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EVIDENCE FOR THE INCORPORATION OF A FLUORESCENT ANTHRACENE FATTY ACID INTO THE MEMBRANE LIPIDS OF *MICROCOCCUS LUTEUS*

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9-(2-Anthryl)-nonanoic acid, a newly synthesized photoactivable molecule, is shown to be incorporated into the membrane lipids of the bacterium *Micrococcus luteus*, through the regular metabolic pathway. This incorporation, which occurs at the *sn*-1 position exclusively and without any degradation or elongation of the anthracene fatty acid, is accompanied by an upward shift of the chain length of the other fatty acids.

As recently advocated by Ji [1] and by Khorana [2], photoreactive groups covalently attached to lipids or to proteins can provide a very useful tool in elucidating some aspects of the structure and functions of biological membranes. Having some interest in studying the dynamics and the topology of lipids in biomembranes, we have prepared a new photoactivable molecule, namely the 9-(2-anthryl)-nonanoic acid (Fig. 1). The synthesis and physical properties of this compound will be described elsewhere. In the present report, this anthracene fatty acid is shown to be incorporated into the membrane lipids of the bacterium *Micrococcus luteus*, through the regular metabolic pathway.

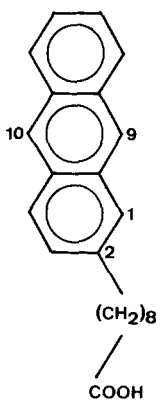

Fig. 1. 9-(2-Anthryl)-nonanoic acid.

Anthracene was chosen as an activable groups because of the interesting properties it displays. This group, which is well suited for labeling the hydrophobic core of membranes, is fluorescent and under strong illumination, it forms 9,9′–10,10′ covalently bound dimers which are no longer fluorescent [3]. Thus, after its incorporation into the membrane lipids, this group can be used: (1) as an indicator of membrane fluidity; (2) for measuring the lateral diffusion rate of the labeled molecules by using the technique of fluorescence recovery after photobleaching or by following the kinetics of the photodimerisation reaction; (3) for studying the topological distribution of lipids in membranes by means of crosslinking reactions between adjacent anthracene-labeled molecules.

Some anthracene derivatives have been used already as extrinsic probes in model and natural membranes for fluorescence polarization masurements [16]. These compounds, which are esters of

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9-anthracene carboxylic acid with various hydroxylated fatty acids [16], are not all the best suited for our purpose, in particular owing to the bulkiness of the anthracene residue when attached perpendicular to an acyl chain. In the molecule we have synthesized, the anthracene moiety and the polymethylenic segment lie along the same axis and, as expected, preliminary monolayer experiments show that with such a configuration, this new anthracene fatty acid is no more bulky than a normal fatty acid.

In the following, we describe the incorporation and the localization of the anthracene fatty acid into the lipids of *M. luteus*.

Micrococcus luteus (ATCC 4698-4) was grown at 30°C under agitation in a peptone-containing medium [4] supplemented with the anthracene fatty acid. As this compound is completely insoluble in water, its incorporation into the bacterial membrane during cell growth required an efficient way of dispersing it in the growth medium. Stable microemulsions were obtained by addition to the growth medium, under gentle stirring, of a solution of the anthracene fatty acid ammonium salt in dimethylormamide (10 mg/ml), followed by two brief sonication periods of 30 s each, at 80 kHz (sonicating bath) and at room temperature. Longer sonication times lead to flocculation of the suspension. Homogeneity and stability of the suspensions were considerably improved if fatty acid-free bovine serum albumin was added to the growth medium at a concentration of 1 mg/ml, which proved to be non toxic for the cells. Efficient anthracene fatty acid incorporation and cell growth were obtained for a fatty acid concentration of 100 μg/ml. This was achieved with a dimethylformamide concentration of 1% (v/v) in the growth medium. This solvent did not inhibit cell growth up to a concentration of 5%.

Cells were harvested at the beginning of the stationary phase. They were washed by centrifugation twice with a 0.2% (w/v) water solution of the non-ionic detergent WR 1339 (from Ruger, U.S.A.), then twice with distilled water in order to remove most of the residual non-incorporated anthracene fatty acid.

Chloroform/methanol extraction [7] of the cells followed by precipitation of the lipid extract by boiling acetone yielded cardiolipin, phosphatidylglycerol, phosphatidylinositol and a dimannosyl diacylglycerol (the major lipids of *M. luteus* [5,6]), still contaminated with triacylglycerols and with a small amount of residual anthracene fatty acid. Chromatography of the mixture on silicic acid according to Vorbeck and Marinetti [8] followed by chromatography on DEAE-cellulose gave first the glycolipid, then the three phospholipids in a pure form, as checked by thin-layer chromatography on Silica gel G.

Phospholipids were sequentially degraded, first by digestion [9] with a commercial preparation of pig pancreatic phospholipase A_2 (Boehringer), then under alkaline conditions. Fatty acids were recovered at each step and converted into their methyl ester. If necessary, the anthracene fatty acid was separated from the other fatty acids by thin-layer chromatography on Silica gel G (solvent: petroleum ether/ether (8:2, v/v)). Gas chromatography identification of the fatty acid methyl esters was carried out with a Girdel apparatus equipped with a 1%-OV1 silicone column, 1 m long. Field desorption mass spectra were obtained with a Varian-MAT 311 A apparatus, using an activated tungsten emitter.

Qualitatively, the growth rate of *M. luteus* in the presence of the anthracene fatty acid was only slightly altered as compared with control cells. A lag period of about 4 h was observed, after which time cell growth reached a density very similar to that of control cultures. On Silica gel G thin-layer chromatograms of total lipid extracts, cardiolipin, phosphatidylglycerol, phosphatidylinositol and the dimannosyldiacylglycerol all exhibited a strong blue fluorescence, when the plates were illuminated at 360 nm. As judged by gas chromatography analysis of the fatty acids recovered after complete degradation of total lipid extracts (free from any trace of residual anthracene fatty acid), the best incorporation we obtained was 9% with respect to total fatty acids.

In the mass spectrometer, operating with a field desorption ionization source which is the best way to obtain the molecular weight of any injected compound [10], the anthracene fatty acid methyl ester recovered from phospholipids, after separation from the other fatty acids, gave a predominant peak at $m/z = 348$. This value is the expected molecular weight for the native compound. This

means that *M. luteus* had incorporated the anthracene fatty acid without any degradation or elongation.

To determine the position of the anthracene fatty acid in the phospholipids, phospholipase A_2 was assayed both on total phospholipid extracts and on isolated phospholipids. On Silica gel G thin-layer plates, the lysophospholipids originating from the enzymatic hydrolysis appeared systematically to be fluorescent, whereas the freed fatty acids were not. Such a result, which leads to the conclusion of an exclusive localization of the anthracene fatty acid at the *sn*-1 position, was confirmed by a gas chromatography analysis of the various fatty acids which were obtained first, after the enzyme digestion, then after chemical degradation of the corresponding lyso derivatives (Table I). Phosphatidylinositol was not assayed by gas chromatography since the amount of purified lipid we obtained was too low.

As compared to control cells [5] (Table I), fatty acids of cells grown in the presence of bovine serum albumin and dimethylformamide were only slightly modified. In contrast, the incorporation of the anthracene fatty acid into the lipids of *M. luteus* resulted in an upward shift of the chain length of the other fatty acids, detrimental to the shorter ones, i.e. the *iso* and *ante-iso* methyl-branched tetradecanoic acids (C15:br). This tendency was particularly marked at the *sn*-2 position where the C15:br acids nearly disappeared at the benefit of palmitic (C16:0), stearic (C18:0), oleic (C18:1) and methyl-branched octadecanoic (C19:br) acids.

Most incorporations of exogenous fatty acids into bacteria so far reported have been carried out with auxotrophic mutants for fatty acids of *Escherichia coli* [11–15]. Greenberg et al. [13] have reported the incorporation of various fatty acids containing photoreactive groups, using an *E. coli* auxotroph strain. In this case, these compounds accounted for 16 to 43% of the total fatty acids and they were located mainly (95%) at the *sn*-2 position. Incorporation of parinaric acid, a fluorescent conjugated polyenene, has been also reported to occur in an *E. coli* auxotroph strain,

TABLE I

FATTY ACID COMPOSITION (%) AT *sn*-1 AND *sn*-2 POSITIONS OF THE MAJOR PHOSPHOLIPIDS OF *MICROCOCCUS LUTEUS*

BSA, bovine serum albumin (1 mg/ml); DMF, dimethylformamide (1%, v/v); C*xx*: br, methyl-branched fatty acids. Concentration of anthracene fatty acid in growth medium 100 μg/ml.

Fatty acids	Anthracene fatty acid + BSA + DMF				BSA + DMF Total phospholipids		Control cells (from Ref. 5)			
	Cardiolipin		Phosphatidyl-glycerol				Cardiolipin		Phosphatidyl-glycerol	
	sn-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2
C12:0					7	6				
C14:1	1		1			2	1	3	tr	1
C14:0	3	6	2		8	8	4	4	1.5	1
C15:br	45	7	49	tr	56	77	71	87	47	82
C16:1	6	12	6	3	3		5	1	6	1
C16:0	12	28	13	54	16	7	9	1	6	0.5
C17:br	20	6	20	13	4		11	1.8	39	14
C18:1	5	13	4	9	3		tr	1.8	tr	tr
C18:0	3	7	2	6	2					
C19:br		22		14						
Anthracene fatty acid	5		3							

although to a small extent (1–2%). Its position on the glycerol backbone was not determined [14]. In the present case, the facile and exclusive incorporation of the anthracene fatty acid at the *sn*-1 position into the lipids of *M. luteus* was obtained with a non-auxotroph strain. Such a preference for a given position, which seems rather unusual, is actually very convenient in view of further studies of the dynamics and of the topology of lipids in this bacterial membrane. This incorporation also demonstrates a great latitude of adaptation of the bacterial membrane. Its response can be understood, not in terms of 'fluidity', since one observes an elongation of the fatty acids, but rather in terms of structure of the lipid matrix. The anthracene fatty acid is two methylene groups longer than the methyl-branched tetradecanoic acid which predominates in normal cells [5]. It is then tempting to suggest that in order to maintain the integrity of the bilayer structure, the elongation of the fatty acids which is mainly observed at position *sn*-2 would occur merely to compensate the elongation at position *sn*-1 which results from the incorporation of the anthracene fatty acid.

Wether this compound is susceptible to be incorporated into the membrane lipids of any other bacteria or cells has to be examined specifically for each organism. Interestingly, preliminary experiments show an incorporation of this acid into the membrane lipids of *Bacillus megaterium* (D. Rigomier and J.F. Tocanne, unpublished results).

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