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The effect of exogenous glycerophospholipids on the fluorescence polarization ratios of *Escherichia coli* cells labelled with diphenylhexatriene

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Saturated, unsaturated, and short acyl chain analogues of phosphatidylcholine and phosphatidylethanolamine were incorporated into a deep heptoseless mutant of *Escherichia coli*, strain D21F2, and into the parent wild-type strain, K12. Normal and lipid-treated cells or lipid extracts from such cells were labelled with diphenylhexatriene and their fluorescence polarization ratios were measured as a function of temperature. Incorporations of dipalmitoyl analogues of phosphatidylethanolamine and/or phosphatidylcholine in the presence of Ca^{2+} caused an increase in polarization ratios over a wide temperature range and the appearance of new phase transitions at 25–30°C as measured in whole D21F2 cells. Incorporation into D21F2 of the dioleoyl analogues of these glycerophospholipids under similar conditions had the opposite effect on the polarization ratios and, in the case of dioleoylphosphatidylethanolamine, caused the occurrence of a new phase transition at 20°C. Incorporation of these same lipids in K12 cells, in the presence of Ca^{2+}, caused changes in the polarization ratios similar to those recorded for D21F2 cells when measurements were made on whole cells. Furthermore incorporation of didecanoyl-phosphatidylcholine in wild-type cells, in the presence of Ca^{2+}, substantially decreased the polarization ratio and broadened the phase transition as could be measured with cell preparations. Since Ca^{2+} stimulates incorporation of lipid, the changes in polarization ratio were always greater when cells had been exposed to exogenous lipid in the presence of this cation. However, even in cells not treated with lipid, Ca^{2+} caused increases in the polarization ratio and affected the thermotropic structural transitions. The polarization ratios of extracted lipids were always reduced when compared to whole cells. Generally there was an attenuation of any differences in polarization ratio between normal and glycerophospholipid-treated samples. Extracted lipids also displayed broadened phase transitions. The results as a whole indicated that *E. coli* cells respond to the uptake of lipid and to the presence of Ca^{2+} by changes in their thermotropic mesomorphic behaviour. These changes reflect to a large extent the fluidity of the incorporated lipid and are exerted on a structural system the phase transitions of which are strongly influenced by the presence of non lipid components in the membrane.

Introduction

Gram-negative bacteria have been shown to incorporate a variety of exogenous lipids into their membranes [1–6]. The process of uptake is most extensive in deep rough mutants [1,3,6] and with some strains [1–6] has been shown to depend on such factors as time, temperature, cell concentration, as well as concentration and composition of added lipid. Evidence was obtained which indicated that the uptake proceeds by a fusion of the lipid vesicles with the membrane [1,2,5,6] and fusogenic divalent cations such as Ca^{2+} were found to enhance to various degrees the incorporation of all lipids tested.

After uptake, the exogenous lipids were recovered in both the inner and outer membranes of the envelope [2,4]. The rate of translocation across the membranes was estimated to be approximately 0.2 nmol/min per nmol of cellular lipid for exogenous phosphatidylserine [3] but has not been assessed for other incorporated lipids. In the case of glycerophospholipid much of this lipid is converted to the diacyl analogue by acyltransferases present mainly in the inner membrane [4].

That the uptake does not involve some adsorption at the surface of the cell cannot be precluded completely. However, when lipid-treated cells were exposed to phospholipase C from *Bacillus cereus.* the endogenous and exogenous pools of lipid were found to be equally susceptible to the action of this enzyme. Consequently the incorporated lipid did not mainly represent a cell surface, more accessible pool [6].

Also, lipid-treated cells examined by electron microscopy following fixation with glutaraldehyde and positive staining [1,5] or following negative staining with phosphotungstate (unpublished results) failed to reveal lipid vesicles adsorbed to the surface of the cells or trapped within the intercellular space.

The amount of exogenous lipid taken up can be relatively large especially in the case of deep rough mutant strains, a likely consequence of such uptake would be noticeable changes in the physical properties of the membranes; however, no studies to date have assessed these changes. The present investigation deals with the effect of incorporating saturated and unsaturated phosphatidylcholines and phosphatidylethanolamines on the structure and fluidity of the envelope membranes studied in whole cells. Changes in this behaviour were assessed by incorporating diphenylhexatriene in the membrane of natural and lipid-treated cells and measuring steady-state fluorescence polarization ratios at different temperatures.

Materials and Methods

Materials. The choline and ethanolamine glycerophospholipid with saturated, unsaturated or short chain acyl groups were purchased from Sigma Chemicals Co. and/or Serdary Research Chemicals and used when found to be chromatographically pure as determined by TLC. 1,6-Diphenyl-1,3,5-hexatriene was purchased from Sigma Chemicals and used without further purification. This probe was chosen because it is generally accepted that it partitions equally between the different lipid phases of the membrane [12].

Cell cultures. Escherichia coli K12 (ATCC23716) and the deep rough, heptoseless strain, D21F2 characterized by Boman et al. [8] and kindly supplied by Dr. Lieve, N.I.H. Bethesda, were grown to the late exponential phase in a medium containing 1.5% Bactopeptone, 0.1% yeast extract, 0.5% NaCl and 2% glucose. The cells were harvested by centrifugation, washed once with 10 mM Tris buffer (pH 7.5) and dispersed in this buffer to yield a suspension containing 15–20 mg protein/ml.

Lipid suspensions. Lipids were dissolved in chloroform and evaporated to dryness as a thin film in a beaker with a stream of nitrogen. 10 mM Tris buffer (pH 7.4) containing 7.2 mM taurocholate was added for lipids with long acyl chains and dispersion was accompanied by sonication in the presence of nitrogen at maximum intensity using the large probe of an Ultrasonics sonicator. For didecanoyl-phosphatidylcholine dispersion, taurocholate was not required and was omitted from the buffer. Sonications were maintained at room temperature for unsaturated and shorter acyl chain aanlogues. The dipalmitoyl analogues were sonicated at 45–50°C. The suspensions were then completely clarified by centrifugation at 10^7 g · min in a Beckman ultracentrifuge equipped with a 70 : 2 Ti rotor. The amount of lipid remaining was estimated from lipid phosphorous [9] analysis of the supernatant.

Lipid uptake. The incubation mixture contained in 0.4 ml, *Escherichia coli* cells (1.5–2 mg protein) 3.5–3.8 mM lipid added as a sonicated suspension and 4 mM Ca^{2+} when indicated. Incubations were for 60 min at 37°C in a shaking water bath. After incubation, mixtures containing Ca^{2+} were treated with 0.04 ml of 0.1 M EDTA, layered over 2.5 volumes of 10% Ficoll containing 5 mM EDTA [1] and centrifuged 15 min at $12000 \times g$. Mixtures containing no Ca^{2+} were centrifuged directly at $12000 \times g$ for 15 min. The sediments were then washed once with 10 mM Tris buffer (pH 7.4). Ca^{2+}-containing mixtures could not be centrifuged

directly because addition of this divalent cation to lipid suspensions resulted in the development of some opalescence. Layering over Ficoll-EDTA assured in this case that the unreacted lipid did not sediment with the cells [1–3]. Examination of the normal and lipid-reacted cells by electron microscopy following treatment with glutaraldehyde and osmic acid and stained with uranyl acetate and lead citrate revealed a complete absence of lipid vesicles either bound to the surface of the bacteria or in the inter-cellular space. Negative staining with phosphotungstic acid of unfixed cells also failed to reveal the presence of surface-adsorbed or inter-cellular lipid. A similar susceptibility to phospholipase C hydrolysis of the endogenous and incorporated lipids of such D21F2 cell preparations had been shown previously [6]. These various results indicated that the bulk of the incorporated lipid penetrates the membrane under the uptake and processing conditions specified in this study.

Spectrofluorimetric measurements. Normal and lipid-treated cells were resuspended in 7.0 ml of 10 mM Tris buffer (pH 7.4) and half the sample was extracted for lipids according to the method of Bligh and Dyer [10]. Diphenylhexatriene in tetrahydrofuran was added to the lipid extract and after evaporation of solvent the residue was sonicated in 7.5 ml of buffer using the large probe of an ultrasonics sonicator at maximum intensity. The sonicate was centrifuged at $5000 \times g$ for 10 min to eliminate titanium particles. The other half of the sample was treated directly with diphenylhexatriene and incubated 30 min at 22°C prior to reading. In each case the glycerophospholipid/fluorescent probe ratio was 100–150. Steady-state polarization ratio measurements were made with a Perkin-Elmer spectrofluorimeter, model MPF-44A. The sample temperature was increased from 0°C at a rate of about 1 degree C per min and was controlled by a circulating water bath. Excitation and emission wavelengths were 360 nm and 420 nm, respectively, and the bandpasses were 6 nm and 8 nm, respectively. Fluorescence intensities were measured parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the vertically polarized excitation and data were expressed as fluorescence polarization ratio defined as

$$\frac{I_{\parallel}}{I_{\perp}}(G)$$

where $G = HH/HV$, a factor which corrects for the instrument anisotropy [11]. Corrections for background fluorescence and scattered light were made using a cell suspension blank which contained no probe.

Results

A previous study established that *Escherichia coli* strain D21F2 and the parent wild-type strain K12, incorporated various exogenous lipids by a Ca^{2+}-stimulated process. The uptake was several fold larger in the heptoseless strain [6], a result which had also been obtained previously with *Salmonella typhimurium* strains [1].

To assess the influence of lipid incorporation on the thermotropic properties of the envelope membranes, the fluorescence polarization ratios displayed by whole cell preparations were measured as a function of temperature. Since the lipids of *E. coli* are located entirely in its envelope membranes [7] and since it is well established that the fluorescence measured with diphenylhexatriene emanates only from that portion of the probe dissolved in the lipid phase [12], the results must then report on the properties of the membrane.

Results illustrated in Fig. 1 indicate that treatment of D21F2 cells with the dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) in the absence of Ca^{2+}, caused a substantial increase in the polarization ratio of the cells throughout most of the temperature range. The difference was especially apparent between 20 and 35°C, with a new phase transition being displayed at approx. 30°C. Incorporation of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) decreased the polarization ratios throughout the entire temperature range and a phase transition became noticeable at 20°C in the case of DOPE-treated cells. The results reveal that the incorporated lipid is not uniformly distributed in the envelope membranes and the new phase transition observed results from this microheterogeneity. The higher polarization ratio in the case of the saturated lipids is in keeping with the greater degree of order and decreased fluidity which they effect in lipid systems.

The melting of the extracted lipids from both

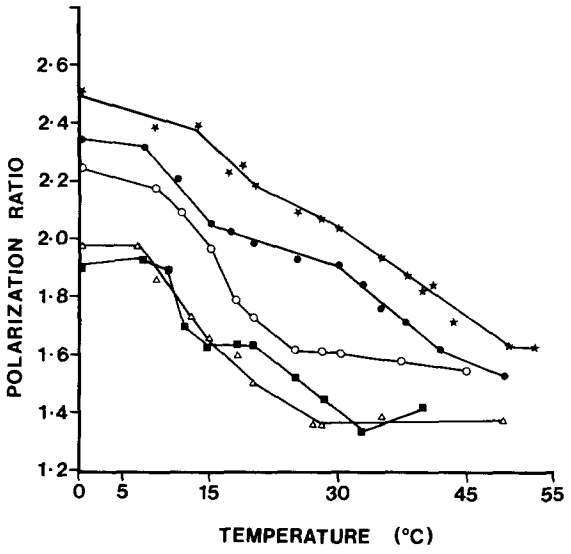

Fig. 1. Temperature dependence of diphenylhexatriene polarization ratios measured in D21F2 cells not reacted with lipid (○) or incubated with DPPC (★), DPPE (●) DOPC (△) or DOPE (■) in the absence of Ca^{2+}.

normal and glycerophospholipid-exposed cells was very gradual as revealed by the almost uniform decreases in polarization ratio with temperature (Fig. 2) which indicated a loss of the microheterogeneity seen with the intact cells. Although the incremental effect of the saturated lipids on the depolarization ratio was still very noticeable, the lowering effect of the unsaturated lipids became less apparent principally because of the substantial decrease in the polarization ratios of the normal cell lipids compared to that of the normal cells particularly in the lower temperature range. This suggests that in normal cells the non lipid components (likely proteins) confer order and rigidity in the membrane. Addition of Ca^{2+} to the incubation medium which increases lipid incorporation [6] manifested itself by the even greater increase in polarization ratio of DPPC-treated cells and the further decrease in polarization ratio of DOPE-treated cells (Fig. 3). A comparison of Figs. 1 and 3 reveals that Ca^{2+} causes an increase in polarization ratios of normal cells and consequently has a solidifying effect in its own right. Exposed to Ca^{2+}, normal cells and those treated with DOPE displayed a substantial fall in the polarization ratios between 0 and 10°C. This fall probably represents a phase transition beginning at a temperature below 0°C and was not detected in cells incubated in the absence of Ca^{2+}. In the presence of this cation, normal and DOPE-treated cells displayed a new phase transition at 15–16°C which was followed by a gradual melting at higher tem-

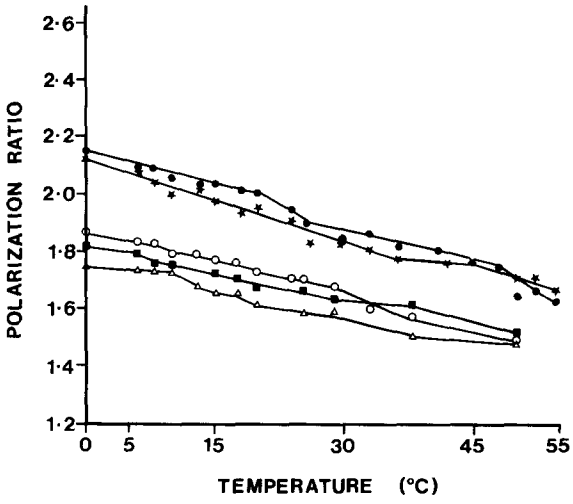

Fig. 2. Temperature dependence of diphenylhexatriene polarization ratios measured in lipid extracts of D21F2 cells treated as described in Fig. 1.

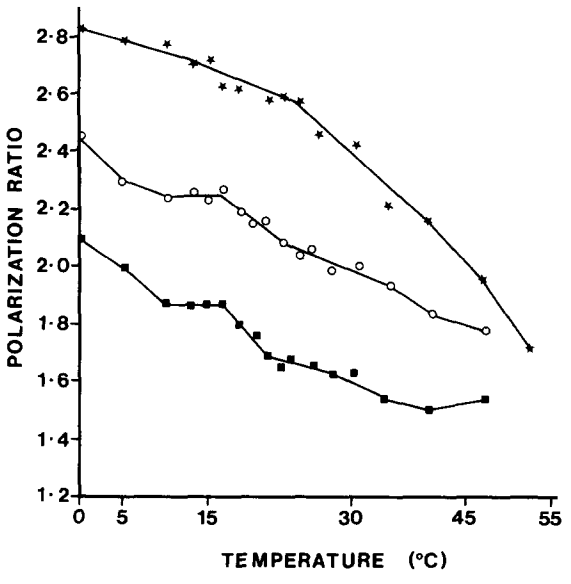

Fig. 3. Temperature dependence of diphenylhexatriene polarization ratios measured in D21F2 cells treated with 4 mM Ca^{2+} alone (○) or together with DPPC (★) or DOPE (■).

peratures. On the other hand, the DPPC-treated cells were characterized by a phase transition at 25–30°C and a gradual melting at more elevated temperatures. Again, the effects of glycerophospholipid incorporation appeared less marked when polarization ratios were measured on the extracted lipid (Fig. 4). In the case of normal and DOPE-treated extracts, no evidence of well-defined phase transitions could be observed. The similarity in the shape of the melting curves of Ca^{2+}, DPPC-treated whole cells and their lipid extracts shows that there was a substantial incorporation of this saturated lipid when the cation was present in the medium.

Because the K12 strain contains complete lipopolysaccharide structures, causing steric hindrance at the cell surface, incorporation of exogenous lipids is substantially less. However, when didecanoylphosphatidylcholine (DDPC) was incubated in the presence of Ca^{2+} with K12 cells enough of this lipid entered their membranes to cause a very substantial decrease in the polarization ratio (Fig. 5). The phase transition that is often seen in normal cells at about 7–8°C appeared to have been replaced by one occurring at a temperature below 0°C. Similar but even more pronounced liquifying effects of DDPC were seen with the D21F2 strain although in this case the polarization ratios were measured at two temperatures only

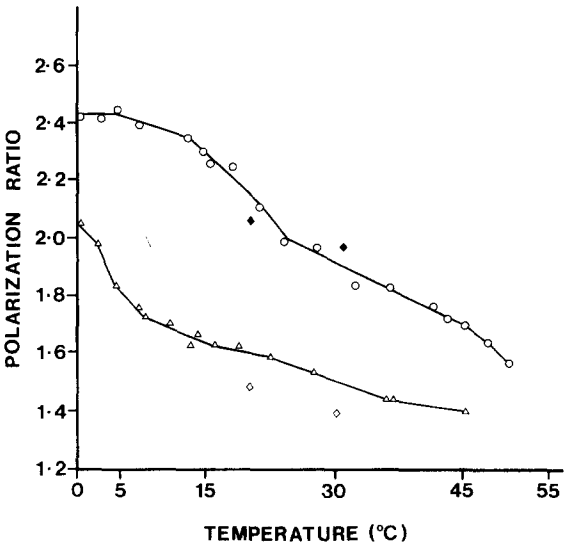

Fig. 5. Temperature dependence of diphenylhexatriene polarization ratios measured in K12 cells treated with 4 mM Ca^{2+} alone (○) or together with DDPC (△), and measured also in D21F2 cells incubated with Ca^{2+} alone (◆) or together with DDPC (◆).

(Fig. 5). Extraction of the lipids once again caused a gradual melting of the samples and no definite phase transition temperatures could be detected (Fig. 6). The difference between the normal and DDPC-treated cells was attenuated mainly be-

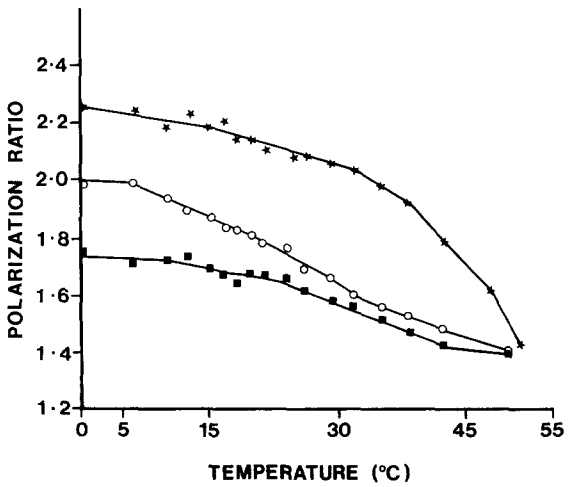

Fig. 4. Temperature dependence of diphenylhexatriene polarization ratios measured in lipid extracts of D21F2 cells treated as indicated for Fig. 3.

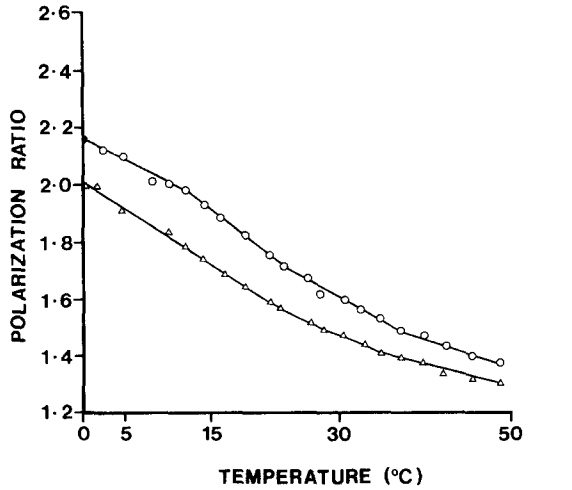

Fig. 6. Temperature dependence of diphenylhexatriene polarization ratios measured in lipid extracts of K12 cells treated as described for Fig. 5.

TABLE I

EFFECT OF GLYCEROPHOSPHOLIPID UPTAKE ON THE POLARIZATION RATIOS OF *ESCHERICHIA COLI* K12 CELLS LABELLED WITH DIPHENYLHEXATRIENE

Polarization ratios are averages ± S.D. of three or four determinations.

Lipid added	Temperature (°C)	Polarization ratio
None	20	2.25 ± 0.03
	30	2.05 ± 0.03
DPPC	20	2.53 ± 0.02
	30	2.45 ± 0.01
DOPC	20	1.91 ± 0.02
	30	1.74 ± 0
DOPE	20	1.47 ± 0.025
	30	1.34 ± 0.01

cause of a decrease in the polarization ratio of the normal cell extracts. Results summarized in Table I indicate that in the presence of Ca^{2+}, both saturated and unsaturated glycerophospholipids incorporated sufficiently in K12 cells to affect the polarization ratios significantly.

Discussion

Part of the effect of Ca^{2+} may be explained on the basis of a greater incorporation of lipids. However, even in normal cells, Ca^{2+} had an overall incremental effect on the polarization ratio. Ca^{2+} and other divalent cations have known fusogenic properties, can cause phase separations [13] and promote formation of hexagonal II mesomorphs in membranes [14]. The precise effects of Ca^{2+} on the membranes of Gram-negative bacteria are not known; however, they appear to be long lasting and yet compatible with survival and growth of the cell [1,15]. For example, following exposure of *S. typhimurium* cells to 10 mM Ca^{2+}, their inner and outer membranes could no longer be separated by the usual procedure and required more rigorous homogenization steps [1,2].

Of interest is the fact that the wild-type strain, can incorporate sufficient exogenous lipids, especially in the presence of Ca^{2+}, to affect the fluidity of its membranes. Even in the absence of Ca^{2+}, (results not shown) sufficient DPPC could be incorporated under the usual incubation conditions to cause an overall increase in the polarization ratio and the appearance of new phase transitions.

Although bacterial cells have been shown to take up various types of lipids, the effects of such uptake on the physical properties of their membranes had not been reported previously. The present study involved mainly an heptoseless strain of *Escherichia coli* because of its greater ability to incorporate exogenous lipids compared to the wild-type K12 strain. However, in both strains, at least in the presence of Ca^{2+}, marked effects of lipid uptake could be noticed. As could be predicted, the glycerophospholipids with long saturated acyl chains, descreased the fluidity of the envelope membranes whereas the diunsaturated and shorter chain analogues increased this fluidity and corresponding changes in the polarization ratios were noticed.

Normally dipalmitoyl lipids have phase transitions at elevated temperatures [16]. The failure to observe transitions characteristic of the pure lipids indicated that there is some mixing of the natural and exogenous lipids. However the new phase transitions observed in the treated cells shows that the mixing of the lipids is incomplete. Likewise, addition of unsaturated lipids such as DOPE caused the appearance of a phase transition in the 15–20°C range which is significantly higher than that of pure DOPE in the lipids which is induced by the incorporation of the unsaturated lipids.

Extraction of the lipids abolished most of the microheterogeneity seen with intact cells except for a phase transition in the 25–30°C range which persisted in extracts of cells treated with DPPC in the presence of Ca^{2+}. It would appear from these results that non lipid elements of the membrane (very likely protein) influence the overall fluidity, since the polarization ratios of the lipid extracts are usually lower than those recorded for the intact cells. They also prevent the lipids from mixing as freely as in the extracts. However some microheterogeneity may well depend on lipid composition rather than the presence of non lipid material. This aspect of our results confirms a previous report by Janoff et al. [17] who showed that changes in the thermotropic structural transition of the outer membrane in response to changes in the

growth temperature were largely controlled by non lipid components.

The results as a whole indicate that the acyl chains and polar headgroup composition of coliform membranes may be variable depending not only on intrinsic regulatory mechanisms responsive to nutrient supply or temperature but also to exogenous lipid availability. Incorporation of lipids in these membranes does affect their thermotropic structural transitions and would be expected to also affect at least some of the fluidity and lipid polar headgroup-dependent functions of the membranes.

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References

1 Jones, N.C. and Osborn, M.J. (1977) J. Biol. Chem. 252, 7398–7404
2 Jones, N.C. and Osborn, M.J. (1977) J. Biol. Chem. 252 7405–7412
3 McIntyre, T.M. and Bell, R.M. (1978) J. Bacteriol. 135 215–222
4 Hellion, P., Landry, F., Subbaiah, P.V. and Proulx, P (1980) Can. J. Biochem. 58, 1381–1386
5 Proulx, P., Hellion, P. and Mackenzie, J. (1982) Can. J. Biochem. 60, 980–986
6 Proulx, P. (1985) Exp. Biol., in the press
7 Lennarz, W. (1966) Adv. Lipid Res. 4, 175–225
8 Boman, H.G. and Monner, D.A. (1975) J. Bacteriol. 121 455–464
9 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
10 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
11 Parker, C.A. (1978) in Photoluminescence of Solutions, p. 51, Elsevier Publishing Co., Amsterdam
12 Johnson, S.M. (1981) in Fluorescent Probes (Beddard, G.S. and West, M.A., eds.), pp. 143–155, Academic Press, London
13 De Gier, J., Blok, M.C., Van Dijck, P.W.M., Mombers, C., Verkley, A.J., Van der Neut-Kok, E.C.M. and Van Deenen, L.L.M. (1978) Ann. N.Y. Acad. Sci. 308, 85–100
14 Cullis, P.R., De Kruijff, B., Hope, M.J., Mayer, R. and Schmid, S.C. (1980) Can. J. Biochem. 58, 1091
15 Jones, N.C., Osborn, M.J. and Schindler, M. (1978) Annal N.Y. Acad. Sci. 308, 215–225
16 Ladbrooke, B.D. and Chapman, D. (1969) Chem. Phys. Lipids 3, 304–356
17 Janoff, A.S., Gupte, S. and McGroarty, E.J. (1980) Boichim. Biophys. Acta 598, 641–644