V. F., Shapiro, Yu, E., Viktorov, A. V., Barsukov, L. I. and Bergelson, L. D. (1972) FEBS Lett. 25, 337–338
22 Peacocke, A. R. and Pritchard, N. J. (1968) Progr. Biophys. Mol. Biol. 18, 187–208
23 Papahadjopoulos, D., Nir, S. and Ohki, S. (1972) Biochim. Biophys. Acta 266, 561–583
24 Ackers, G. K. (1967) J. Biol. Chem. 242, 3237–3238
25 Small, D. M. (1967) J. Lipid Res. 8, 551–557