Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102-1106.
Elder, J. H., & Alexander, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4540-4544.
Folch, J., Lees, M., & Sloane Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497-509.
Hediger, M. A., Coady, M. J., Ikeda, T. S., & Wright, E. M. (1987) *Nature 330*, 379-381.
Iversen, L. L. (1971) *Br. J. Pharmacol. 41*, 571-591.
Iversen, L. L. (1973) *Br. Med. Bull. 29*, 130-135.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477-5487.
Kanner, B. I. (1978) *Biochemistry 17*, 1207-1211.
Kanner, B. I. (1983) *Biochim. Biophys. Acta 726*, 293-316.
Kanner, B. I., & Schuldiner, S. (1987) *CRC Crit. Rev. Biochem. 22*, 1-38.
Keyan, S., & Kanner, B. I. (1988) *Biochemistry 27*, 12-27.
Kuhar, J. M. (1973) *Life Sci. 13*, 1623-1634.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
McConahey, P. J., & Dixon, F. J. (1980) *Methods Enzymol. 70*, 210-213.
Merril, C. R., Goldman, D., & Van Keuren, M. L. (1984) *Methods Enzymol. 104*, 441-447.
Messner, D. J., & Catterall, W. A. (1985) *J. Biol. Chem. 260*, 10597-10604.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346-356.
Radian, R., & Kanner, B. I. (1983) *Biochemistry 22*, 1236-1241.
Radian, R., & Kanner, B. I. (1985) *J. Biol. Chem. 260*, 11859-11865.
Radian, R., Bendahan, A., & Kanner, B. I. (1986) *J. Biol. Chem. 261*, 15437-15441.
Schofield, P. R., Darkson, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H., & Barnard, E. A. (1987) *Nature 320*, 221-227.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.

Transverse and Lateral Distribution of Phospholipids and Glycolipids in the Membrane of the Bacterium *Micrococcus luteus*

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ABSTRACT: The photodimerization of anthracene was used to investigate the transverse and lateral distribution of lipids in the membrane of the Gram-positive bacterium *Micrococcus luteus*. 9-(2-Anthryl)nonanoic acid (9-AN) is incorporated at a high rate into various membrane lipids of *M. luteus*. On irradiation of intact bacteria at 360 nm, anthracene-labeled lipids form stable photodimers which can be extracted and separated by thin-layer chromatography. We present here the results of a study on the distribution of two major lipids, phosphatidylglycerol (PG) and dimannosyldiacylglycerol (DMDG), within each leaflet of the membrane lipid bilayer. After metabolic incorporation of a tritiated derivative of 9-AN in *M. luteus*, the radioactivity associated with the photodimers issued from PG and DMDG was counted. In the bacterial membrane, the ratio of PG-DMDG heterodimer with respect to PG-PG and DMDG-DMDG homodimers is around half of what should be obtained for a homogeneous mixture of the two lipids. In order to find out whether this was due to an asymmetric distribution of the two lipids between the two membrane leaflets or a heterogeneous distribution of the two lipids within the same membrane leaflet, the transverse distribution of PG and DMDG was also investigated. This was carried out by following the kinetics of oxidation of the two lipids by periodic acid in the membrane of *M. luteus* protoplasts. PG predominated slightly in the outer layer (60%), while DMDG was found to be symmetrically distributed between the two leaflets. By itself, this lipid asymmetry cannot account for the lipid distribution determined from the photodimerization experiments. This indicates that PG and DMDG are not homogeneously distributed in the plane of the bacterial membrane.

One of the most challenging problems in membrane biology is to get a clear picture of lipid and protein organization and mobility at a microscopic and even molecular level. Surprisingly, biological membranes contain a large variety of lipids, and such a diversity is not required for maintaining the bilayer membrane assembly and fluidity. Despite extensive studies on the structure of biological membranes during the last decade, we have no direct information on the potential specificity of lipid distribution around membrane proteins or on the molecular organization of those lipids which are not in contact with proteins. A transverse asymmetry of lipids has been observed in red blood cells (Op den Kamp, 1979) and in various cytoplasmic (Sandra & Pagano, 1978) and intracellular membranes (Herbette et al., 1984). This asymmetry is related to the nature of the polar head group and the degree of unsaturation of the constituent fatty acids (Bick et al., 1987).

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In some instances, membranes of certain specialized cells with highly regionalized functions, such as epithelial and sperm cells, have been shown to have regional differences in protein and lipid composition and mobility (Van Meer & Simons, 1982; Wolf & Voglmayr, 1984). However, these observations rely on the ability to isolate and purify different fragments of the same membrane.

A direct approach for studying lipid–lipid and lipid–protein interactions involves labeling membrane components with site-directed ligands bearing chemically reactive photoactivatable groups (Middaugh et al., 1983). Various types of phosholipids carrying a hydrophobic photoreactive group have been synthesized for photo-cross-linking experiments (Khorana, 1980). They have been used to characterize integral membrane proteins and to identify which subunits form the lipid–protein boundary in a multisubunit membrane protein (Sigrist et al., 1984; Giraudat et al., 1985).

Photoreactive phospholipids have also been employed to investigate lipid organization in model membranes (Gupta et al., 1979; Khorana, 1980; Curatolo et al., 1981). However, the results are difficult to interpret due to specific interactions of the photoreactive group (Bayley, 1982; Bisson & Montecucco, 1985, 1986), and at the present time there is no report on the lipid distribution in natural membranes. In fact, it is difficult to find a photolabel in which the reactivity and the lifetime of the excited state are such that it will cross-link exclusively with its nearest neighbor.

In order to investigate both the lateral distribution and diffusion rate of lipids in membranes at the molecular level, we have employed anthracene as a fluorescent and photoactivatable group. This group, which is well suited for labeling the hydrophobic core of the membrane, is fluorescent, and under illumination (360 nm) it forms 9–9′, 10–10′ covalently bound dimers which are not fluorescent. After incorporation into membrane lipids, this group can be used (i) as an indicator of membrane fluidity, (ii) to measure the lateral diffusion rate of the labeled molecules from the fluorescence recovery after photobleaching or by following the kinetics of the photodimerization reaction, and (iii) to study the topological distribution of lipids in membranes by identification of the photoproducts formed in cross-linking reactions between adjacent anthracene-labeled molecules.

The synthesis and characterization of 9-(2-anthryl)nonanoic acid (9-AN)[1] have been described in previous publications (de Bony & Tocanne, 1983; Vincent et al., 1985). The polymethylenic chains are only slightly influenced by the presence of anthracene with its long axis parallel and attached at the ω-position of nonanoic acid (de Bony & Tocanne, 1982). 9-AN is incorporated at a high rate into the lipids of prokaryotic (Welby & Tocanne, 1982) and eukaryotic cells (Dupou et al., 1986) via normal metabolic pathways. Various anthracene phospholipid derivatives have been synthesized, and a new photo-cross-linking method has been devised for the identification and analysis of the photoproducts in various model membranes (de Bony & Tocanne, 1984). The method has been extended to the study of the lipid distribution in the membrane of the Gram-positive bacterium *Micrococcus luteus* after metabolic incorporation of 9-AN (de Bony et al., 1984). Various types of photoproducts were identified, and a qualitative analysis of several lipid photodimers was reported.

[1] Abbreviations: 9-AN, 9-(2-anthryl)nonanoic acid; PG, phosphatidylglycerol; DMDG, dimannosyldiacylglycerol; PI, phosphatidylinositol; CL, cardiolipin; NL, neutral lipids; TLC, thin-layer chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; NI, nonidentified lipids; FA, fatty acids; MG, monoglycerides; DG, diglycerides.

We describe here the synthesis of a tritiated derivative of 9-AN and its application for quantitative analysis of the lateral distribution of phosphatidylglycerol and dimannosyldiacylglycerol in the membrane of *M. luteus*. The transverse distribution of these two lipids was also studied by analysis of the kinetics of their oxidation by periodic acid.

Materials and Methods

Synthesis of 9-(2-Anthryl)[9-$^3H(N)$]nonanoic Acid. The synthesis of 9-(2-anthryl)nonanoic acid has been described elsewhere (de Bony & Tocanne, 1983). The corresponding tritiated derivative on the C_9 methylene group was obtained from Wolf–Kischner reduction of the intermediate 9-(2-anthryl)-9-ketononanoic acid methyl ester (de Bony & Tocanne, 1983), involving conversion of the keto group into a hydrazone followed by pyrolytic conversion into a methylene group. The intermediate hydrazone was prepared by treating 10 mmol of the keto ester with 0.8 mL of anhydrous hydrazine (15% v/v) rehydrated with ^3H_2O (5550 GBq/mL) in triethyleneglycol mono(butyl ether) (3 mL). After 2 h at 100 °C, potassium hydroxide (0.4 g) was added to the crude mixture. After a 30-min reflux, water was eliminated by evaporation, and the temperature was progressively increased to 210 °C. The resulting pyrolytic complex was then hydrolyzed in tritiated water (0.6 mL, 5550 GBq/mL) to give the reduced tritiated anthracene fatty acid. After purification (overall yield 85%), [^{3}H]-9-AN exhibited spectral characteristics in agreement with those of the predicted structure. Its specific radioactivity was 5.18 GBq/mmol.

Bacterial Culture. Micrococcus luteus (ATCC 4698-4) was grown in a peptone medium at 30 °C under agitation as described by Salton and Freer (1965) and harvested at the end of the exponential growth phase (A_{650nm} = 12–14).

For 9-AN metabolic incorporation experiments, the culture medium was supplemented with fatty acid free bovine serum albumin (1 mg/mL). 9-AN, dissolved in dimethylformamide at a concentration of (25 mg/mL), was slowly added to the culture medium with a syringe, under gentle stirring. Stable microemulsions were obtained by brief sonication (30 s) of the culture medium in a water bath type sonicator. The final concentration of 9-AN in the culture medium was 0.04 mg/mL for the lipid distribution studies, and it ranged from 0.02 to 0.07 mg/mL for the lipid incorporation rate studies.

Photoirradiation of Bacteria. Bacteria were collected and washed by centrifugation to remove most of the nonincorporated anthracene derivative. They were then put into fresh culture medium at 4 °C and divided into two identical batches, one for the photodimerization studies and the other for control experiments. For photodimerization, 2-mL samples of the bacterial suspension (A_{650nm} = 5) were illuminated for 3 min, at 15 °C, in a fluorescence cuvette. An optical bench made in the laboratory was equipped with a 200-W mercury arc lamp (HBO 200 W, Osram, West Germany) and a heat and UV filter (aqueous $CuSO_4$, 0.3 M, in a glass reservoir) transmitting light above 340 nm (de Bony & Tocanne, 1983).

After photoirradiation, bacteria were washed six times by centrifugation at 4 °C, twice with fresh culture medium, twice with the same medium in the presence of the nonionic detergent W.R. 1339 (0.2% v/v), and the last two times with fresh culture medium. This treatment eliminated around 60% of the radioactivity initially added to the culture medium. The radioactivity released during subsequent washes was negligible (0.6% of the total radioactivity).

Lipid Extraction and Estimation of the Rate of Photodimerization. Lipids were extracted from the bacterial pellet at 4 °C (Bligh & Dyer, 1959). In order to prevent cardiolipin

synthesis, the bacteria were solubilized in the chloroform/methanol mixture for only 30 min.

The rate of photo-cross-linking could be estimated by measuring the ratio of the intensities at 380 nm of two lipid extracts equivalent in weight dissolved in chloroform/methanol (9:1 v/v), issued from irradiated and nonirradiated bacteria, respectively.

Thin-Layer Chromatography and Autoradiography. The total lipid extract from one batch of photodimerized bacteria was spotted at one edge of a TLC plate (PF_{254}, Merck) and submitted to migration with chloroform/methanol/acetic acid/water (65:25:10:4 v/v/v/v). The plate was dried and irradiated at 254 nm (Bioblock UV lamp) for 1 min in order to partially dissociate the photodimers into their monomers. The plates were then run in the second dimension. Lipid extracts from nonirradiated bacteria were spotted at both edges of the plate for identification of the lipids monomers. The chromatograms were observed by fluorescence or by autoradiography (^3H Hyperfilm, Amersham; −70 °C).

Lipid Counting. After migration in the first direction, the photodimer spots were scraped off, and the silica gel powder was put into separate tubes. Lipids were hydrolyzed by an incubation for 1 h, at 50 °C, in the presence of 12 N HCl (0.2 mL), and the fatty acids released were extracted in ether. Control experiments showed that after four consecutive extractions over 80% of the radioactivity associated with the silica gel was recovered, whatever the initial amount deposited. The radioactivity associated in the spots ranged from 10000 to 50000 cpm. Under these conditions, the experimental error was estimated to be less than 5% for relative values.

Rate of Incorporation of 9-AN into Phosphatidylglycerol and Dimannosyldiacylglycerol. Bacteria were cultured in the presence of 9-AN at a concentration of 0.02, 0.04, or 0.07 mg/mL in the growth medium. After lipid extraction, PG and DMDG were separated on a silicic acid column and weighed. The anthracene concentration in each extract was evaluated from the counts and by UV spectroscopy.

Kinetics of Photodimerization. Bacteria cultured in the presence of 9-AN were extensively washed and diluted in water to reach an absorbance of less than 0.04 at 358 nm. The kinetics of the photodimerization of anthracene-labeled lipids in the bacterial membrane was monitored by recording the decrease in fluorescence intensity with time from the illuminated bacteria. Experiments were carried out directly in the spectrofluorometer (JY3D, Jobin Yvon, France) under gentle stirring at 20 °C. The excitation and emission wavelengths were 358 and 415 nm, respectively.

Preparation of Protoplasts and Membrane Vesicles. For determination of lipid transverse asymmetry, the culture medium was supplmented with sodium [1-^{14}C]acetate (specific activity 1.98 GBq/nmol; CEA France) at a concentration of 0.1 MBq/mL. Cells were harvested at the end of the exponential growth phase (200 mL, A_{640nm} = 12–14). They were centrifuged (3500*g*, 15 min), resuspended in 20 mL of a glycine buffer (0.2 M, pH 9) containing sucrose (30%), and then digested by lysozyme (400 μg/mL; Sigma; 50000 units/mg) in the presence of DNase (10 μg/mL; Sigma; 2400 units/mg) for 15 min at 4 °C to produce protoplasts (100% digestion).

Protoplasts were pelleted (1800*g*, 30 min) and then washed by centrifugation in 20 mL of an isotonic Tris buffer (10 mM Tris, 3 mM SO_4Mg, 700 mM NaCl, pH 7) at 4 °C. A pH of 7 was attained after three washes.

For the preparation of membrane vesicles, half of these protoplasts (10 mL) was suspended in 10 mL of a hypotonic Tris buffer (10 mM Tris, 3 mM SO_4Mg, pH 7) at 4 °C. After lysis of the protoplasts (10 min), membranes were washed twice by centrifugation (3500*g*, 20 min) in 10 mL of the same buffer. They were homogenized by suction into a syringe. Around 14 mg of total lipid extract was obtained from the protoplast and membrane vesicle preparations.

Periodate Oxidation. Protoplasts (10-mL suspension) and membrane vesicles (10-mL suspension), originating from the same bacterial culture were oxidized in their original suspension medium at pH 7 and 4 °C. At time $t = 0$, a solution of concentrated sodium periodate (2 M) was diluted in the suspension medium to the desired final concentration (10–50 mM). Membrane vesicles were continuously homogenized by syringe suction to favor simultaneous oxidation of lipids in both membrane leaflets. Samples (0.8 mL) were taken every 5 min, and the reaction was stopped by addition of a 10% (w/v) solution of sodium thiosulfate.

Lipids were immediately extracted (Bligh & Dyer, 1959) and separated by thin-layer chromatography on nonactivated Kieselgel H (Merck, Darmstad, West Germany) plates impregnated with 1 mM sodium carbonate to enable separation of phosphatidylglycerol from its oxidized form. The eluting solvent was chloroform/methanol/water (65:25:4 v/v). The various spots were identified by staining with iodine and by scanning the plates with a radioscanner (Berthold, Germany). After complete removal of iodine (16 h), silica gel corresponding to each delineated spot of interest was scraped off the plate and suspended in a scintillation cocktail (toluene in ButylPBD 8 g/L) and counted (Intertechnique, France). Chromatography and radioactivity counting were carried out in duplicate or triplicate for each oxidation experiment.

Results

M. luteus Lipid Composition and Metabolic Incorporation of 9-AN

The lipid composition of *M. luteus* has been described elsewhere (Welby et al., 1976). For the major phospho- and glycolipids, it contains around 60% phosphatidylglycerol (PG) and 30% dimannosyldiacylglycerol (DMDG). Two minor lipids, phosphatidylinositol (PI; around 5%) and cardiolipin (CL; around 5%) are also present. For the neutral lipids, there are essentially diglycerides and smaller amounts of monoglycerides and fatty acids.

As described for most bacterial systems (Krebs, 1982), the lipid composition of *M. luteus* can vary significantly from batch to batch. Moreover, depending on the culture and the membrane isolation and lipid extraction conditions, the proportion of CL can increase dramatically at the expense of PG. This phenomenon, which has been observed in both *M. luteus* (Johnson & Grula, 1980) and other bacterial strains (Rigomier et al., 1978), is due to deregulation of cardiolipin synthetase which occurs when the cells are deprived of optimal culture or incubation conditions. Thus, during protoplast formation, up to 80% of PG can be converted into CL (Lacombe & Lubochinsky, 1988). However, if lipids are extracted rapidly at 4 °C, the proportion of CL does not exceed 10%. Moreover, when a significant amount of CL has been formed, it becomes difficult to extract by regular lipid extraction procedures, and it remains associated with the cell wall (Filgueiras & Op Den Kamp, 1980; Lacombe & Lubochinsky, 1988).

When *M. luteus* is cultured in the presence of 9-AN, the various phospho- and glycolipids incorporate this fatty acid at a rate which depends on the lipid type and on the concentration of 9-AN in the culture medium. Thus, at a concentration of 0.02 mg/mL, the rate of incorporation of the probe

in PG and DMDG is 7% and 25%, respectively. For higher probe concentrations (0.07 mg/mL), the rate of incorporation in PG can reach 40%. As previously reported (Welby & Tocanne, 1982), 9-AN is specifically incorporated at the *sn*-1 position in PG.

Photodimerization of Lipids in Intact Cells and Identification of Photodimers

Anthracene Photochemistry. The photochemistry of anthracene can be briefly summarized as follows:

absorption

$$A + h\nu\ (360\ nm) \rightarrow A^* \quad (1)$$

fluorescence

$$A^* \rightarrow A + h\nu'\ (410\ nm) \quad (2)$$

dimerization

$$A^* + A \rightarrow A\text{–}A \quad (3)$$

photolysis

$$A\text{–}A + h\nu\ (280\ nm) \rightarrow A + A \quad (4)$$

Anthracene absorbs light in the 240–290-nm and 310–390-nm regions (eq 1), and a fluorescence emission can be observed between 390 and 450 nm (eq 2). On irradiation in the near-ultraviolet (around 360 nm), a dimerization reaction can also occur leading to dimers in which the covalently joined rings at 9–9′ and 10–10′ are no longer conjugated (eq 3). Interestingly, the dimerization reaction can be reversed by illumination at around 280 nm (eq 4). Anthracene monomers are partially recovered with restoration of their characteristic absorption and emission spectra above 300 nm. The kinetics of photodimerization of anthracene can be followed by measuring the changes in absorption and emission intensity during irradiation (de Bony & Tocanne, 1983).

Photodimerization of Lipids in Intact Cells. Figure 1 shows the autoradiographic pattern of a total lipid extract from regular (lane A) and photoirradiated (lane B) bacteria. On lane A one can readily distinguish PI, PG, DMDG, and a small amount of CL. It should be remembered that, in fact, each spot was a mixture of anthracene and host lipid and that radioactivity was a reflection of the amount of anthracene–lipid molecules present in such a mixture. In this experiment, the radioactivities associated with the four spots were in the following ratios: CL/CL, 1/1; PI/CL, 1/6; PG/CL, 7/2; DMDG/CL, 13/1. Neutral lipids (monoglycerides, diglycerides, and fatty acids) were also present and accounted for around 45% of the total radioactivity contained in the lipid extract. Minor spots at R_f lower and slightly higher than that of PI have not yet been identified. In total, they contained less radioactivity than PI. Since the rate of incorporation of 9-AN in DMDG is about 4-fold higher than that in PG, the slight differences in the proportions of these two lipids observed from batch to batch lead to large changes in the corresponding radioactivity counts.

After photoirradiation of the bacteria, the spots corresponding to anthracene monomer–lipids vanished, and new spots appeared at lower R_f, corresponding to various photodimers, in agreement with our previous observations on model (de Bony & Tocanne, 1984) and *M. luteus* membranes (de Bony et al., 1984). Three main photodimers were formed which, from bottom to top, were unambiguously attributed to PG–PG, PG–DMDG, and DMDG–DMDG (de Bony et al., 1984).

A clear-cut identification of these photodimers and of others was made possible by partially dissociating the anthracene

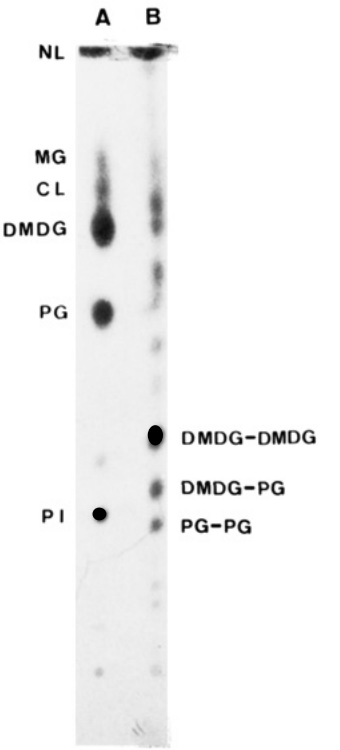

FIGURE 1: Autoradiogram of a total lipid extract from *M. luteus* cultured in the presence of [^{3}H]-9-AN before (lane A) and after (lane B) photoirradiation (3 min, 360 nm) of intact bacteria.

photodimers directly on the TLC plate. The following procedure was used: (a) migration of the photodimerized lipid extract in the first dimension in order to separate the various lipid dimers from the residual monomers; (b) partial photolysis of the dimers by direct illumination of the chromatogram at 256 nm; (c) chromatography in the second dimension to separate the restored lipid monomers from the corresponding dimers.

Figure 2A shows an autoradiogram of the corresponding TLC plate after 4-day exposure of the film. Since the same solvent system was used for both migrations, the spots on the main diagonal reflect the position of the residual photodimers while the others, above the diagonal, correspond to the restored monomers. From bottom to top, three main spots were attributed to PG–PG, PG–DMDG, and DMDG–DMDG from their dissociation products. The heterodimer gave rise to two spots at the PG and DMDG locations, while each homodimer produced a single spot at the corresponding monomer R_f. In order to be able to identify most of the other spots, the film had to be exposed for a longer period (40 days, Figure 2B). This autoradiogram can thus only be interpreted qualitatively due to saturation of the denser spots. At low R_f, two spots were identified as PI–PG and PI–DMDG. They were not systematically counted as their radioactivity was only slightly above potential contamination by others compounds.

Several scattered spots were observed in the upper middle part of the autoradiogram, some located at R_f values corresponding to PG, DMDG, and CL. Many of these spots corresponded to photodimers including monoglycerides, diglycerides, and fatty acids associated with phospho- and glycolipids. A complete identification of those compounds was not undertaken. However, the presence of several photoproducts containing mono- and diglycerides demonstrates that these lipid intermediates are inserted in the bacterial membrane.

Three minor spots at an unexpected location below the main diagonal could also be identified. Photoirradiation of the plate

Table I: Relative Distribution (%) of [^{3}H]-9-AN-PG (A) and [^{3}H]-9-AN-DMDG (B) Monomers and Photodimers Measured in Liposomes and Intact Bacteria[a]

	exptl distribution values (%)					calcd distribution values (%) for a homogeneous distribution of the two lipids					
	monomers		dimers			monomers		dimers			
expt	A (I)	B (II)	A–A (III)	A–B (IV)	B–B (V)	A (VI)	B (VII)	A–A (VIII)	A–B (IX)	B–B (X)	$A\text{–}B_{exptl}/A\text{–}B_{calcd}$ (XI)
liposomes											
1	32	68	13	46	41	36	64	13	46	41	1
bacteria											
2	35	65	24	31	45	40	60	16	48	36	0.65
3	65	35	46	30	24	61	39	37	48	15	0.62
4	66	33	44	24	32	56	44	32	49	19	0.49
5	54	46	46	27	27	59	41	35	48	17	0.56
6	54	46	44	21	35	61	39	37	48	15	0.44

[a] Columns I–V correspond to the percentage of radioactivity associated with the two monomers and the three photodimers, respectively. Columns VI and VII correspond to the calculated percentages of monomers from the experimental dimer distributions. Columns VIII–X show the recalculated distribution of the dimers, on the basis of the recalculated monomer distribution, assuming a homogeneous distribution of the two lipids. Column XI shows the ratio between experimental and calculated heterodimer distribution values. Entry 1: mixture of [^{3}H]-9-AN-PG and [^{3}H]-9-AN-DMDG extracted from bacteria and reconstituted in the form of liposomes and irradiated for 3 min. Entries 2–5: different bacterial batches irradiated for 3 min. Entry 6: same as entry 5 but with an irradiation time of 15 s. The percentages are given to within ±10% as relative values. For details, see the last paragraph in the appendix.

at 256 nm induced a partial dimerization of anthracene–lipid directly on the TLC plate. This can be explained by overlap of the anthracene monomer and photodimer absorption spectra in this region. Therefore, the three spots located on the left part below the diagonal corresponded to PI–PI, PG–PG, and DMDG–DMDG produced by photodimerization (irradiation at 256 nm) of a small proportion of the residual lipids which had not cross-linked at 360 nm. They migrated at R_f values corresponding to PI, PG, and DMDG in the first direction and to PI–PI, PG–PG, and DMDG–DMDG in the second direction. Similarly, two light spots on either side of the PG–DMDG photodimers corresponded to recombined PG–PG and DMDG–DMDG photodimers.

Surprisingly, we only observed one photodimer of CL which was identified as CL–CL at an R_f close to that of DMDG–DMDG. The absence of other observable photodimers containing CL cannot be attributed to clusters of cardiolipin in the membrane. This lipid is known to be formed from PG by cardiolipin synthetase. CL-containing photodimers were unstable on silicic acid and tended to dissociate into monomers. Consequently, we concentrated on the two main lipids, PG and DMDG, and we carried out a quantitative analysis of the corresponding three photodimers.

PG and DMDG Distribution in Liposomes and in M. luteus Membranes

To validate the method, the photodimerization of anthracene was exploited to quantitate lipid distribution in liposomes. From bacteria cultured in the presence of [^{3}H]-9-AN, PG and DMDG were extracted and reconstituted in the form of a liposomal dispersion. Radioactivity counting indicated the presence in this mixture of 32% anthracene–PG and 68% anthracene–DMDG. After irradiation at 360 nm and separation of the photoproducts by TLC in one direction, the relative proportions of the three photodimers were PG–PG = 13%, PG–DMDG = 46%, and DMDG–DMDG = 41% (cf. Table I) as expected for a regular distribution of the two lipids. This result is in total agreement with previous work in this laboratory showing good miscibility properties of PG and DMDG in monolayers and liposomes (Lakhdar-Ghazal & Tocanne, 1981).

The ratio of initial PG and DMDG which were transformed into these three photodimers can be directly recalculated from this relative distribution, giving 36% PG and 64% DMDG in the mixture. Within experimental errors, these values are in

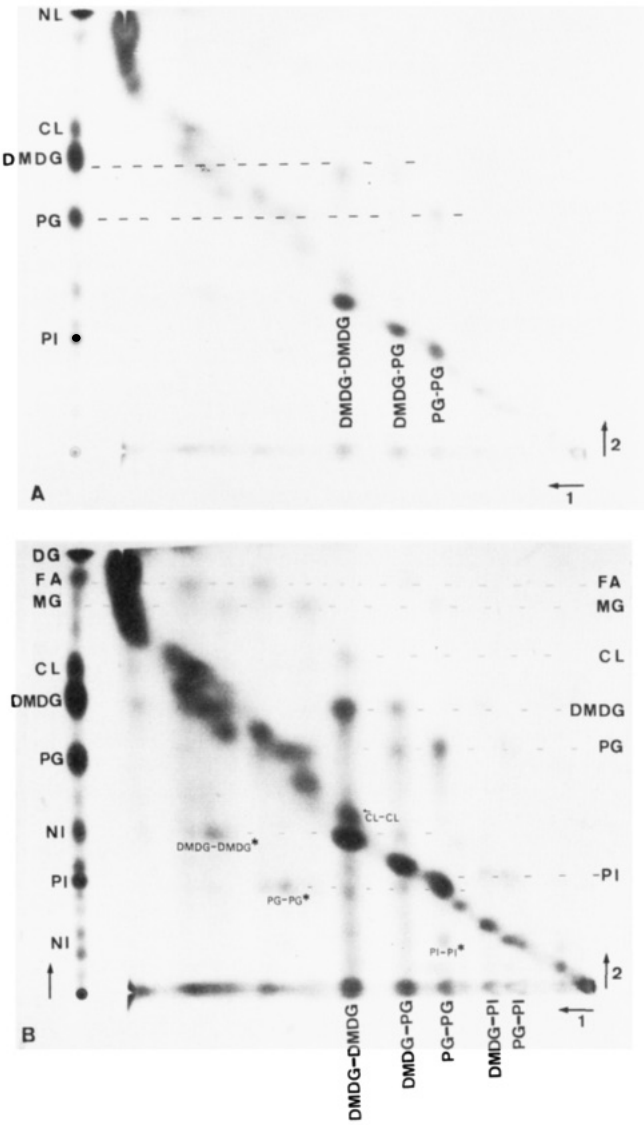

FIGURE 2: Autoradiogram of a total lipid extract from photoirradiated bacteria obtained after migration in the first direction, partial dedimerization by illumination of the plate at 256 nm, and migration in the second direction: plates A and B correspond to 4 and 40 days of film exposure, respectively. (*) indicates photodimers which were formed on the TLC plate during the photolysis illumination step (see Results).

good agreement with the percentages of the two lipids measured before photoirradiation. On the basis of these calculated values, assuming a homogeneous distribution of the two lipids, the expected distribution of the three types of photodimers is PG–PG = (36%)(36%) = 13%; PG–DMDG = 2(36%)(64%) = 46%, and DMDG–DMDG = (64%)(64%) = 41%. These calculated values are in excellent agreement with the experimental one (Table I) indicating that anthracene–PG and anthracene–DMDG were homogenously distributed in liposomes, confirming the validity of the method for quantitative analysis of lipid distributions.

Similar experiments were carried out on intact bacteria. As described in the appendix, not all the homo- and heterodimers formed need to be counted. Only the three dimers from two out of all the lipid species present in the membrane need to be taken into account. In the first batch, the percentage of radioactivity associated with the two lipids in the absence of irradiation was PG = 35% and DMDG = 65% as indicated in Table I. After photoirradiation of bacteria and extraction of the lipids, the radioactivity associated with the three types of dimers was PG–PG = 24%, PG–DMDG = 31%, and DMDG–DMDG = 45%. From these figures, the percentages of the corresponding lipid monomers are PG = 40% and DMDG = 60% in good agreement with the original monomer values. The expected distribution of the three photodimers for a homogeneous mixture of the two lipids will be PG–PG = (40%)(40%) = 16%, PG–DMDG = 2(40%)(60%) = 48%, and DMDG–DMDG = (60%)(60%) = 36%. Comparison between the experimental and calculated values reveals an increase in homodimers and a decrease in heterodimers for the photoproducts formed in intact bacteria.

We carefully checked for contamination of the photodimer spots on the chromatogram which might have accounted for the discrepancy between the experimental and calculated distributions. For instance, the two homodimers might contain impurities. As can be seen in Figure 1, PG–PG migrated at a position close to that of PI. However, since the photodimerization rate was close to 90% and since PI is a minor lipid (PG/PI = 4.5), possible contamination of PG–PG by residual PI would only modify the PG–PG radioactivity count by less than 1%. On the other hand, as can be seen in Figure 2, DMDG–DMDG may contain some CL–CL. Under our experimental conditions, the amount of CL present in the membranes when bacteria are irradiated is low (DMDG/CL = 13), and as previously mentioned, CL–CL photodimers tend to dissociate on silicic acid. The contamination of DMDG–DMDG by CL–CL, which was not observed consistently, can be evaluated at less than 5%. Even though both homodimers were slightly overestimated with respect to the corresponding heterodimer, the differences between the observed and exact distributions are within the range of experimental error. They thus cannot account for the discrepancy between the experimentally observed distribution and the corresponding calculated distribution for a homogeneous mixture of the two lipids in the membrane.

The distribution of PG and DMDG was investigated in three other independent bacterial batches. As can be seen in Table I, the percentage of radioactivity associated with each lipid type was found to vary from one batch to the other due to differences in the rate of incorporation of 9-AN between the bacterial cultures. However, in each case, the experimental distributions for the two homodimers were higher than the calculated ones. In contrast, the experimental distributions for the heterodimer were around half that expected for an even distribution of the two lipids (see last column in Table I). This

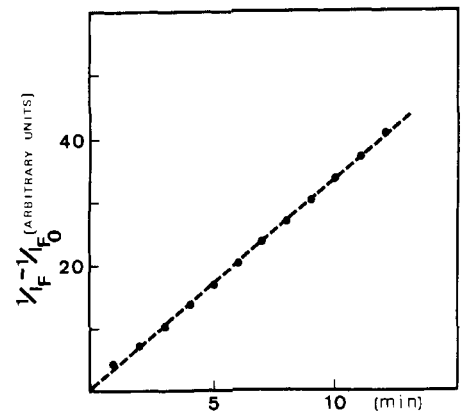

FIGURE 3: Time course of the photodimerization of anthracene–lipids in intact bacteria. Experiments were carried out directly in a spectrofluorometer. Ordinates represent the reciprocal of the recorded fluorescence intensity. λ_{ex} = 358 nm; A_{358} = 0.04; λ_{em} = 415 nm.

is considerably above experimental errors which were estimated to be around 10% and is indicative of a heterogeneous distribution of PG and DMDG in the bacterial membrane. This heterogeneity may stem either from an asymmetric distribution of the two lipids between the two membrane lipid leaflets or from a heterogeneous distribution of the two lipids within each lipid leaflet.

Kinetics of Lipid Photodimerization in Intact Bacteria and Lipid Distribution in Partially Photodimerized Bacteria. We also wanted to determine the kinetics of the anthracene photoreaction in intact bacteria. Since anthracene photodimerization is accompanied by loss of its characteristic UV absorption and fluorescence emission spectra, the photodimerization of anthracene-labeled lipids could be followed directly in a fluorescence spectrometer equipped with wide excitation slits. As shown in Figure 3, the reciprocal plot was linear over a considerable period of time, indicating that in these bacteria anthracene photodimerization is a result of a single bimolecular reaction, as was previously observed in liposomes (de Bony & Tocanne, 1983). This strongly suggests that the anthracene-labeled lipids are distributed homogeneously in the nonlabeled lipids.

We also analyzed the photoproducts after a 15-s irradiation of the bacteria in the optical bench, which leads to the cross-linking of around one-third of the total anthracene lipids. As can be seen in Table I, entry 6, the discrepancy between the experimental and calculated distributions for a homogeneous distribution of the two lipids was even more marked than that after 3-min irradiation. However, the experimental values may have been slightly distorted by contamination of the PG–PG and DMDG–DMDG dimers with residual monomers. Taken together, the kinetic (Figure 3) and distribution (Table I) data strongly suggest that the anthracene-labeled lipids are homogeneously distributed within the host lipids in the bacterial membrane.

Transverse Distribution of PG and DMDG in M. luteus Protoplasts

Preliminary Remarks. Transverse lipid asymmetry in biological membranes is generally investigated by degrading the constituent phospholipids with phospholipases (Op den Kamp, 1979; Etemadi, 1980). However, this cannot be applied to glycolipids, and so we decided to use periodate which reacts with both PG and DMDG. This reagent is known to oxidize α-diols rapidly, and a 4 °C it is relatively impermeant across membranes (Gahmberg & Andersson, 1977). Nevertheless, almost complete oxidation of the outer layer needs to be

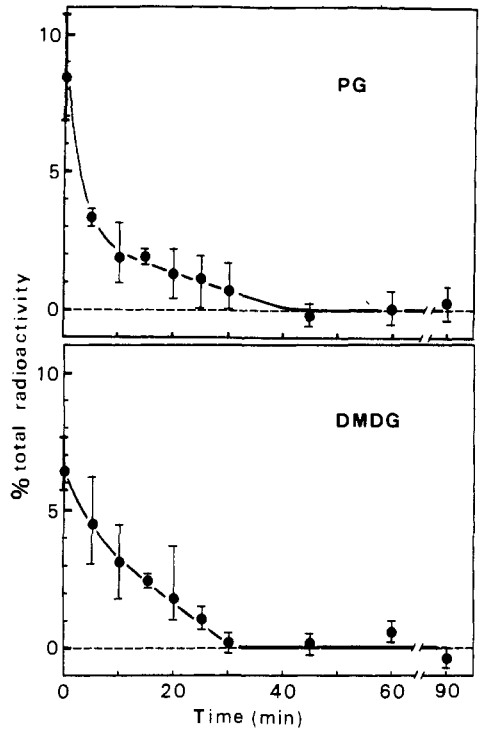

FIGURE 4: Time course of the oxidation of phosphatidylglycerol (PG) and dimannosyldiacylglycerol (DMDG) by periodate in membrane vesicles obtained after lysis of protoplasts. The ordinate represent the percentage of radioactivity associated with these lipids after thin-layer chromatography of a total lipid extract, with respect to the total radioactivity found in the same lane and normalized to 100. Full circles indicate the average value of three determinations, and the bars indicate the dispersion of the data. Periodate concentration was 50 mM, and the temperature was 4 °C.

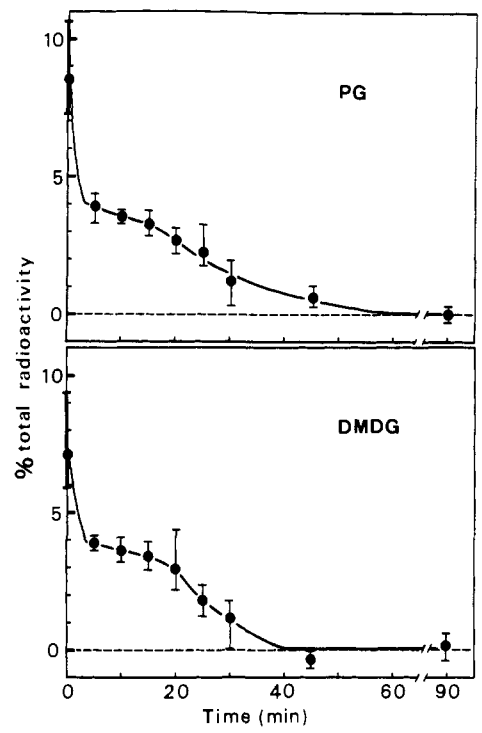

FIGURE 5: Time course of the oxidation of PG and DMDG by periodate in the membrane of intact *M. luteus* protoplasts. For details, see legend to Figure 4.

achieved before a significant amount of the reagent penetrates through the membrane. These conditions are met when one observes a clear-cut biphasic time course for lipid oxidation.

Preliminary experiments carried out with suspensions of PG and DMDG extracted from the bacterium showed that periodate concentrations in the range 10–50 mM completely oxidized these lipids relatively quickly (less than 30 min at 50 mM). The oxidized forms of the two lipids could also be separated from the native lipids by thin-layer chromatography on silica gel after impregnation of the gel with 1 mM sodium carbonate.

As mentioned above, there is a high level of cardiolipin synthetase activity during protoplast preparation. This results in the conversion of 80–90% of PG into cardiolipin, a lipid which is not a substrate for periodate oxidation. Attempts to reduce this conversion showed that the best results were obtained by preparing the protoplasts at high pH (pH 9). Under these conditions, conversion of PG into CL could be limited to 60%.

Results. To control the extent of the oxidation process, *M. luteus* membrane vesicles were treated with 50 mM periodate. As can be seen in Figure 4, a monophasic oxidation process was observed for both PG and DMDG, which reached completion after about 30 min. Oxidation was always faster with PG than with DMDG.

In contrast, the profiles obtained for intact protoplasts (Figure 5) indicated a clear-cut biphasic process for both PG and DMDG. There was a marked change in the slopes of the curves after the first 5 min, and a plateau was observed over the following 10 min. Oxidation then accelerated again to reach completion after about 45 min. Both lipids were oxidized in synchrony. From these profiles and from the quantity of oxidized lipids at the midplateau, 60% of the PG and 49% of the DMDG were deduced to be in the outer layer of the membrane of *M. luteus*.

This experiment was repeated with another batch of bacterial protoplasts with a lower level of conversion of PG into CL. In this case, the radioactivity associated with PG amounted to around 20% of the total lipid extract instead of 10% in the previous experiment. Nevertheless, the kinetics of PG and DMDG oxidation by periodate were similar to those shown in Figures 4 and 5, giving 62% of the PG and 42% of the DMDG in the outer layer of protoplast membranes. The asymmetric distribution of PG was identical for these two batches with different rates of interconversion of PG into CL (60% and 80%, respectively). This is a good indication that the distribution of residual PG reflects that of the total PG in the intact membrane.

Since periodate did penetrate the membrane to some extent, experiments were repeated at lower periodate concentrations (40, 20, and 10 mM). Decreasing the reagent concentration led to a progressive slowing of both oxidation reactions and periodate penetration inside the protoplasts. However, these concentrations were sufficient to oxidize all lipid molecules in the membrane vesicles. Overall, the results of the various experiments indicated that 60 ± 10% PG and 45 ± 10% DMDG reside on the outside of the bacterial membrane. Although PI contains an α-diol, this lipid was a very poor substrate for periodate oxidation under our experimental conditions.

We checked that periodate did not lyse the protoplasts. Protoplasts were oxidized as described above, and samples were removed at regular intervals and diluted in an isotonic buffer. In the dilution conditions used, an absorbance of 1 was measured at 640 nm for these protoplast suspensions, which remained unchanged after 90-min incubation of the protoplasts with 50 mM periodate. On the other hand, the absorbance fell to 0.02 after lysis of the same suspension with a hypotonic buffer.

Discussion

The ability of the anthracene photodimerization reaction to characterize the lateral distribution of lipids in biological membranes is based on the following requirements: (1) 9-AN must be metabolically incorporated into all lipids of interest. This is due to the fact that the photo-cross-linking reaction is specific for anthracene. (2) The anthracene dimerization constant is not affected by the nature of the lipid polar head groups. (3) Anthracene–lipids are homogeneously distributed throughout the host lipids. (4) The lifetime of the excited state of the probe is negligible compared to the time scale of lateral movements of lipids in the membrane. (5) Photodimerization occurs exclusively within the same membrane leaflet (head-to-head photodimers). (6) The distribution of lipids between the two membrane lipid leaflets is known.

Our previous results on 9-AN indicated that anthracene–lipids behave as normal lipids, in agreement with reports using other anthryl derivatives (Stoffel & Michaelis; 1976; Bergelson et al., 1985). The fact that their rate of incorporation can reach 40% is a good indication that anthracene lipids are readily miscible with host lipids. Moreover, the kinetics of anthracene photodimerization in intact bacteria is monophasic, demonstrating that the probe is homogeneously distributed among the host lipids.

It is to be noted that, in *M. luteus*, the incorporation rate of 9-AN is higher in DMDG than in PG. Consequently, the relative amount of the two anthracene–lipids is different from that of the corresponding host lipids. However, considering their regular phase behavior and their good miscibility properties with host lipids, it seems reasonable to assume that the distribution of anthracene–phospholipids and anthracene–glycolipids reflects that of host lipids.

On irradiation, anthracene-labeled lipids can only form head-to-head photodimers. Cross-linkages between two lipids located in opposite leaflets (head-to-tail photodimers) is unlikely since it would force the two lipids to overlap by six carbon atoms, corresponding to a highly unstable conformation. Our previous experiments showing a clear-cut calcium-induced phase separation in phosphatidylcholine/phosphatidic acid mixtures strongly suggested that head-to-tail lipid dimerization is not a significant phenomenon (de Bony & Tocanne, 1983).

The lifetime of the anthracene excited state is of the order of 10^{-9} s, which is negligible compared to the time scale ($\sim 10^{-6}$ s) of lipid lateral movements (Vincent et al., 1985).

After photoirradiation of intact cells, various types of photodimers are formed which can be readily separated and analyzed. The bidimensional autoradiogram indicates that fatty acids and mono- and diglycerides are inserted into the bacterial membrane. There are few data on the intracellular location of this class of lipid in prokaryotes, essentially based on the location of their corresponding synthetic enzymes (Bell et al., 1971). Since a membranous insertion of anthracene lipids is required for photodimerization, this method might be of value for obtaining more direct information on the location and relative amounts of lipid intermediates.

We initially studied the distribution of the two major lipids PG and DMDG in the membrane of *M. luteus*. Photoirradiation of several different bacterial batches induced the formation of PG–DMDG heterodimers at a rate which was approximately 60% of that expected for a homogeneous distribution of the two lipids. This was not due to an artifact since the technique showed a homogeneous PG–DMDG distribution in liposomes prepared from the two lipids. These differences could be due to transverse or lateral asymmetry, or both.

The distribution of PG and DMDG over the two membrane leaflets was subsequently investigated by periodate oxidation. The distribution of DMDG was found to be nearly symmetrical over the two membrane leaflets (45 ± 10% in the outer layer). Although it is clearly established that sphingoglycolipids are located preferentially in the outer leaflet of eukaryotic cell membranes (Op den Kamp, 1979; Etemadi, 1980), data on glycolipid distribution in prokaryotes are scarce, even though bacterial membranes are rich in neutral glycolipids. It has been suggested that neutral glyceroglycolipids are located on the outside of the plasma membrane in bacteria (Owen & Salton, 1977). However, the distribution of neutral glycolipids in bacteria has not been studied quantitatively.

PG was observed to be slightly preponderant in the outer leaflet (60 ± 10%). However, during protoplast formation a major part of PG is transformed into CL which is not a substrate for periodate. It is thus important to know whether cardiolipin synthetase activity is evenly distributed between the two sides of the membrane. In fact, periodate oxidation of *M. luteus* protoplasts differing in the rate of interconversion of PG into CL gave identical results, indicating that the distribution of residual PG probably reflects that of the total PG.

M. luteus phospholipid distribution has also been studied with phospholipases and phospholipid exchange proteins (Barsukov et al., 1976), giving PG proportions of 50% and 70%, respectively, on the outer leaflet. In the above study, around 70% of PG was converted into CL. The distribution of this lipid was also investigated. Forty percent of the CL was found in the outer leaflet by use of phospholipid transfer proteins, and 50% was found by use of the phospholipases. Our data and those of Barsukov et al. indicate that even though most of the PG is transformed into CL, the distribution of the remaining PG reflects that in intact bacteria and is nearly symmetrical on both faces of the membrane.

In order to account for the observed differences between the experimental and calculated distributions of the PG–DMDG heterodimers (Table I) in terms of lipid transverse asymmetry, a simple calculation shows that about 80–90% PG on one side and 80–90% DMDG on the other side of the membrane would be required. This degree of asymmetry is much greater than we observed. The observed deficiency in PG–DMDG heterodimer cannot thus be accounted solely by a transverse asymmetry in the distribution of the two lipids. In fact, it points more to a heterogeneous lateral distribution of the two lipids in the membrane of *M. luteus*.

Lateral phase separation of lipids in model membranes is well described and characterized (Van Dijck, 1978; Mc Elhaney, 1982). Lateral heterogeneities in the distribution of lipids and proteins have been clearly identified at a macroscopic scale in some cells with membrane regionalization and in which different parts of the same membrane have different structural and functional properties. They include epithelial cells (Chapelle & Gilles-Baillien, 1983), thylakoidal membranes (Murphy & Woodrow, 1983), and sperm cells (Wolf & Volgmayr, 1984).

For natural membranes, a number of studies indicate the existence of lateral heterogeneities in distribution and mobility of lipids and proteins (Karnovsky et al., 1982). This has been observed with various membrane systems by NMR (Albert & Yeagle, 1983; Selinsky & Yeagle, 1985), ESR (Zachowski & Devaux, 1983; Stier & Sackmann, 1973), freeze–fracture (Severs & Robenek, 1983), fluorescence (Barrow & Lentz, 1985; Klausner et al., 1980; De Laat et al., 1979), and other methods (Bridgman & Nakajima, 1981; Puplin & Bloch, 1983; Humphries & Lovejoy, 1984). Even though these

studies suggest the existence of different lipid pools in membranes, microscopic lipid heterogeneities have yet to be clearly demonstrated. This is mainly due to the lack of techniques enabling selective identification or specific extraction of lipids in the putative microdomains.

Ideally this problem needs to be investigated with methods giving information on both the topological and dynamic properties of the molecules as these two aspects are interrelated. In this respect a metabolically incorporated probe would throw considerable light on the behavior of lipids in the membrane. For example, recent studies in CHO cells reveal the existence of a lipid pool of lower mobility. This was only detected after metabolic incorporation of 9-AN into lipids. It was not observed after direct insertion of the probe in the membrane (Dupou et al., 1988). In addition, a number of studies have indicated the presence of lipid pools which are inaccessible to phospholipases (Bevers et al., 1978). It has also been shown that exogenous lipids do not intermix freely with the major pool of endogenous lipids (D'Souza et al., 1983). Recently, it has been suggested that micrometer-scale domains exist in fibroblast plasma membranes (Yechiel & Edidin, 1987). Considering the lateral distribution and mobility of lipids, our results can be interpreted in terms of a lipid compartmentation in the plane of the membrane with certain zones enriched in one particular lipid, possibly around proteins. It is also possible that some proteins may trap lipids, thereby forming domains which are no longer exchangeable with the bulk of the membrane.

Appendix

The lipid phase in a membrane can be considered as a collection of n molecules which distribute between x_1, ... x_i, ... x_n molecules ($\sum i_1 x_i = n$) of M_1, ... M_i, ... M_n different molecular species, respectively.

If one considers only the two molecular species M_i and M_j, the formation of M_i–M_i or M_j–M_j homodimers is strictly equivalent to the stepwise selection of two M_i or two M_j molecules among the n lipid molecules which are present in the membrane. The probability for such an event is equal to the ratio of favorable over possible cases:

$$p(M_i\text{–}M_i) = (x_i/n)(x_i/n) = x_i^2/n^2$$

$$p(M_j\text{–}M_j) = (x_j/n)(x_j/n) = x_j^2/n^2$$

Now, to form a M_i–M_j heterodimer, either a M_i or a M_j molecule can be selected first, and the probability of such an event is

$$p(M_i\text{–}M_j) = 2x_ix_j/n^2$$

It should be stressed that this probability calculation is valid only for a very large collection of objects, as is the case for lipids in a biological membrane. It should be also emphasized that the important parameter which is actually to be considered is not the absolute value of these probabilities but rather their relative values. This last parameter remains unchanged after normalization of n to unity. It remains also unaffected if, for example, one restricts the analysis of the photoproducts to only the two molecular species M_i and M_j. In this case, the probabilities for obtaining the homo- and hetero-dimers are

$$p(M_i\text{–}M_i) = x_i^2/(x_i + x_j)^2$$

$$p(M_i\text{–}M_j) = 2x_ix_j/(x_i + x_j)^2$$

$$p(M_j\text{–}M_j) = x_j^2/(x_i + x_j)^2$$

To the proportionality factor $n^2/(x_i + x_j)^2$, these probabilities distribute as those calculated above in the general case.

Conversely, from the relative distribution of the homo- and heterodimers which have formed between the two molecular species M_i and M_j, it is possible to go back to their relative distribution in the original mixture. Indeed, and in the general case, x_i^2/n^2 and x_j^2/n_2 molecules of the M_i–M_i and M_j–M_j homodimers are equivalent to $2x_i^2/n^2$ and $2x_j^2/n^2$ molecules of M_i and M_j monomer, respectively, while $2x_ix_j/n^2$ molecules of the heterodimer M_i–M_j are equivalent to the same number $2x_ix_j/n^2$ molecules of M_i and M_j monomers. As a whole, $2x_i(x_i + x_j)/n^2$ and $2x_j(x_i + x_j)/n^2$ molecules of M_i and M_j monomers are counted, respectively. They distribute effectively in the x_i/x_j molar ratio which characterizes these molecules in the initial mixture. It can be easily checked that this back-calculation stands as well for regular as for irregular repartition (lateral or transverse asymmetry) of the lipid molecules in the membrane. This means that when the complex lipid mixture which exists in a natural membrane is being considered, it is not necessary to count all the homo- and heterodimers which are formed. One can simply focus on the three dimers (two homo- and one heterodimer) which originate from two out of all the lipid species present in the membrane.

The knowledge of the relative distribution of these three dimers is enough by itself to recalculate the relative distribution of the two native lipid monomers initially present in the membrane. With this in mind, the following procedure was applied in the present study: (1) Determine the relative weight of the homo- and heterodimers originating from two M_i and M_j molecular species. (2) From these values, calculate back the relative distribution of the M_i and M_j monomers. (3) Compare this relative distribution with that found when counting the lipid monomers in the membrane before the photodimerization reaction. (4) Then, use this relative distribution to recalculate the relative dimer distribution which is expected to result from a regular repartition of the monomers molecules in the membrane. (5) Finally, compare this calculated relative dimer distribution to the experimental one.

References

Albert, A. D., & Yeagle, P. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7188–7191.

Barrow, D. A., & Lentz, B. R. (1985) *Biophys. J. 48*, 221–234.

Barsukov, L. I., Kulikov, V. I., & Bergelson, L. D. (1976) *Biochem. Biophys. Res. Commun. 71*, 704–711.

Bayley, H. (1982) in *Membranes and Transport* (Martonosi, A. N., Ed.) Vol 1, pp 185–195, Plenum, New York.

Bell, R. M., Mavis, R. D., Osborn, M. J., & Vagelos, P. R. (1971) *Biochim. Biophys. Acta 249*, 628–635.

Bergelson, L. D., Molotkovsky, J. G., & Manevich, Y. M. (1985) *Chem. Phys. Lipids 37*, 165–195.

Bevers, E. M., Op Den Kamp, J. A. S., & Van Deenen, L. L. M. (1978) *Eur. J. Biochem. 84*, 35–42.

Bick, R. J., Van Winkle, W. B., Tate, C. A., Entmann, M. L., Blasie, J. K., & Herbette, L. G. (1987) *Biochemistry 26*, 4831–4836.

Bisson, R., & Montecucco, C. (1985) in *Progress in Protein-Lipid Interaction* (Watts & De Pont, Eds.) pp 259–287, Elsevier, Amsterdam.

Bisson, R., & Montecucco, C. (1986) in *Techniques for the Analysis of Membrane Proteins* (Ragan & Cherry, Eds.) pp 153–184, Chapman and Hall, London.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. 378* 911–917.

Bridgman, P. C., & Nakajima, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1278–1282.

Chapelle, S., & Gilles-Baillien, M. (1983) *Biochim. Biophys. Acta 753*, 269–271.

Curatolo, W., Radhakrishnan, R., Gupta, C. M., & Khorana, H. G. (1981) *Biochemistry 20*, 1374–1378.
de Bony, J., & Tocanne, J. F. (1983) *Chem. Phys. Lipids 32*, 105–121.
de Bony, J., & Tocanne, J. F. (1984) *Eur. J. Biochem. 143*, 373–379.
de Bony, J., Martin, G., Welby, M., & Tocanne, J. F. (1984) *FEBS Lett. 174*, 1–6.
De Laat, S. W., Van der Saag, P. T., Elson, E. L., & Schlessinger, J. (1979) *Biochim. Biophys. Acta 558*, 247–250.
D'Souza, C. J. M., Clarke, J. T. R., Cook, H. W., & Spence, M. W. (1983) *Biochim. Biophys. Acta 752*, 467–473.
Dupou, L., Teissié, J., & Tocanne, J. F. (1986) *Eur. J. Biochem. 154*, 171–177.
Dupou, L., Lopez, A., & Tocanne, J. F. (1988) *Eur. J. Biochem. 171*, 669–674.
Etemadi, A. H. (1980) *Biochim. Biophys. Acta 604*, 347–475.
Gahmberg, C. G., & Andersson, L. C. (1977) *J. Biol. Chem. 252*, 5888–5894.
Giraudat, J., Montecucco, C., Bisson, R., & Changeux, J. P. (1985) *Biochemistry 24*, 3121–3127.
Gupta, C. M., Costello, C. E., & Khorana, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3139–3143.
Herbette, L., Blasie, J. K., Deefor, P., Fleischer, S., Bick, R. J., Van Winkle, W. B., Tate, C. A., & Entman, M. L. (1984) *Arch. Biochem. Biophys. 234*, 235–242.
Humphries, G. M. K., & Lovejoy, J. P. (1984) *J. Membr. Biol. 80*, 249–256.
Johnson, J. H., & Grula, E. A. (1980) *Can. J. Microbiol. 26*, 658–665.
Karnovsky, M. J., Kleinfield, A. M., Hoover, R. L., & Klausner, R. D. (1982) *J. Cell. Biol. 94*, 1–6.
Khorana, H. G. (1980) *Bioorg. Chem. 9*, 363–405.
Klausner, R. D., Kleinfield, A. M., Hoover, R. L., & Karnovsky, M. J. (1980) *J. Biol. Chem. 255*, 1286–1295.
Krebs, J. J. R. (1982) *J. Bioenerg. Biomembr. 14*, 141–157.
Lacombe, C., & Lubochinsky, B. (1988) *Biochim. Biophys. Acta 961*, 183–187.
Lakhdar-Ghazal, F., & Tocanne, J. F. (1981) *Biochim. Biophys. Acta 644*, 284–294.
Mc Elhaney, R. D. N. (1982) *Chem. Phys. Lipids 30*, 229–259.
Middaugh, C. R., Vanin, E. F., & Ji, T. H. (1983) *Mol. Cell. Biochem. 50*, 115–141.
Murphy, D. J., & Woodrow, I. E. (1983) *Biochim. Biophys. Acta 725*, 104–112.
Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.
Owen, P., & Salton, M. R. J. (1977) *J. Bacteriol. 132*, 974–985.
Pumplin, D. W., & Block, R. J. (1983) *J. Cell. Biol. 97*, 1043–1054.
Rigomier, D., Lacombe, C., & Lubochinsky, B. (1978) *FEBS Lett. 89*, 131–135.
Salton, M. R. J., & Freer, J. M. (1965) *Biochim. Biophys. Acta 107*, 531–536.
Sandra, A., & Pagano, R. E. (1978) *Biochemistry 17*, 332–338.
Selinsky, B. S., & Yeagle, P. L. (1985) *Biochim. Biophys. Acta 813*, 33–40.
Severs, N. J., & Robenek, H. (1983) *Biochim. Biophys. Acta 737*, 373–408.
Sigrist, H., Allegrini, P. R., Stauffer, K., Schaller, J., Abdulaev, N. G., Rickli, E. E., & Zahler, P. (1984) *J. Mol. Biol. 173*, 93–108.
Stier, A., & Sackmann, E. (1973) *Biochim. Biophys. Acta 311*, 400–408.
Stoffel, W., & Michaelis, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 925–935.
Van Dijck, P. W. M., De Kruijff, B., Verkleij, A. J., Van Deenen, L. L. M., & De Gier, J. (1978) *Biochim. Biophys. Acta 512*, 84–96.
Van Meer, G., & Simons, K. (1982) *EMBO J. 1*, 847–852.
Vincent, M., Gallay, J., de Bony, J., & Tocanne, J. F. (1985) *Eur. J. Biochem. 150*, 341–347.
Welby, M., & Tocanne, J. F. (1982) *Biochim. Biophys. Acta 689*, 173–736.
Wolf, D. E., & Volgmayr, J. V. (1984) *J. Cell Biol. 98*, 1678–1684.
Yechiel, E., & Edidin, M. (1987) *J. Cell Biol. 105*, 755–760.
Zachowski, A., & Devaux, P. F. (1983) *FEBS Lett. 163*, 245–249.