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Evolution

Molecular evolution of biomembranes: Structural equivalents and phylogenetic precursors of sterols

(triterpenes/tetraterpenes/prokaryotes/pre-aerobic evolution)

MICHEL ROHMER*†, PIERRETTE BOUVIER*†, AND GUY OURISSON*‡§

*Laboratoire de Chimie Organique des Substances Naturelles, associé au Centre National de la Recherche Scientifique, Université Louis Pasteur, Strasbourg; †Institut de Botanique, 28 rue Goethe, 67083 Strasbourg; and ‡Institut de Chimie, 1 rue Blaise Pascal, 67008 Strasbourg, France

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ABSTRACT **Derivatives of one triterpene family, the hopane family, are widely distributed in prokaryotes; they may be localized in membranes, playing there the same role as sterols play in eukaryotes, as a result of their similar size, rigidity, and amphiphilic character. Their biosynthesis embodies many primitive features compared to that of sterols and could have evolved toward the latter once aerobic conditions had been established. Membrane reinforcement appears to be achieved in other prokaryotes by other mechanisms, involving either ≈40-Å-long rigid hydrocarbon chains terminated by one polar group acting like a peg through the double-layer or similar chains terminated by two polar groups acting like tie-bars across the membrane. These inserts can be tetraterpenes (e.g., carotenoids). The biophysical function of membrane optimizers appears to have evolved toward sterols by changes limited to only a few enzymatic steps of the same fundamental biosynthetic processes.**

Sterols are always present in the membrane of eukaryotic cells (1), whereas they are normally absent in prokaryotes (2, 3). We shall show that, in many bacteria and cyanobacteria, triterpenes of the hopane family ("hopanoids") are present, that they are structural analogs of the sterols of eukaryotes, and that they qualify as phylogenetic precursors of sterols. We shall also show that, in other prokaryotes including the most primitive ones, tetraterpenoids may play a similar role through different biophysical mechanisms.

Hopanoids in prokaryotes

The wide distribution and variety of structures of hopanoids was first revealed by their molecular fossils, ubiquitous in sediments (4). In currently living prokaryotes, three series of 3-deoxyhopane derivatives are known (Fig. 1). The simplest ones are C_{30} derivatives of hopane itself, diploptene and diplopterol. A second family comprises C_{35} derivatives of bacteriohopane, a skeleton carrying at C-29 a heavily oxygenated n-C_5 chain (5–7). These C-pentosyltriterpenes are usually preponderant (Table 1). The third family derives also from bacteriohopane but carries additional methyl groups on the ring system, either at C-3 in an *Acetobacter* (8) or probably at C-6 in *Nostoc* (9).

An extensive survey of the distribution of hopanoids in prokaryotes, still underway, shows already that many microorganisms, belonging to widely separated taxonomic groups, contain these triterpene derivatives (Table 1).

Hopanoids as structural equivalents of sterols

We shall assume that hopanoids, in prokaryotes, are localized in membranes. They possess a quasi-planar, rigid, amphiphilic structure similar to that of sterols, with similar molecular dimensions (Fig. 2). This should be compatible with their mutual replacement. Indeed, when the protozoon *Tetrahymena pyriformis* (a eukaryote) is grown on a medium containing sterols, it uses them for its membranes. However, when the culture medium is deprived of sterols, *Tetrahymena* biosynthesizes diplopterol and mainly a hopanoid-like isomer, tetrahymanol (Fig. 1), in amounts comparable to the sterols that disappear (10). Tetrahymanol is then localized in the membranes (11), which adjust their phospholipid composition to maintain a proper fluidity (12). This shows that hopanoids play a sterol-like role in membranes of *Tetrahymena*, and it suggests clearly, although indirectly, that this may also be true in the prokaryotes that contain hopanoids.

It is accepted that the n-C_{16-18} chains of eukaryotic phospholipids fit well with sterol molecules, leading to cohesive van der Waals cooperative interactions (1). Sterols are thus assumed to act, by virtue of their parallel orientation, correct dimensions, and rigidity, as reinforcers of the fluid matrix of the n-acyl chains. The presence of axial groups on both sides of the molecule, making it thicker, lessens the cooperative interactions with the n-acyl chains, as shown by *in vitro* experiments with 14α-methylsterols (13) and with tetrahymanol (R. A. Demel, personal communication) in membrane models.

However, a better intermolecular fit could be restored with these molecules, the cross sections of which are larger, provided the acyclic lipids had themselves a larger cross section than did n-acyl chains. Increased effective cross sections can be achieved by the introduction of *cis* double bonds; this is partly how *Tetrahymena pyriformis* adjusts the composition of its phospholipids in its sterol-free, tetrahymanol-containing state (12). Another way to obtain thicker lipids is to have branched chains. Whereas only n-acyl lipids have been reported in *Tetrahymena* (14) and in cyanobacteria (15), branched-chain fatty acids are frequent in bacteria, and so are cyclopropyl or ω-cyclohexyl acids (16, 17). These thicker acyl chains are often located in various membranes (16) and, although hopanoids are present in some of them such as *Bacillus acidocaldarius* (6), they are absent from others such as *Bacillus subtilis*. However, in several groups of prokaryotes, neither sterols nor hopanoids are present.

Other mechanisms of membrane reinforcement

A priori, the acyclic lipids of the membrane could be stabilized into a double-layer membrane by many types of interactions, in particular with the membrane-bound, partially lipophilic proteins. However, in at least some prokaryotes, other biophysical mechanisms of reinforcement are discernible, making use of polyterpenoids other than hopanoids (or sterols).

Fig. 3 depicts schematically four such hypothetical mechanisms, different from the one just discussed which involves rigid inserts fitted to one half of the double layer (mechanism *A*). Mechanism *B* would involve a rigid insert about 40 Å long in

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§ To whom reprint requests should be addressed.

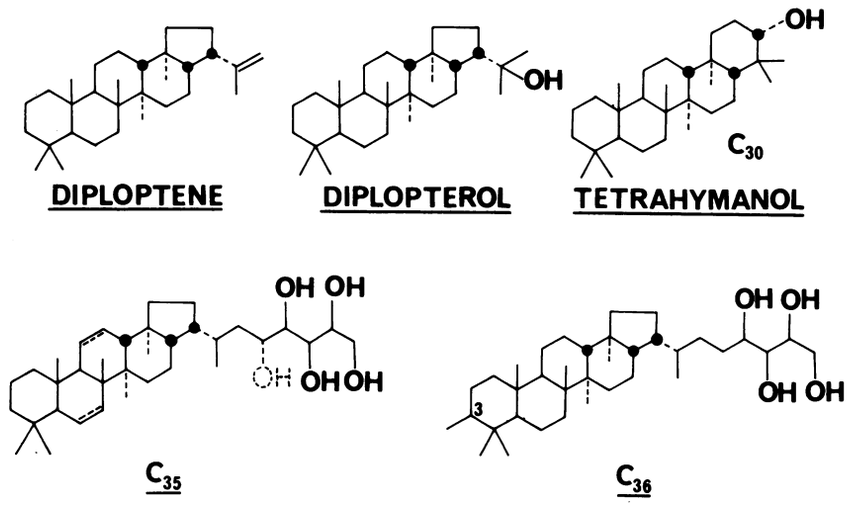

FIG. 1. Hopanoids in prokaryotes.

its lipophilic part, terminated at one end by a hydrophilic group and acting as a peg to keep both halves of the lipid membrane rigid. Mechanism *C* implies a rigid insert of similar size but with hydrophilic groups at both ends bracing together the two layers like a tie-bar. Mechanism *D* makes use of 40-Å-long lipophilic chains terminated at both ends by hydrophilic groups; however, it involves intrinsically nonrigid chains stretched and made taut by the inclusion of their end groups in the water environment. Finally, mechanism *E* suggests that a rigid, elongated hydrocarbon about 40 Å long could, if maintaining an orientation perpendicular to the membrane interfaces, also keep the lipid assembly rigid.

There is indirect or direct evidence for all of these mechanisms, and in each case it implies polyterpenes. Fig. 4 shows the structures of some of the many bacterial carotenoids that could play the role of inserts in mechanisms *B* and *C*. In some cases, the localization of bacterial carotenoids in membranes has been proved (18, 19); however, there was no indication of their internal orientation. In the sterol-free form of the mycoplasm *Acholeplasma laidlawii*, carotenoids (of unknown structures) have been shown to act as reinforcers of the membrane double layer, in the place of sterols (20).

In model liposomes, carotenoid organization has been tested both with β-carotene itself (shown to be probably mostly parallel to the *n*-acyl chains, as mechanism *E* postulates) or with hydroxylated derivatives. Even though the latter are shorter than the bacterial carotenoids considered as spanning through the membrane, they appear also to be oriented parallel to the lipid chains (21). The polar carotenoids of *Sarcina flava* have been postulated to straddle the lipid double layer, even though the biophysical consequences of this orientation were not recognized (22).

Finally, amphiphilic carotenoids (such as required for mechanism *B*) are also abundant in prokaryote-like organelles of the eukaryotes, the chloroplasts (23), which do not contain significant amounts of sterols.

For mechanism *D*, an excellent model has been found in the lipids of the extreme thermoacidophilic archaebacteria *Thermoplasma* (which contains the ethers shown in Fig. 4) of a dimer of phytanol (24). These are associated, not with *n*-acyl lipids but with variable amounts of the phytanyl ethers of glycerol; in this case, both the fluid and the rigid partners have the same segmental structure, and their intermolecular interactions should be optimal.

Table 1. Distribution of hopanoids in prokaryotes*

A. Hopanoids present (10^2–10^3 ppm, bacteriohopane polyols usually preponderant):
Cyanobacteria: *Anabaena* sp., *Nostoc* (2 strains), *Synechocystis* (2 strains)
Purple non-sulfur bacteria†: 6 strains of *Rhodopseudomonas, Rhodospirillum, Rhodomicrobium*
Methylotrophs: 8 strains of *Methylococcus, Methylomonas, Methylocystis, Methylosinus, Hyphomicrobium*
Other bacteria: *Nitrosomonas europaea, Pseudomonas cepacia, Azotobacter vinelandii, Acetobacter* (12 strains, covering 9 species); Gram-positive, *Bacillus acidocaldarius, Streptomyces chartreusi*

B. Hopanoids not detected (<10 ppm):
Cyanobacteria: *Synechococcus* sp., *Spirulina* sp.
Purple sulfur bacteria†: *Chromatium* sp., *Amoebobacter* sp., *Thiocapsa* sp.
Green sulfur bacteria†: *Chlorobium* (2 strains)
Archaebacteria: *Halobacterium cutirubrum, Sulfolobus acidocaldarius, Thermoplasma acidophilum;* Gram-positive: *Methanobacterium thermoautotrophicum*†
Other bacteria: *Thiobacillus* (2 strains), *Pseudomonas fluorescens, P. aeruginosa, P. stutzeri, P. maltophilia, P. diminuta, Xanthomonas campestris, Rhizobium lupini, Agrobacterium tumefaciens, Caulobacter crescentus, Moraxella* (2 strains), *Escherichia coli, Proteus vulgaris, Desulfovibrio desulfuricans*†; Gram-positive: *Bacillus subtilis, Sporosarcina lutea, Clostridium paraputrificum*†, *Streptococcus faecalis*†, *Micrococcus luteus, Micromonospora* sp., *Actinoplanes brasiliensis*

* The strains studied are all defined by a proper collection number (available on request) and will be described in a later publication, together with extraction procedures and quantitative data. Unless indicated otherwise, they are Gram negative.

† Grown anaerobically; the others are grown aerobically.

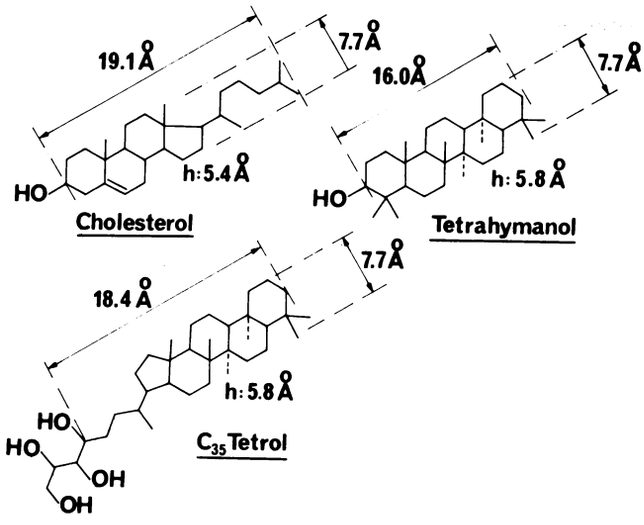

FIG. 2. Comparison of the dimensions of sterols and structural equivalents.

Although the C_{40} ethers have been found so far only in one archaebacteria, of peculiar ecological characteristics, we know from other evidence that they must be much more widely distributed: unmistakable molecular fossils of these substances have been found in sediments deposited in quite ordinary conditions (W. Michaelis and P. Albrecht, personal communication), which could have harbored for instance methanogenic bacteria but not the thermoacidophilic bacteria studied so far (24). Similar molecular fossils have also been found in several petroleums (W. Seifert, personal communication).

An interim summary of the suggestions made here is that a series of polyterpenoids—C_{30}–C_{50}, acyclic or polycyclic, conformationally mobile or rendered rigid by conjugation or by polycyclization—can play the same biophysical role of membrane stabilizers by various mechanisms. We shall now see that a hypothetical molecular evolution can be discerned, leading ultimately to the sterols.

Hopanoids as sterol precursors

Hopanoids can well have led, by a plausible phylogenetic evolution, to sterols. They are cyclization products of squalene, by an enzyme-mediated, acid-catalyzed process similar to but more primitive than the one leading from squalene to sterols (Fig. 5). The following characteristics can be considered as primitive compared to sterol biosynthesis.

(*i*) Hopanoid biosynthesis cyclizes a simpler substrate, squalene, instead of its derivative, the 2,3-epoxide (25, 26).

(*ii*) It implies a conformation of the substrate (all-*pre*-chair squalene) less constrained thermodynamically than that required for the precursor of sterols, which must be a partly *pre*-boat conformation (27, 28). Thus, the cyclization is brought about by the enzyme on squalene in the least constrained, and therefore the most favorable, of all the completely *pre*-cyclized conformations.

(*iii*) It implies only a simple polycyclization, without rearrangement or further degradations as in sterol biosynthesis (28–30).

(*iv*) The two squalene cyclases studied so far, in *Acetobacter rancens* (29) and *Tetrahymena pyriformis* (3) are not highly substrate-specific: they cyclize squalene and both enantiomers of its 2,3-epoxide, whereas the squalene epoxide cyclase of eukaryotes acts specifically on the 3(*S*) enantiomer of the epoxide only, and neither on the 3(*R*)-epoxide nor on squalene itself (30).¶

These criteria single out the hopane family from all other groups of triterpenes; furthermore, most of these are bent molecules, not suitable as reinforcers of a phospholipid matrix despite their amphiphilic character.

In his speculations on the evolution of sterols, Bloch (31) recently suggested that, in the prebiotic atmosphere, "chemical evolution of the sterol pathway, if it did indeed occur, must have stopped at the stage of squalene" because of lack of molecular oxygen, "an obligatory electron acceptor in the contemporary biosynthesis of sterols." However, hopanoid biosynthesis proceeds from acetate to end product without molecular oxygen—i.e., it can have occurred under primitive anaerobic conditions: it is a hydration of squalene, whereas sterol biosynthesis requires the oxidation of squalene by molecular oxygen and, in the final stages, oxidative removal of methyl groups. The discussion summarized above thus shows that, under anaerobic conditions, further chemical evolution could have occurred up to the hopanoid stage—i.e., up to molecules probably able to fill a sterol-like role in reinforcing membrane structures.

The function of membrane reinforcer can thus have been obtained from squalene under anaerobic conditions and maintained as such even in present-day aerobic bacteria or cyanobacteria. However, once aerobic conditions had developed, the formation of squalene epoxide had become possible, as well as its cyclization to 3β-hydroxy triterpenoids (probably hopane derivatives at first) under the influence of the squalene cyclase. Minor mutations of this cyclase, involving small changes in the relative positions of the active centers initiating the cyclization and stopping it, can then have led to the successive opening of the various routes of triterpene biosynthesis. At no point in this evolutionary drift had any vital function to be maintained, as long as 3-deoxyhopanoids were still present. However, once the genuine precursors of sterols, lanosterol in vertebrates and fungi and cycloartenol in plants (32), were obtained, their degradation to sterols may have paved the way toward the eukaryotic membrane, with its efficient *n*-acyl chains/sterol combination.

One microorganism is relevant for this hypothetical scheme: *Methylococcus capsulatus* produces, besides C_{30} and C_{35} ho-

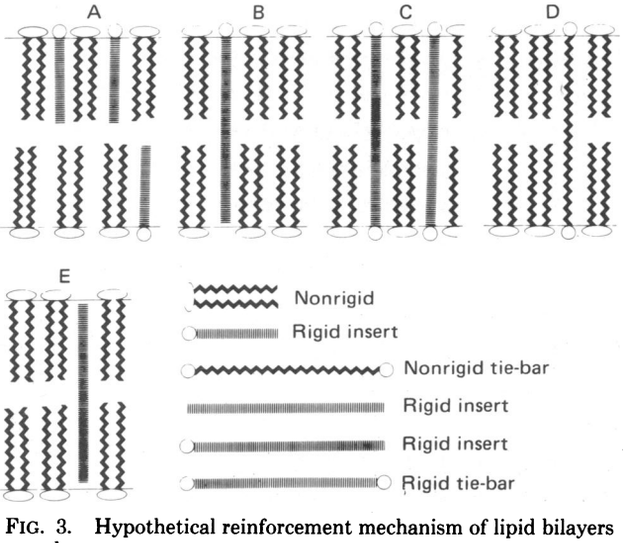

FIG. 3. Hypothetical reinforcement mechanism of lipid bilayers in membranes.

¶ The cell-free system of *Acetobacter rancens* cyclizes 3(*RS*)-squalene epoxide into hopane-3α- and -3β,22-diols (29); that of *Tetrahymena pyriformis* cyclizes the same racemic substrate into gammacerane-3α- and -3β,21α-diols (3). These cyclases are therefore not highly substrate-specific but are product-specific.

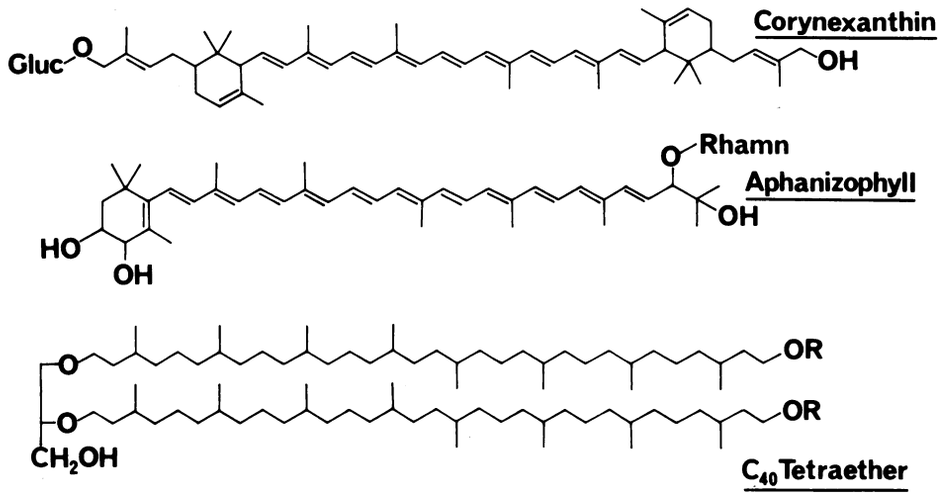

FIG. 4. Bacterial carotenoids and "equivalent" tetraterpenes.

panoids, not sterols proper but 4α-methylsterols (33, 34) and their precursor, lanosterol. In a cell-free system prepared from this bacterium, 3(*RS*)-squalene oxide is cyclized to give a mixture of lanosterol and 3β-hydroxydiploptene [derived from the 3(*S*) enantiomer] and of 3-epilanosterol and 3α-hydroxydiploptene [derived from the 3(*R*) enantiomer]. Thus, the squalene oxide cyclases of *Methylococcus*, like the squalene cyclase of *Acetobacter*, are "not yet" as highly substrate-specific as the squalene oxide cyclase of eukaryotes. However, in *Methylococcus*, squalene itself gives only diploptene and diplopterol, and no 3-deoxylanosterol. Thus, in this organism, at least two cyclases operate: one cyclizing squalene or its epoxides to hopanoids, and the other cyclizing squalene epoxides to lanosterol derivatives. Furthermore, the absence of 3-hydroxyhopanoids in *Methylococcus* implies that they be localized in separate cellular compartments, of which one contains 3(*S*)-squalene epoxide and the lanosterol-producing cyclase (3).

Phylogenetic precursors of hopanoids

The tetraterpenes involved in the other reinforcement mechanisms proposed in Fig. 3 present still more primitive characteristics: they are certainly less sensitive to a close cooperative molecular fit between the fluid matrix and the reinforcing partner, and they eschew the need for enzyme-mediated cyclization. Furthermore, they share with hopanoids the compatibility with an anaerobic biosynthesis.

It is therefore tempting to assume a phylogenetic sequence in which hopanoids were produced from squalene, once a suitable enzyme had been formed, but in which the same biophysical function was, up to that point, maintained only by mechanisms *B–E* of Fig. 3.

Of these, one may even speculate that *D* is the most primitive one, not only because it occurs in archaebacteria, considered as the most primitive organisms living today (35), but also because it is the simplest one, implying as it does only polyterpene

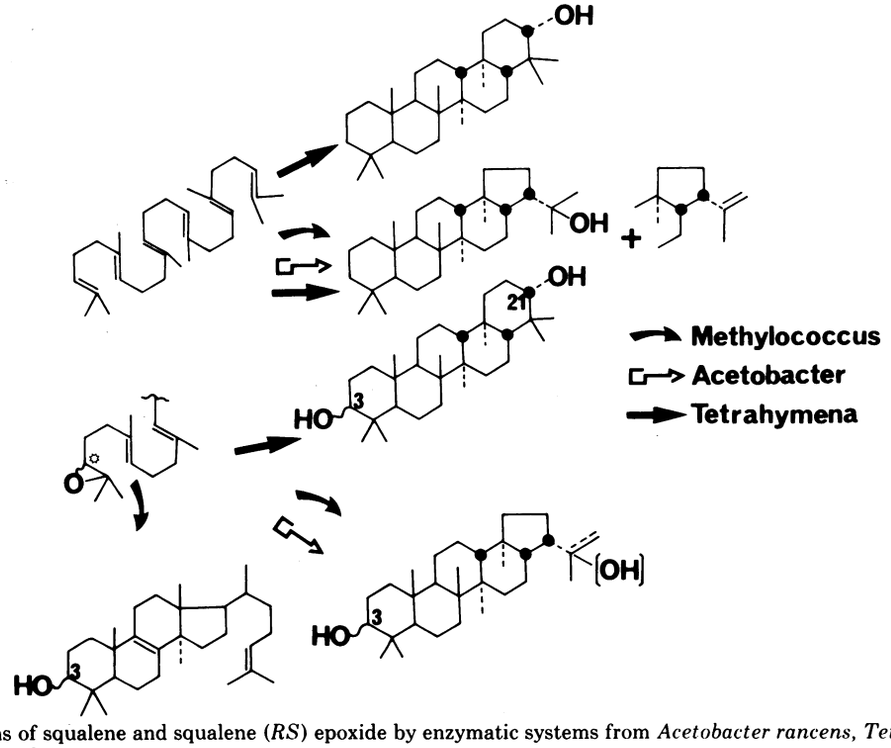

FIG. 5. Cyclizations of squalene and squalene (*RS*) epoxide by enzymatic systems from *Acetobacter rancens*, *Tetrahymena pyriformis*, and *Methylococcus capsulatus*.

partners and only one type of link (ethers) of the lipids with the polar heads.

This evolutionary model points to work required in many fields to give it more substance and to define it critically or refute it. We have already mentioned several questions still open. It would also be possible and interesting (*i*) to investigate more systematically the distribution of hopanoids and of specific carotenoids among prokaryotes; (*ii*) to define the intracellular localization of hopanoids and of hydroxylated carotenoids in prokaryotic cells and their orientation in membranes; (*iii*) to investigate the behavior of hopanoids and of the various membrane "bracers" on different types of artificial membranes of phospholipids, in particular those containing branched chains; and (*iv*) to study the intracellular localization of hopanoids in the only eukaryotes found regularly to contain 3-deoxyhopanoids along with sterols—the ferns (36, 37), together with a few bryophytes (38, 39), lichens (40), and fungi (41, 42). Are their hopanoids perhaps localized in some of their organelles, like chloroplasts or mitochondria? If so, why have they not, like the corresponding organelles of other eukaryotes, "lost" their ability to synthesize hopanoids?

In the study of all these problems, a perception of the potential significance of hopanoids and of other membrane reinforcers might provide useful clues.

After acceptance of this paper, we were made aware of the similarity of some of the above ideas with earlier proposals by Nes (43), in particular in regard to the structural equivalence of tetrahymanol and sterols. One of us (G.O.) had attended a lecture on this topic by W. R. Nes and, aware of the possibility of cryptomnesia, acknowledges that this may have led to his initial interest in the subject. However, the views presented in both papers are so fundamentally divergent that any plagiarism is excluded.

The ideas developed here, in response to a challenge proposed by Mrs. M. C. Dillenseger, have gained their initial momentum from discussions with Professors Konrad Bloch and Roger Y. Stanier at various stages of the evolution of this manuscript. Our work has been subsidized by grants from the Centre National de la Recherche Scientifique, Université Louis Pasteur, Hoffmann-La Roche (Basel, Switzerland), Roure-Bertrand (Grasse, France), and Rhône-Poulenc (Paris).

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