Biochimica et Biophysica Acta 820 (1985) 115–121
Elsevier

BBA 72767

A differential scanning calorimetry and fluorescence polarisation study of membrane lipid fluidity in a psychrophilic bacterium

Linda McGibbon [a], Andrew R. Cossins [b], Peter J. Quinn [c] and Nicholas J. Russell [a,*]

[a] *Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL,* [b] *Department of Zoology, University of Liverpool, Liverpool L69 3BX, and* [c] *Department of Biochemistry, King's College London (KQC), Campden Hill, London W8 7AH (U.K.)*

(Received June 19th, 1985)

Key words: Differential scanning calorimetry; Fluorescence polarisation; Membrane fluidity; Psychrophilic bacterium; Phospholipid; (*M. cryophilus*)

The liquid-crystalline to gel phase transition temperature (T_m) of phospholipids extracted from the psychrophilic bacterium *Micrococcus cryophilus* was approx. −30°C, as measured by differential scanning calorimetry (DSC). The T_m value was little affected by bacterial growth temperature, despite a 4-fold decrease in the fatty acyl C18/C16 chain length ratio between 20°C and 0°C. The neutral lipids, which are comprised mainly of a mixture of wax esters that are more saturated than the phospholipids, did not alter the T_m values or form separate domains. It is suggested that the wax esters may be interpolated between the halves of the bilayer. Fluorescence polarisation measurements of membranes and extracted lipids using 1,6-diphenyl-1,3,5-hexatriene as the probe, confirmed the absence of a bulk lipid phase transition over the growth temperature range, and showed that the protein in membranes decreased lipid fluidity. The diphenylhexatriene polarization of membranes or their extracted lipids from bacteria grown at 0 or 20°C showed that, like DSC, fluorescence polarisation is insensitive to the acyl chain length change that occurs in this organism. A surprising observation was that the membrane lipids of *M. cryophilus* were considerably more ordered than was expected from studies of other organisms, or synthetic lipids with similar fatty acid compositions.

Introduction

The ways in which lipid fatty acid composition influences membrane fluidity and its importance in membrane structure and function are well documented [1]. We have studied membrane fluidity regulation in the psychrophilic bacterium *Micrococcus cryophilus* not only because of this organism's ability to grow at low temperatures, but because it has a simple lipid composition and the temperature-dependent changes are especially clear-cut. Two fatty acids, palmitoleic and oleic, comprise > 95% of the phospholipid fatty acids, and there is a 4-fold decrease in the ratio of C18/C16 fatty acids when the growth temperature is lowered from 20 to 0°C [2,3].

There are no concomitant changes in unsaturation. However, despite this fact, the activity of a membrane-bound Δ^9-desaturase that is responsible for desaturating the products of fatty acid synthetase [4,5] is influenced by temperature, apparently via alterations in phospholipid fluidity [6,7]. In an ESR study using nitroxide-labelled

* To whom correspondence should be addressed.

stearic acid probes dissolved in the bacterial membranes, inflexions in plots of log (order parameter) versus $1/T$ were observed at temperatures that correlated with similar inflexions in Arrhenius plots of desaturase activity [7]. Moreover, the inflexions depended on growth temperature and occurred at temperatures that were within the growth temperature range [7]. The most common explanation invoked for such inflexions is that they are caused by liquid-crystalline to gel phase lipid transitions. But that seems unlikely in this instance, because the transition should be well below zero in *M. cryophilus* in view of its fatty acid and phospholipid composition. For example, the T_m values of synthetic dioleoylphosphatidylglycerol and dioleoylphosphatidylethanolamine are -18 and -16°C, respectively [8,9].

In the ESR experiments mentioned above, the T_m of *M. cryophilus* phospholipids was not measured directly, nor was it established what was responsible for the observed inflexions. The ESR order parameters of membranes were only 12% greater than those of the extracted lipids [6], and although two membrane lipid environments could be detected using 12-NS, the mobility of 5-NS appeared to reflect that of the bulk lipid phase in the membrane. We report now the use of DSC to measure the T_m of extracted lipids. The fluidity of membranes and extracted lipids has also been measured using fluorescence polarisation as an alternative to ESR in an attempt to provide further insight into the regulation of membrane fluidity in this psychrophilic bacterium.

Materials and Methods

Materials

1,6-Diphenyl-1,3,5-hexatriene (Aldrich, puriss grade) was made up and stored as a 2 mM solution in tetrahydrofuran. All other reagents and solvents were of analytical reagent grade.

Methods

Bacterial growth. Micrococcus cryophilus (ATCC 15174) was grown in a Casamino acids/salts medium at 0 or 20°C as described previously [10]. Bacteria were acclimatised by at least two sub-culturings at the required temperature, and harvested in mid- to late-exponential phase [7].

Lipid extraction. Lipids were extracted from bacterial pellets, obtained by centrifugation, using the method of Bligh and Dyer [11] as described by Kates [12]. Non-lipid impurities were removed using Sephadex G-25 [13]. Total lipid was fractionated into phospholipid and neutral lipid using silicic acid column chromatography [12]. Total lipid and phospholipid were quantitated by measurement of phosphorus content [14], on the basis of known compositions [10]. Lipids were stored at -20°C as a solution in chloroform/methanol (1 : 1, v/v).

Formation of liposomes. Lipid solutions were transferred to a small round-bottom flask and dried as a film in vacuo. Water was added (0.1 ml per mg of lipid), the lipid allowed to hydrate for 1 h at room temperature (18–23°C) and sonicated using a bath sonicator, until a clear, opalescent suspension was obtained (1–10 min); the formation of multilamellar liposomes was checked by phase contrast light microscopy.

Preparation of membranes. Bacteria were converted to sphaeroplasts using lyzozyme and EDTA [15]. The sphaeroplasts were lysed by two passages through a French pressure cell (American Instrument Co. Inc., Silver Springs, MD 20910, U.S.A.) operated at $4.14 \cdot 10^4$ kPa; residual unbroken cells were removed by centrifugation at $4068 \times g$ ($R_{av} = 9.17$ cm) for 5 min, and membranes collected from the supernatant by centrifugation at $143\,000 \times g$ ($R_{av} = 6.30$ cm) in a 60 Ti rotor using a Beckman L2-65B ultracentrifuge for 2 h at 4°C. The membrane pellet was resuspended in phosphate buffer (pH 7.4), and stored frozen at -20°C. Protein was estimated by a modification of the procedure of Lowry et al. [16].

Differential scanning calorimetry. Lipid samples (1–2 mg dispersed in 10 μl of water) were sealed in aluminium sample pans, and thermal analysis performed using a Perkin-Elmer DSC-2 differential scanning calorimeter, using 10 μl of water as reference. Samples were cooled or heated at 5 Cdeg per min between -63 and 27°C (i.e., well below and above the upper and lower limits, respectively, of the transition temperature range). At least two heating and cooling scans were performed on each sample.

It was observed that the endothermic and exo-

thermic peaks produced by consecutive heating and cooling, respectively, overlapped, but did not coincide: the completion temperatures of exothermic peaks were on average 7.8 Cdeg lower than the start temperatures of endothermic peaks. The explanation is probably that, in a mixed lipid system, cooling drives a time-dependent phase separation of the lipids according to their gel phase transition temperature. Heating simply melts these already phase-separated domains. Also, supercooling of the sample could lower the exotherms, or the cooperativity of a sample may be greater when changing from a higher energy state (liquid-crystalline phase) to a lower energy state (gel phase) compared with driving the phase change in the reverse direction.

Fluorescence polarisation. Samples (approx. 50 μl) of membranes or liposomes were diluted in 2 ml of 20 mM phosphate buffer (pH 7.4) in a quartz fluorimeter cell to give a final A_{500} = 0.1–0.4. To each 2-ml sample was added 2 μl of 2 mM diphenylhexatriene in tetrahydrofuran. After mixing, the suspensions were allowed to equilibrate for 15 min at room temperature before making fluorescence polarisation measurements using a T-format fluorescence polarisation spectrometer as described previously [17]. Cuvette temperature was measured immediately prior to measurement of polarisation using a thermistor.

The excitation and emission spectra of sphaeroplast membranes were recorded using a Hitachi Perkin-Elmer MPF-2A fluorescence spectrometer.

Results and Discussion

Differential scanning calorimetry

The liquid-crystalline to gel phase transition of liposomes made with phospholipids extracted from *M. cryophilus* grown at 0 or 20°C occurred at temperatures well below zero for both exothermic and endothermic transitions. The DSC cooling curves for phospholipids and total lipids are shown in Fig. 1, and the data for cooling and heating thermograms are collated in Table I. The highest onset temperature observed was −22°C for endothermic transitions, whilst the lowest completion temperature was −45°C for exothermic transitions. There was little difference between 0 and 20°C phospholipid samples (Fig. 1), and the onset temperatures in particular were very similar (Table I). Gas-liquid-chromatographic analysis confirmed that the phospholipid fatty acid compositions were the same as those previously recorded for similar samples [27]. There was a greater difference in the completion temperatures, that of phospholipids from 0°C-grown bacteria being lower by approx. 6 Cdeg (Table I). This probably reflects not only the shorter average chain length of phospholipids from bacteria grown at the lower temperature, but also the greater fatty acyl chain heterogeneity compared with phospholipids from cells grown at 20°C.

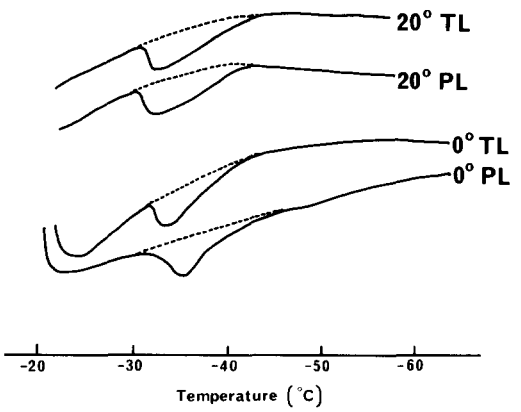

Fig. 1. DSC cooling curves for total lipid (TL) and phospholipid (PL) extracted from *M. cryophilus* grown at 0 or 20°C.

TABLE I

A SUMMARY OF THE GEL-LIQUID-CRYSTALLINE PHASE TRANSITION TEMPERATURE RANGES OF TOTAL LIPID AND PHOSPHOLIPID EXTRACTED FROM *M. cryophilus* GROWN AT 0° OR 20°C

PL, phospholipid; TL, total lipid.

Sample	Bacterial growth temperature (°C)	DSC scan mode	Transition temperature range (°C)
PL	20	Heating	−42 to −32
		Cooling	−41 to −31
TL	20	Heating	−35 to −25
		Cooling	−43 to −31
PL	0	Heating	−40 to −24
		Cooling	−45 to −32
TL	0	Heating	−39 to −26
		Cooling	−42 to −31

Thus the phospholipids contain 52% oleoyl and 48% palmitoleoyl residues, and 72% oleoyl and 28% palmitoleoyl residues in 0°C- and 20°C-grown bacteria, respectively. Studies with synthetic, miscible phospholipid mixtures have demonstrated that a 50 : 50 mixture gives the broadest transition [18]. This fact explains why the phospholipids of *M. cryophilus* grown at 0°C gave a broader transition (16 Cdeg) compared with phospholipids from 20°C-grown organisms (10 Cdeg). The asymmetric shape and relative broadness of the peaks indicate that the lipids are not homogeneously dispersed, probably due to the mixed headgroup composition [10]. The observed broad, sub-zero thermal transition of *M. cryophilus* is not only what one would predict (see Introduction), but is similar to that of phospholipids extracted from fatty acid auxotrophs of *Escherichia coli* containing 42% palmitoleate and 51% *cis*-vaccenate in their membranes (i.e. very similar to *M. cryophilus*), which gave a broad thermal transition from −10.5 to −23°C [19].

Phospholipids comprise 85% of the total lipid of *M. cryophilus*; the second most abundant lipid is a mixture of wax esters which accounts for 13–14% of the total [10]. Based on a consideration of their composition, conformation and possible location within the membrane, it had been postulated previously that the wax esters could 'stiffen' the membrane [20,21] (i.e. they, would increase the liquid-crystalline to gel phase transition temperature). However, the present study found no evidence of this on the basis of a comparison of exotherms and endotherms of liposomes made with phospholipid or total lipid (i.e., phospholipids + wax esters) extracted from *M. cryophilus* grown at 0 or 20°C. DSC cooling curves of total lipid are shown in Fig. 1 and data from cooling and heating thermograms are collated in Table I. The differences between all phospholipid and total lipid samples were within the normal temperature variation (< 10%) observed for different preparations of the same kind of sample. It appears that the wax esters affect neither the position nor the cooperativity of the phospholipid phase transition, suggesting that wax esters do not disrupt the bilayer packing. Nor do the wax esters separate into distinct domains at temperatures close to the phospholipid T_m, because there was no broadening of the phase transition range of total lipid compared with phospholipid (Fig. 1). Wax esters may be interpolated between the halves of the bilayer, as suggested for alkanes [22] and ubiquinone-10 [23].

The growth temperature range of *M. cryophilus* is from −4 to +25°C. Therefore, the membrane lipids are in the liquid-crystalline phase at all growth temperatures, and this organism does not experience a 'phase crisis' [24] if the growth temperature is changed suddenly. Lipid phase transitions could not be observed directly in membrane preparations. Instead an alternative technique, fluorescence polarisation, was used to investigate membranes; this also served as a comparison with previous experiments on *M. cryophilus* using ESR to measure fluidity [6,7].

Fluorescence polarisation

Preliminary experiments showed that the absorbance of sphaeroplast membrane suspensions significantly influenced the fluorescence polarisation value; this effect was more pronounced in membranes prepared from bacteria grown at 0 than at 20°C (Fig. 2a). The magnitude of the decrease in 20°C sphaeroplasts was only slightly greater than that observed with liposomes (cf. Figs. 2a and 2b); the latter was probably due to the scattering of fluorescent light [25]. There are no known lipid chromophores in the lipids of *M. cryophilus* apart from ubiquinone-8 [10]; however, this has an absorption maximum of 275 nm [26] that is considerably less than the excitation wavelength (360 nm) used in the present experiments.

Apparently the membranes of *M. cryophilus* grown at 0°C contain a chromophore with a fluorescence emission polarisation which is considerably below that of diphenylhexatriene. This was confirmed by analysing membranes for their intrinsic fluorescence, which, although only 3–4% of the level plus diphenylhexatriene, was detectable. The excitation and emission spectra (Fig. 3) demonstrate that the endogenous membrane fluorophore has excitation and emission maxima at 345 nm and 440–450 nm, respectively. Both of these values are close to the corresponding values for diphenylhexatriene [27]. They are also similar to those of NADH; the membranes of *M. cryophilus* contain tightly-bound cofactor(s) [5], but the identity is unknown. We favour the assumption that

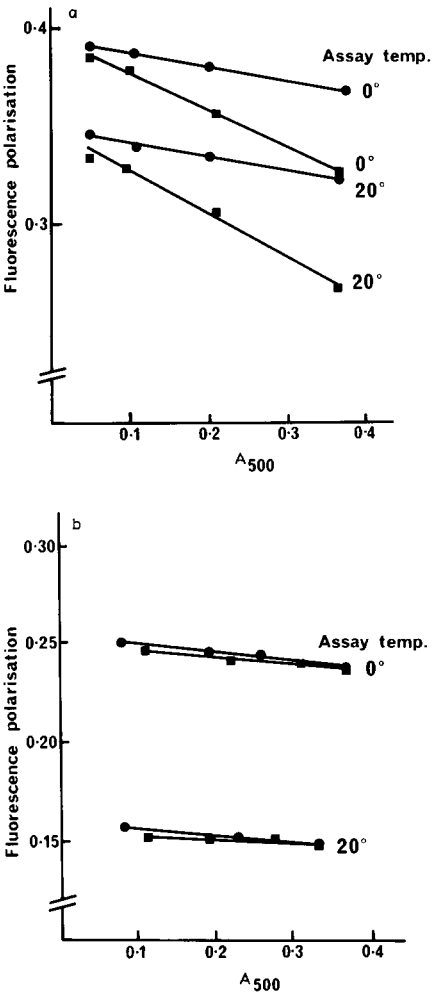

Fig. 2. (a) The effect of membrane suspension absorbance on diphenylhexatriene polarisation. *M. cryophilus* was grown at 0 (■) or 20°C (●), and polarisation measurements were made at 0 and 20°C. (b) The effect of liposome suspension absorbance on diphenylhexatriene polarisation. Liposomes were formed using total lipid extracted from *M. cryophilus* grown at 0 (■) or 20°C (●).

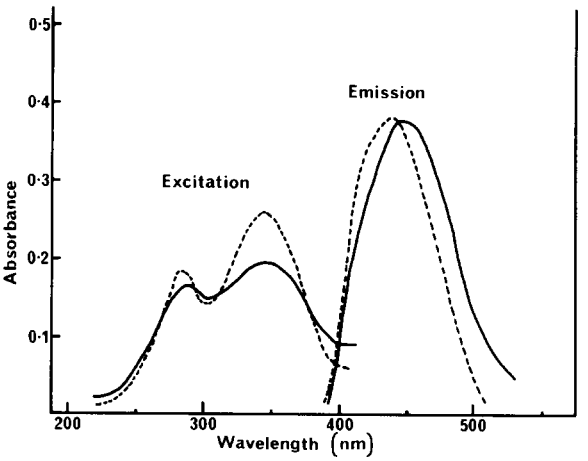

Fig. 3. The excitation and emission spectra of sphaeroplast membranes of *M. cryophilus*. Membranes were prepared from bacteria grown at 0 (— — —) or 20°C (———). The samples were matched for absorbance ($A_{500} = 0.1$). The excitation and emission wavelengths were 360 and 430 nm, respectively.

the endogenous chromophore is a protein for the following reasons. First, liposomes showed no such maxima. Second, membranes of *M. cryophilus* grown at 0°C contain 325 ± 14 μg of protein per ml (at a suspension density of $A_{500} = 0.1$; there is little or no intrinsic chemical absorption at this wavelength). This value is more than twice that of membranes in cells grown at 20°C (155 ± 17 μg protein per ml). It is not known if there is a general increase in all proteins or in one or a few protein species, but this altered protein content could account for the observed effect of membrane density on fluorescence polarisation values. In order to avoid artifacts, the reported values of fluorescence polarisation have been extrapolated to a theoretical value corresponding to zero absorbance, unless stated otherwise.

When fluorescence polarisation was corrected in this manner, there was no significant difference in the values for 0 and 20°C sphaeroplast membranes, irrespective of the assay temperature between 0 and 20°C (Fig. 4). As expected, the fluorescence polarisation value decreased with increasing temperature, but there were no discontinuities between 0 and 20°C (Fig. 4), which represents the bulk of the growth temperature range. As a further check, the fluorescence polarisation of some samples was measured at 2.5 Cdeg increments over the temperature range 0–30°C, but no discontinuities were observed. The same was true of liposomes made with lipids extracted from bacteria grown at 0 or 20°C (Fig. 4). These results confirm the absence of bulk phase changes in the extracted lipids or in the native membranes at temperatures within the growth temperature range.

Both DSC and fluorescence polarisation provide information about the average fluidity of the membrane, and appear to be insensitive to the

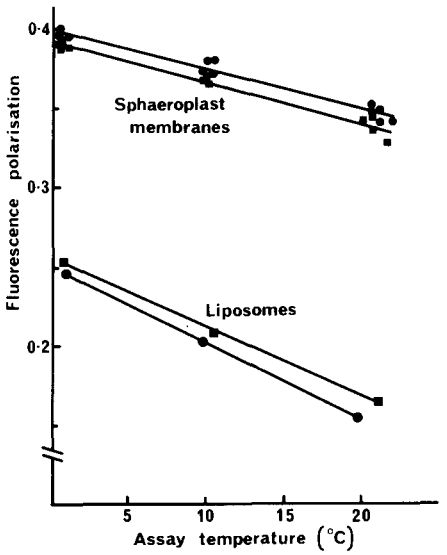

Fig. 4. The effect of temperature on diphenylhexatriene polarisation in liposomes and sphaeroplast membranes of *M. cryophilus* grown at 0 (■) or 20°C (●). The data have been corrected to theoretical zero absorbance (see text).

TABLE II

A COMPARISON OF THE FLUORESCENCE POLARISATION OF DIPHENYLHEXATRIENE IN SOME SYNTHETIC PHOSPHOLIPID LIPOSOMES AND NATURAL MEMBRANES

DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine.

Membrane system	Assay temp. (°C)	Polarisation value	Ref.
M. cryophilus			
(growth temp. = 20°C)	20	0.395	[a]
DPPC (T_m = 41°C)	10	0.43	28
	60	0.09	
DSPC (T_m = 58°C)	10	0.43	28
	60	0.10	
DOPC (T_m = −17°C)	23.5	0.115	29
Tetrahymena microsomes	15	0.231	30
(growth temp. = 39°C)	39	0.118	
E. coli K12 outer membrane	10	0.20	31
(growth temp. = 20°C)	35	0.10	

[a] Data taken from Fig. 4.

fatty acyl chain length change that occurs in *M. cryophilus*. In contrast, ESR spin probes are sensitive to the fatty acyl chain length change that occurs in *M. cryophilus* when growth temperature is altered. In a previous ESR study of *M. cryophilus* [7], growth-temperature dependent inflexions were observed in plots of log (order parameter) of nitroxide-labelled fatty acids versus $1/K$. The present study shows that these inflexions were not due to bulk lipid phase transitions.

The data in Fig. 4 also show that the fluorescence polarisation values of sphaeroplasts are 1.5–2.1-times those of the extracted lipids, depending on the bacterial growth temperature, indicating that the presence of protein decreases lipid fluidity. A similar result was obtained using ESR to monitor acyl chain motions [6].

The absolute values of fluorescence polarisation of *M. cryophilus* lipids and membranes are also of interest, because they show that despite the very high proportion of monounsaturated fatty acyl chains the membrane was ordered by comparison with both synthetic lipids and other natural membranes, including some microbial examples (Table II). For saturated and unsaturated phospholipids above their transition temperature, the diphenylhexatriene polarisation value is < 0.10. The comparison with *E. coli* outer membrane is particularly striking, because this bacterium contains 55% unsaturated fatty acid [32] yet has a diphenylhexatriene polarisation value much less than that of *M. cryophilus* membranes which contain approx. 97% unsaturated fatty acid. The fluorescence polarisation values for *M. cryophilus* liposomes are consistent with a liquid-crystalline lipid phase, but the lipid and the membrane are relatively 'stiff'. This may result from the rather homogeneous fatty acid composition, because the membranes of *E. coli* auxotrophs containing large proportions of a single fatty acid supplement also show reduced fluidity [19] as well as inactivation of some membrane-bound enzymes [33]. A similar relative lack of acyl chain mobility has been observed by fluorescence polarisation of triacylglycerols containing three *cis* double-bonded fatty acyl chains at temperatures above the phase transition [34]. In *M. cryophilus* there is a preference for the longer oleoyl acyl chains in the *sn*-1 position of phospholipids [35]. This produces a lower melting point isomer, but also exaggerates the difference in the depth to which the two acyl chains penetrate the

bilayer [36]. In turn, this leads to the possibility that the terminal methyl regions of the two monolayers could interdigitate [37], which has been suggested for some saturated mixed-acid lipids [38]. This effect has not been investigated in phospholipids containing two monounsaturated fatty acyl chains. If interdigitation did occur, it would restrict the wobbling motion of this region of the acyl chains, thereby causing an apparent 'stiffening' of the bilayer motion as monitored by diphenylhexatriene polarisation.

References

1 Kates, M. and Manson, L.A., eds. (1984) Membrane Fluidity, Biomembranes, Vol. 12 (Manson, L.A., series ed.), Plenum Press, New York
2 Russell, N.J. (1971) Biochim. Biophys. Acta 231, 254–256
3 Russell, N.J. and Sandercock, S.P. (1980) in Membrane Fluidity: Biophysical Techniques and Cellular Regulation (Kates, M. and Kuksis, A., eds.), pp. 181–190, The Humana Press, New Jersey
4 Russell, N.J. (1978) Biochim. Biophys. Acta 531, 179–186
5 Foot, M., Jeffcoat, R. and Russell, N.J. (1983) Biochem. J. 209, 345–353
6 Russell, N.J. (1984) in Membrane Fluidity, Biomembranes, Vol. 12 (Kates, M. and Manson, L.A., eds.), pp. 329–347, Plenum Press, New York
7 Foot, M., Jeffcoat, R., Barratt, M.D. and Russell, N.J. (1983) Arch. Biochem. Biophys. 224, 718–727
8 Findlay, E.J. and Barton, P.J. (1978) Biochemistry 17, 2400–2405
9 Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576–587
10 Russell, N.J. (1974) J. Gen. Microbiol. 80, 217–225
11 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
12 Kates, M. (1972) Techniques in Lipidology. Isolation, Analysis and Identification of Lipids, North-Holland Publishing Company, Amsterdam
13 Wells, M.A., and Dittmer, J.C. (1963) Biochemistry 2, 1259–1263
14 Marinetti, G.V. (1962) J. Lipid Res. 10, 141–145
15 Lloyd, G.M. and Russell, N.J. (1984) Can. J. Microbiol. 30, 1357–1366
16 Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210
17 Cossins, A.R. and Macdonald, A.G. (1984) Biochim. Biophys. Acta 776, 144–150
18 Chapman, D. (1975) Q. Rev. Biophys. 8, 185–235
19 Baldassare, J.J., Rhinehart, K.B. and Silbert, D.F. (1976) Biochemistry 15, 2986–2994
20 Russell, N.J. and Volkman, J.K. (1980) J. Gen. Microbiol. 118, 131–141
21 Russell, N.J. (1978) FEMS Microbiol. Lett. 4, 335–338
22 White, S.H., King, G.I. and Cain, J.E. (1981) Nature (London) 290, 161–163
23 Katsikas, H. and Quinn, P.J. (1981) FEBS Lett. 133, 230–234
24 Silvius, J.R. and McElhaney, R.N. (1980) Proc. Natl. Acad. Sci. USA 77, 1255–1259
25 Johnson, S.M. and Nicolau, C. (1977) Biochem. Biophys. Res. Commun. 76, 869–874
26 Lester, R.L. and Crane, F.L. (1959) Biochim. Biophys. Acta 32, 492–498
27 Shinitsky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657
28 Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257–1261
29 Chong, P.L.-G. and Cossins, A.R. (1984) Biochim. Biophys. Acta 772, 197–201
30 Martin, C.E. and Thompson, G.A., Jr. (1978) Biochemistry 17, 3581–3586
31 Janoff, A.S., Gupte, S. and McGroarty, E.J. (1980) Biochim. Biophys. Acta 598, 641–644
32 Lugtenberg, E.J.J. and Peters, R. (1976) Biochim. Biophys. Acta 441, 38–47
33 Baldassare, J.J., Brenckle, G.M., Hoffman, M. and Silbert, D.F. (1977) J. Biol. Chem. 252, 8797–8803
34 Berlin, E. and Sainz, E. (1984) Biochim. Biophys. Acta 794, 49–55
35 McGibbon, L. and Russell, N.J. (1983) Curr. Microbiol. 9, 241–244
36 Keough, K.M.W. and Davis, P.J. (1984) in Membrane Fluidity, Biomembranes, Vol. 12 (Kates, M. and Manson, L.A., eds.), pp. 55–97, Plenum Press, New York
37 Small, D.M. (1984) J. Lip. Res. 25, 1490–1500
38 Mason, J.T., Huang, C. and Biltonen, R.L. (1981) Biochemistry 20, 6086–6092