The terpenoid theory of the origin of cellular life: the evolution of terpenoids to cholesterol

Terpenoids have an apparently essential function in modern cellular membranes, reinforcing them against shear stresses. Primitive membranes could initially have been formed by simple terpenoids, and vesicles formed from these membranes may have evolved into progressively more complex units, more and more similar to protocells.

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Life, as we know it, is cellular, and a physical boundary separates the living organism from the outside world. Yet most discussions on the molecular origins of life center solely around questions such as how nucleotides, amino acids and sugars were formed, on Earth or in interstellar space [1], how proteins, and in particular enzymes originated and how nucleic acids arose and became able to self-replicate. Here we will consider a fourth question, rarely debated (but see [2] for a perceptive exception): how did membranes originate?

Understanding the origin of membranes is central to an understanding of the origin of life. Life may, perhaps, have begun as a two-dimensional system limited by a mineral surface [3,4], but, in a three-dimensional system like water, segregation of a separate compartment is achieved by a closed lipidic membrane or vesicle, which is essentially impermeable to water. The vesicle thus defines what is inside as the living organism, what is around as the membrane, and what is outside as the rest of the world (Fig. 1). This review will focus on the prebiotic formation of membrane-bound protocells, and the molecular evolution of the lipidic part of these membranes. We shall show that it is likely that terpenoids, a varied set of molecules characterized by structures formed out of branched C_5 prenyl units, were uniquely important in the formation of closed vesicles and hence membranes. Later, although the terpenoids developed many other roles (insect development hormones, plant growth hormones, and mammalian sex hormones are all terpenoids, as is the anti-cancer drug taxol), they remained essential to reinforce the membranes of all living organisms. Here we will describe how the terpenoids probably evolved to fulfill this reinforcement function. Finally, we shall show how the self-association of identical terpenoid amphiphilic molecules into vesicles could have automatically resulted in much more complex molecular systems.

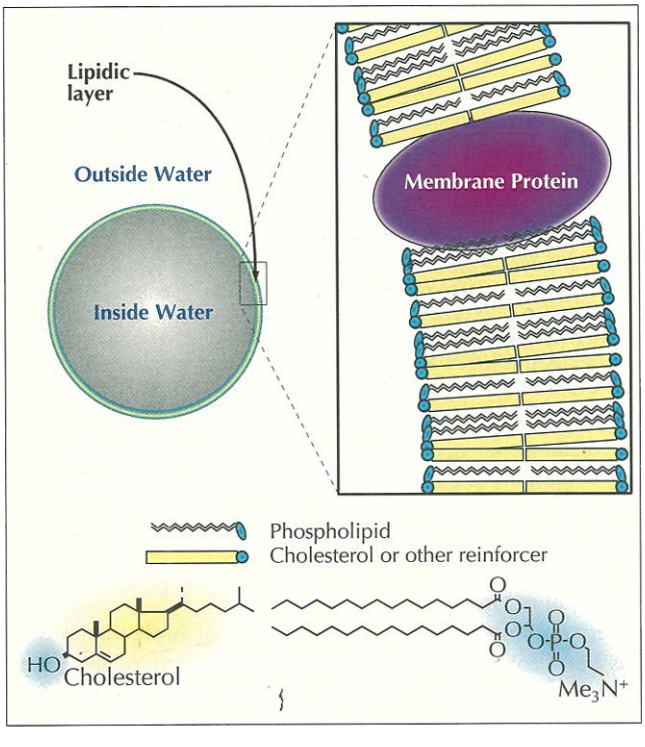


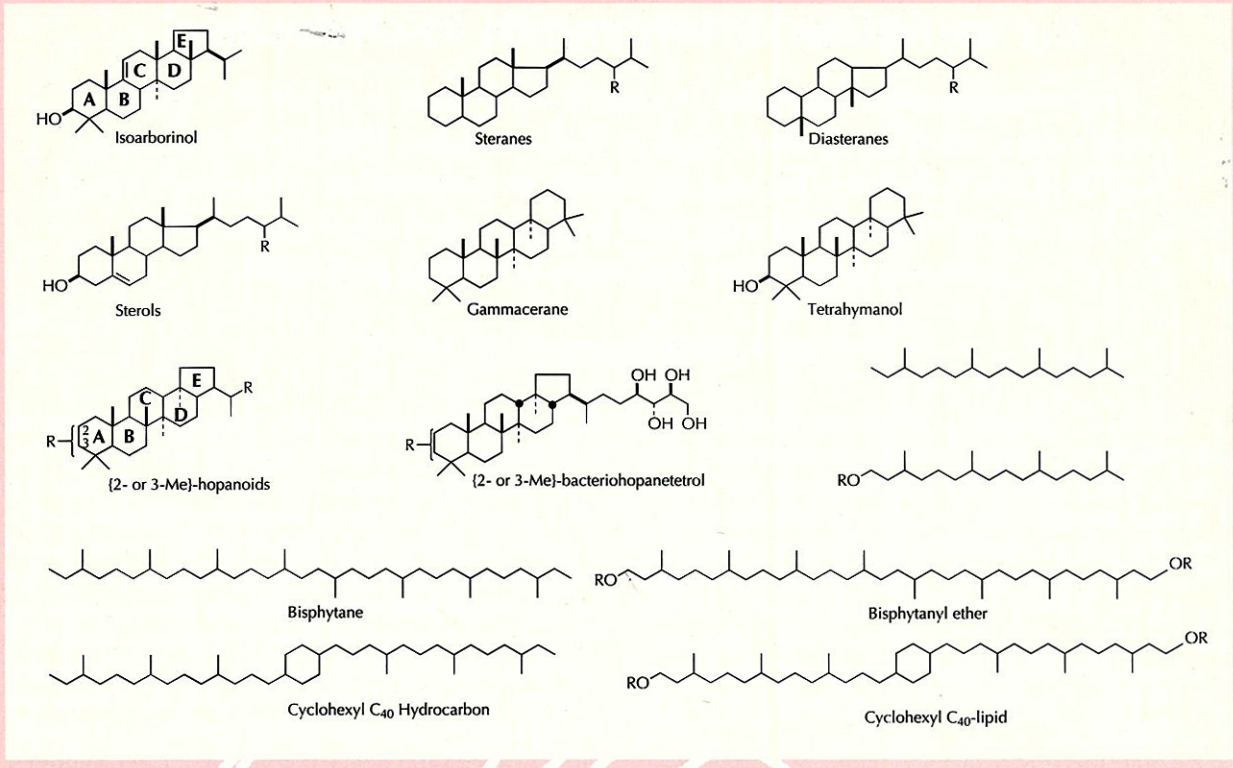
Fig. 1. Membranes separate 'inside' from 'outside'. Eukaryotic membranes usually consist of a bilayer of diacyl phospholipids and cholesterol. Amphipathic proteins are also present in the bilayer.

The cellular membrane

Biochemistry text-books describe membranes as being formed by the self-assembly of amphipathic di-acyl phospholipids, in which membrane proteins 'float' (Fig. 1). Some books mention that such membranes contain large amounts of cholesterol, but very few note that this is true only of eukaryotes, not bacteria. Bacterial membranes rarely contain cholesterol, but often contain hopanoids instead [5]. Also, archaebacterial membranes lack conventional *n*-acyl lipids and instead consist of highly branched, polyprenic diphytanylglyceryl phospholipids (Fig. 2) and other similar polyterpenyl ethers, with a wide variety of polar heads [6]. Given the wide range of constituents that are present in modern membranes, how can we discover what the first membranes were made of?

One way of deducing the origins of membrane constituents is to examine how they are built up. The biosynthesis of cholesterol, for example, requires many steps, all of course enzymatically controlled, and some having no

The terpenoid



The structures shown here have either been found in sediments of different ages or are the predicted precursors of sedimentary molecular fossils. Some are also found in extant organisms. For example, isoarborinol, isolated 25 years ago from a shale [11], is a known constituent of some higher plants, and its presence in shale could have meant that plants of the same families lived in that particular palaeoenvironment. More recently, however, isoarborinol has been found in many diverse sediments, and it now seems more likely that it also occurs in an unidentified microorganism; this microorganism must be aerobic, as the biosynthesis of isoarborinol requires oxygen [12].

Steranes and the rearranged diasteranes are frequently found in sediments. They probably arose in the sediment by maturation from sterols (and 4α-methyl sterols), which are ubiquitous components of eukaryotic cell membranes (or, in the case of the 4α-methyl sterols, of methylotrophic bacteria [13]). Their maturation from sterols can indeed be simulated by heating cholestene in clay [14]. It is possible, but unlikely, that diasteranes derive from diasterols, which are still unknown, but biogenetically plausible.

Gammacerane has been found in several sediments. Its obvious precursor is tetrahymanol, a membrane constituent of some ciliates [15]. The pentacyclic triterpene skeleton of hopanoids, and of their 2- and 3-methyl derivatives, has been found in more than 250 compounds isolated in petroleums, shales and other sediments [7]. These are the so-called geohopanoids, which were orphan lipids when they were first discovered. They are probably the most important molecular fossils, and it has now been shown that they are ubiquitously present in all sediments [7]. Their precursors, the novel class of biohopanoids, are frequently found in bacteria [5]. Similarly, the 2- and the 3-methyl hopanoids were first found in a few sediments, postulated to come from still unknown methyl-biohopanoids, and isolated soon later in some bacteria [5]. For a short time the C_{40} hydrocarbons such as bisphytane, which were isolated from several sediments, were also orphan lipids, [16]. Their significance was a puzzle until the first bisphytanyl-phospholipids were found in thermophilic archaea [6], and later, also intact, in a young sediment [17].

The precursors of some other orphan lipids are still unknown. The least problematic one, a cyclohexyl C_{40} hydrocarbon, is obviously derived from bisphytane by a C-C cyclization [18], the second C-C bonding being just a repeat of the central Me-Me one.

close analogy in abiotic chemistry. The phospholipids of eukaryotes require the separate biosyntheses of the *n*-acyl chains, of glycerol, and of the phosphorus-containing head-groups, and their combination. Since modern membrane constituents (cholesterol, hopanoids, phospholipids, and so on) could not have been formed without multiple specific enzymatic systems, we must look elsewhere for the earliest membrane constituents.

The fossil record

Over the past 25 years a large variety of molecular fossils has been found by studying the constituents of sedimentary organic matter. The structures of many of these fossils are now precisely known, making it possible, given an understanding of biosynthetic processes and the maturation mechanisms that occur in the sediments, to deduce the probable structures of their 'living' precursors. Many

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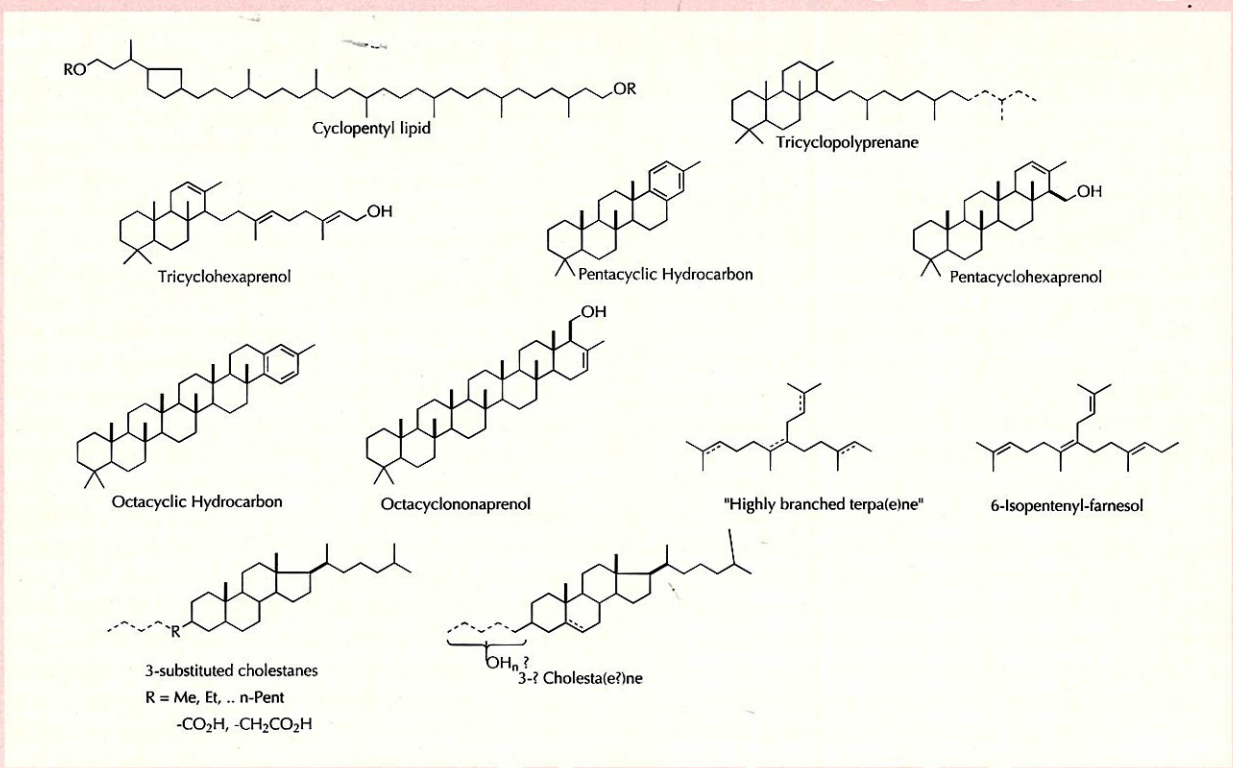

It is related to the formation, in many thermophilic archaea grown at the highest temperatures they tolerate (above 100 °C!), of cyclopentane rings (for example, the cyclopentyl lipid shown) along the chain by C-C bond formation between methyl and methylene groups [6]. We trust that our prediction that some archaeal species will be found to contain phospholipids with this novel cyclohexyl chain will be vindicated.

A similar prediction concerns the orphan tricyclopolyprenanes found in many sediments [19,20]. Their structures have been unambiguously identified, at least up to that of tricyclohexaprenane, and derive obviously from one of the stereomeric tricyclohexaprenols. Several isomers have been synthesized [21–23]; they have however not yet been found in nature, but could be present in some anaerobic bacteria. Tricyclopolyprenanes are in fact the first members of an extraordinary series of sedimentary polycyclopolyprenane derivatives (the examples shown here are a pentacyclic hydrocarbon, pentacyclohexaprenol, an octacyclic hydrocarbon and octacyclononaprenol), isolated as monoaromatic hydrocarbons [24]. Their precursors are obviously the corresponding polycyclopolyprenols, formed by cyclization of linear polyprenols (for instance, of the C_{45} solanesol), and eventually aromatized in the sediments in their terminal ring with loss of two carbon atoms. They are optically active, which demonstrates that their precursors were products of enzymatic syntheses (this information is still lacking for the tricyclopolyprenanes).

Another case is that of the highly branched terpenoids (such as 6-isopentenyl-farnesol), saturated hydrocarbons and their polyunsaturated precursors, isolated from many sediments [25] .They can reasonably be derived only from the corresponding polyprenylated polyprenols, known so far neither from sediments nor from microorganisms.

A final, very intriguing, case is that of the 3-substituted cholestane derivatives (carboxylic acids [26] or hydrocarbons [27]) which have recently been found in several sediments, up to 3-pentyl-cholestane. The striking analogy with the existence of the C_5 side chain in bacteriohopane derivatives suggests that a novel family of cholesterol derivatives may exist that carry a C_5 chain at C-3, and may be unsaturated at C-5/C-6. In this case, unlike all others so far, we can propose no simple biosynthetic mechanism for their derivation.

classes of natural products are represented in these sediments, but those that derive from lipids are the most abundant, because they are resistant to degradation [7,8]. Of course, there are many other classes of abundant, recognizable and informative molecular fossils; the sedimentary porphyrins are a particularly important example [9]. The molecular fossils that are prominent in extracts of sediments can often be related to microbial precursors. This does not imply that bacteria are always the only contributors to sedimentary organic matter, even though dead organisms are normally promptly processed by bacteria; other microorganisms, in particular microplankton, are also important.

Although these sedimentary molecular fossils are varied and complex, their value is limited by the simple fact that

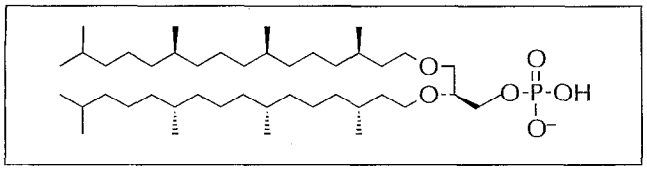

Fig. 2. A diphytanylglyceryl phospholipid. These phospholipids are found only in archaebacteria, and differ from eukaryotic lipids in two important ways. First, the lipid portion is highly branched, coming from the terpenoid family. Second, the head group is very variable.

they are not old enough. The ideal molecular fossil for our purposes would be at least 3 000 million years old. It is probably unreasonable to hope for such extreme chemical stability; on top of this, for us to find such fossils the sediments in which they reside would need to be uncontaminated by leaching from younger sediments. Studies of the constituents of very old sediments have not so far led to the discovery of derivatives of obviously primitive lipids, no longer extant, and there is no reason to believe that any relatively young sediment will contain molecular fossils derived from precursor organisms now extinct. On the contrary, we assume that the fossil record is only an overview of metabolites of organisms still present today. In some cases, these organisms are obviously plants; in most, they are certainly microorganisms, bacteria, protozoa, planktonic algae, and so on, representing the usual scavengers of larger dead organisms. Not all of the precursors of the molecules found in the fossil record have been identified in present-day organisms, however. There is no systematic approach to identifying such molecules in modern species, just patience and luck [10]. The available data on the molecular fossils found to date are summarized in the box on the previous two pages. In our view, although these molecules form the fossil record, they are at least as valuable for what they teach us of the range of membrane constituents that are present in extant organisms as for the information they give us on the membrane composition of older organisms.

Terpenoids are essential

No terpenoid-free cellular membrane is known, suggesting that terpenoids are important for membrane function. Let us first consider the effect of cholesterol on eukaryotic phospholipid membranes; it is incorporated in the bilayer, its OH group forms a hydrogen bond with the headgroup of a phospholipid, and its dimensions allow cooperative van der Waals attractive forces to elongate and order the lipidic chains [28]. This reinforcement of membrane architecture has been shown by a wide variety of physical methods, many of which we have used to characterize the various cholesterol surrogates discussed below.

What happens in cells devoid of cholesterol? This was first studied with a eukaryote, the ciliate *Tetrahymena pyriformis*. When exogenous sterols are absent, this organism synthesizes the simple quasi-hopanoid, tetrahymanol [29]. Tetrahymanol (also present in the lipids of other microorganisms) shares with cholesterol its amphiphilic character, its approximate dimensions and its rigidity; it is a structural analog, and also a functional equivalent. The biosynthesis of tetrahymanol in *T. pyriformis* is suppressed by cholesterol and some other polyterpenoids [30]; the same is true in the anaerobic ciliate *Trimyema compressum* [31]. Similarly, when the mycoplasm *Acholeplasma* is grown *in vitro* in the absence of exogenous cholesterol it produces carotenols, polar carotenoids which are incorporated in its membranes [32]. This is the only mycoplasm known to be able to survive without cholesterol. Other highly polar carotenoids (sulfates of ω,ω′-trihydroxylated β-carotene ketones) are present in photosynthetic bacteria but do not function as light-harvesting pigments [33], and we have postulated that at least some of the characteristic α,ω-dipolar carotenoids so often present in bacteria could be incorporated perpendicularly to the membrane, and be cholesterol surrogates as rivet-like reinforcers [34]. Early models for carotenoid insertion into membranes proposed that the molecules are inserted into only one leaflet of the bilayer, which is inconsistent with the *trans* configurations of the double bonds [35,36]. We have examined the

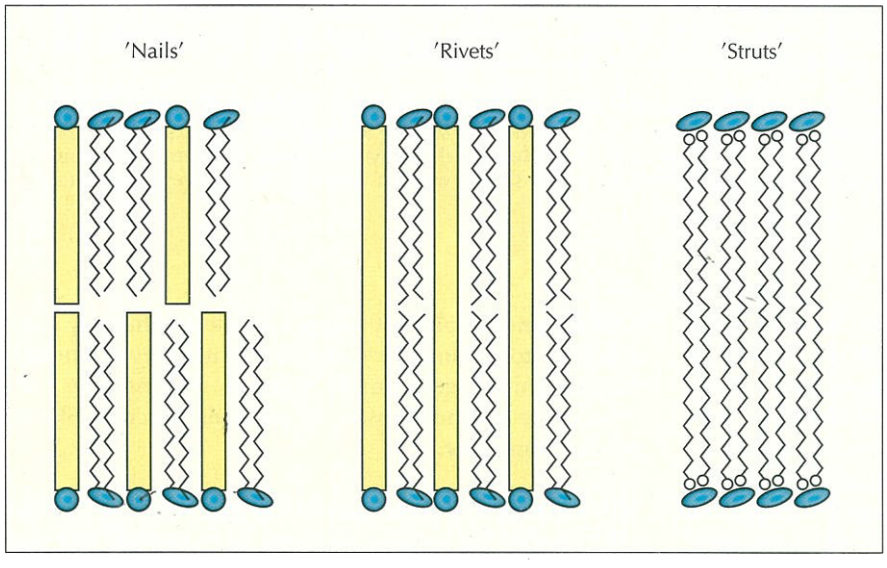

Fig. 3. Different methods for membrane reinforcement. In most eukaryotic membranes, sterols such as cholesterol and other compounds of similar dimensions (such as hopanoids) act as 'nails', inserting into only one half of the lipid bilayer. Carotenoids cross both halves of the bilayer, however, like 'rivets'. Some archeal lipids, from thermophilic bacteria, form both halves of the bilayer themselves, and because they are kept taut by the fact that the heads are hydrophilic, these membranes are automatically reinforced, as if by 'struts'.

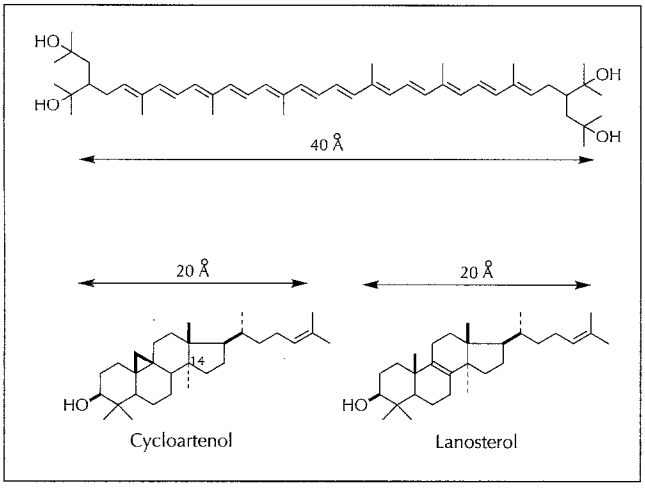

Fig. 4. Top, α,ω-dipolar carotenoid. This molecule presumably acts as a 'rivet' (see Fig. 3) since it is long enough to span both halves of the membrane. Bottom, cycloartenol, which does act as a membrane reinforcer, and its isomer lanosterol, which does not. The reasons for this difference will be discussed later in the review.

insertion of a variety of carotenoids into membranes by a variety of methods; the reinforcement is, as expected, optimal when the length of the lipidic chain of the carotenoid fits the thickness of the phospholipid bilayer [37] (Fig. 3). Thus, the carotenoids appear to span the entire width of the bilayer. It therefore seems likely that all membranes require reinforcement of one kind or another, from a terpene or a molecule of similar shape and general characteristics.

In archaea, the membrane itself is formed by a double layer of the di-phytanyl ethers, or by a double-face monolayer of double thickness, formed by the di-bisphytanyl ethers of thermophiles [38]. These peculiar lipids, which have two polar heads separated by a hydrophobic portion of about 40 Å, form membranes that are more stable than those made from ordinary lipids and do not appear to need further reinforcement. In a sense, these membranes behave as if they are already reinforced by 'struts', because their transmembrane chains are kept taut by the fact that their polar heads are held in the aqueous compartments; they thus become surrogates for rigid additives like cholesterol.

Several of the amphiphilic substances mentioned above can be shown to have a cholesterol-like reinforcing effect on artificial phospholipid membranes, for example bacteriohopanetetrol and other biohopanoids [39], α,ω-dipolar carotenoids, in particular bacterioruberins in *Halobacterium* membranes [37], and tricyclohexaprenol [40,41]. This effect is very sensitive to structural details; although cycloartenol, the biosynthetic precursor of cholesterol in plants, does reinforce phospholipid membranes, its closely related isomer lanosterol, the precursor of cholesterol in animals and fungi, does not [42] (Fig. 4). The observations made so far led us to propose a general structural rule: "an amphipathic substance whose lipophilic part is about 20 x 6 x 6 Å, or which carries two polar groups separated by a lipophilic segment about 40 x 6 x 6 Å large, and which is either partially rigid, or can be rendered rigid if its hydrophilic head-groups are blocked in the opposite water compartments, could be a membrane constituent or membrane reinforcer" [43]. We predict, therefore, that the parents of all the orphan lipids described above and in the box that meet these dimensional criteria were once part of the membranes of still unknown organisms. Even the extended series of polycyclopolyprenols (see box), which must have been the precursors of the corresponding monoaromatic hydrocarbons found in sediments, have the correct dimensions to stabilize a membrane. In Fig. 5, the dimensions of tricyclohexaprenol and octacyclononaprenol are compared with the dimensions of common lipids; clearly, these polycyclopolyprenols fit our criteria for membrane reinforcers. The hypothesis that they stabilize membranes has not yet been tested, however, as they have not yet been isolated or synthesized.

Although other important polyterpenic cell components, such as dolichols and ubiquinones, chlorophyll and retinal (in the rhodopsins), are also associated with membranes, it is unlikely that these components have a significant structural role, as they are present in the membrane in very small amounts, and they are essential for other important functions. The same is true of lipids that anchor proteins to the membrane, such as a polyprenyl (C_{15} or C_{20}) chain (or, in other cases, an *n*-acyl chain such as a myristoyl group). The important role of the lipid in these cases is certainly to localize the protein near the membrane, rather than to have a mechanical effect on the membrane. Having examined the structural characteristics that make terpenoids important to the function of membranes, we are now in a position to discuss their evolution.

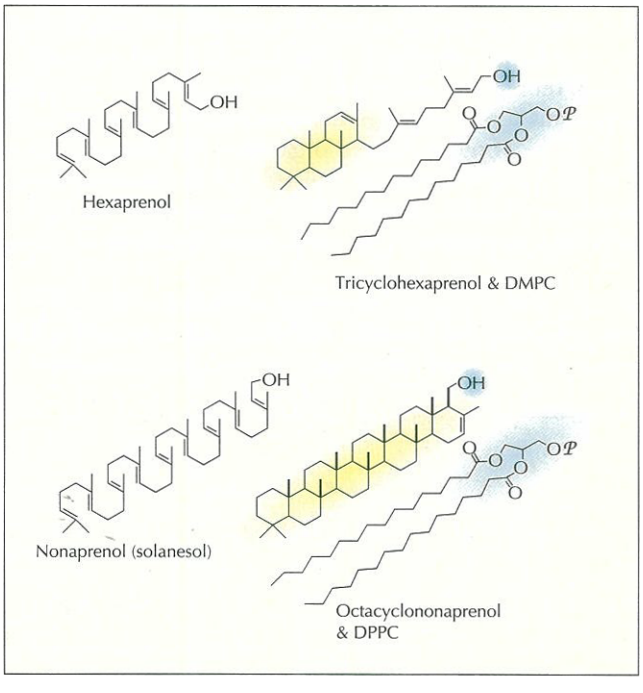

Fig. 5. Overall dimensions of polycycloprenols in comparison with two common membrane lipids, dimyristoylphosphatidylcholine (DMPC) and dipalmitylphosphatidylcholine (DPPC). *P*, polar head.

The evolution of membrane terpenoids: general principles

Discussing the evolution of a family of substances that has always had an essential function amounts to discussing how the members of this family are arranged in order of primitive character. Florkin [44] was the first to express the principle that was later enunciated by Granick [45] as "Biosynthesis recapitulates Biogenesis", the molecular equivalent of Haeckel's Rule that "Ontogeny recapitulates Phylogeny". When two biosynthetic pathways achieve the same function, the one that requires the fewer and the simpler enzymatic steps is expected to be the more primitive of the two. By a 'simple' enzymatic system we mean one that accelerates a reaction along its spontaneous course, in other words, the course that would be followed without an enzyme. The spontaneous reaction is typically slower and lacks enantioselectivity, but has identical regio- and diastereo-selectivities. For instance, any (enzyme-mediated) electrophilic addition to C=C bonds following Markovnikof's Rule (Fig. 6, lower scheme) would be more 'primitive' than a pathway that forces the addition to run against the constraints of electronic factors (Fig. 6, upper scheme). Similarly, the enzyme-catalyzed cyclization of a 1,5-diene that proceeds by a chair-like transition state (Fig. 7, upper scheme) is more 'primitive' than one that proceeds by a more energy-demanding boat-like pathway (Fig. 7, lower scheme). In both cases, the selectivity of the non-enzymatic reactions is attributable to intrinsic activation energy differences, which can only be overcome by enzymatic constraints on the entropy of the system.

Another common trend is that abiotic reactions can use a variety of substrates with a given structural unit (for instance a C=C double-bond, or an amino group); this is the basis of the functional group approach which is so useful in organic chemistry. Enzymatic reactions are usually much more substrate-specific, but enzymes of primitive organisms are sometimes demonstrably more permissive than those of more evolved organisms. We shall see below that the cyclases acting on squalene or on epoxysqualene, which are highly specific for the 3S-epoxide in higher organisms, are widely tolerant in microorganisms.

Finally, any pathway involving molecular dioxygen is less primitive than a similar one that leads to products that achieve the same function by anaerobic processes.

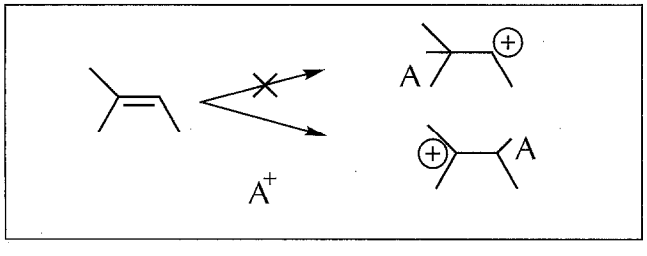

Fig. 6. The preferred mode of electrophilic addition to carbon–carbon double bonds follows Markovnikof's Rule to give the more substituted and more stable carbenium ion (lower pathway). The upper pathway, giving the less substituted and less stable carbenium ion, requires more energy.

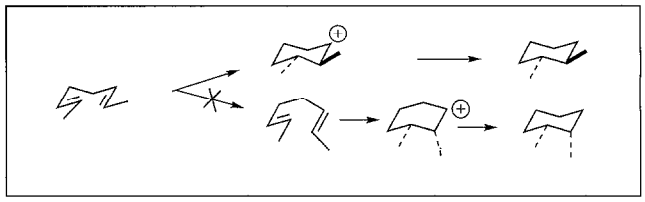

Fig. 7. The preferred mode of cyclization of a 1,5 diene proceeds via a chair-like transition state (upper scheme), not via a boat (lower scheme).

Dioxygen was very scarce in the atmosphere until after the development of photosynthetic organisms, which was probably as early as ~3.5 x 10^9 years ago [46].

According to these criteria, the eukaryotic membrane components are all quite modern. As noted above, biosynthesis of the *n*-acylphospholipids and cholesterol requires many diverse steps. The synthesis of cholesterol in particular requires many highly tuned enzymatic reactions, including the requirement for dioxygen to obtain epoxysqualene, anti-Markovnikof additions, boat-like cyclizations and rearrangements to cycloartenol or lanosterol, and oxidative degradations. The synthesis of the *n*-acylphospholipids also requires a number of enzymatic steps which cannot be simulated in the chemical laboratory, and even their postulated abiotic synthesis [4] is not free of such problems.

We were led by the above considerations to postulate that primitive membranes could have been more readily formed from the simplest possible terpenoids, the acyclic polyprenols, linked to an appropriate and simple polar head-group like a phosphate anion. Once we had recognized the essential role of terpenoids in membranes, we proposed that sodium di-polyprenyl phosphates might form membranes; we synthesized them, and showed that they do have the expected properties [47]. We had not, however, expected them to be very efficient vesicle builders, but to our surprise we found that even the simple sodium di-geranyl phosphate, with C_{10} chains, forms regular vesicles. Sodium di-farnesyl phosphate, its higher isoprenolog the di-geranylgeranyl phosphate, and phosphates with mixed chains, such as C_{10}/C_{15} or C_{15}/C_{20}, also form vesicles, whereas the smaller C_5 phosphates are soluble in water [V. Birault, G. Pozzi, Y.N. and G.O., unpublished data]. We therefore propose that the most primitive membrane-building units were the polyprenyl phosphates. Here we show how the polyterpenoids appear to form a phylogenetic series, starting with these simple terpenes and successively recruiting novel enzymatic steps to produce the various structures involved in the reinforcement of modern membranes.

The formation of polyprenols

The abiotic formation of polyprenyl phosphates requires the formation of the polyprenols, followed by their phosphorylation. Going backwards, the synthesis of the polyprenols requires only the recurrent acid-catalyzed

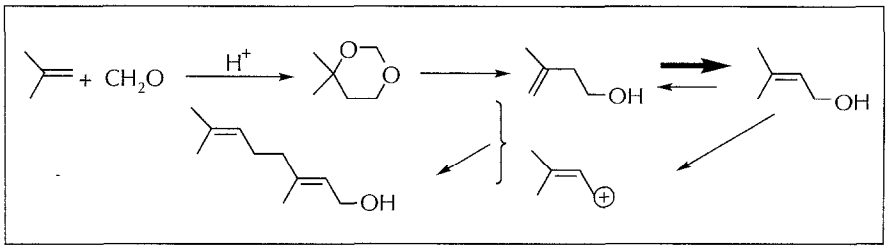

Fig. 8. Formation of C_5 units (isopentenol) from formaldehyde and isobutene. Once formed, two C_5 units can react to form a C_{10} unit (geraniol), and this sequential acid-catalysed addition of C_5 units forms the polyprenols.

condensation of the C_5 units of isopentenol by a reaction of the general type:

$$C{=}C + C^+ \longrightarrow C{-}C{-}C^+ \qquad \text{Scheme A}$$

These C_5 units can themselves be derived from simpler precursors, for instance from formaldehyde and isobutene (via an acid-catalysed Prins reaction; Fig. 8). All these reactions are simple, and their regulation could be quite easy, as the head-to-tail elongation proceeds by C_5 increments. There are several possible side reactions, however, such as cyclizations, isomerizations, double-bond migrations and rearrangements, and it is possible that these were avoided by performing the reaction on a surface. In fact, solid acidic catalysts are used in some industrial syntheses of isopentenol.

In contemporary organisms, this stepwise condensation of C_5 units is achieved by specific prenyl-transferases. It is now known that the C_5 isoprenic unit is made by at least two distinct biosynthetic pathways, in animals, plants or fungi [48], and in bacteria [49].

The polyprenyl phosphates

The phosphorylation of the polyprenols may have initially involved phosphorus pentoxide, produced by volcanoes, or polyphosphates (especially cyclo-triphosphate), which

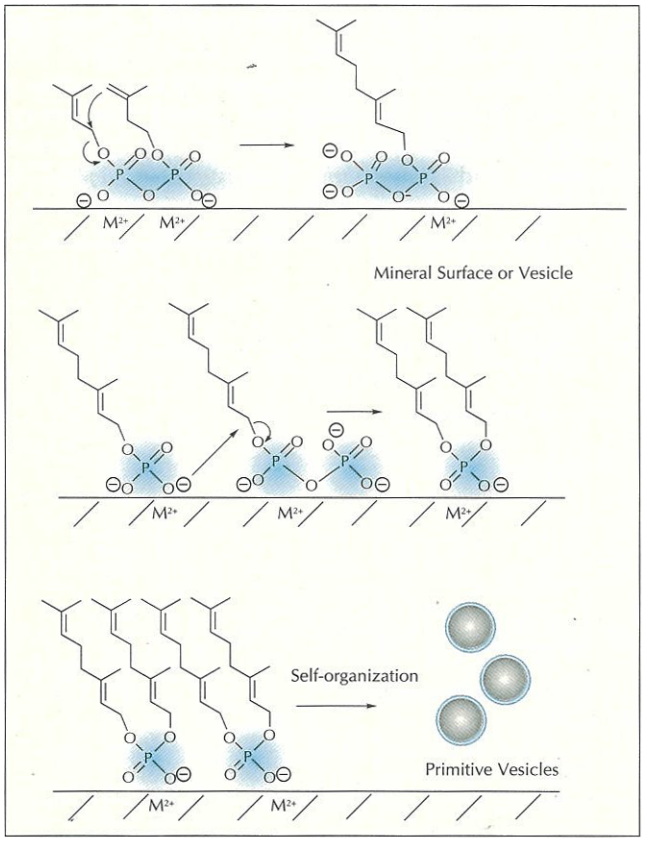

Fig. 9. Possible prebiotic synthesis of polyprenol phosphates on a mineral surface, forming primitive vesicles. Prenyl phosphate units are attached to the mineral surface by electrostatic forces. Prenyl groups condense in much the same way as shown in Fig. 8 until a critical concentration has been reached and they become able to form vesicles.

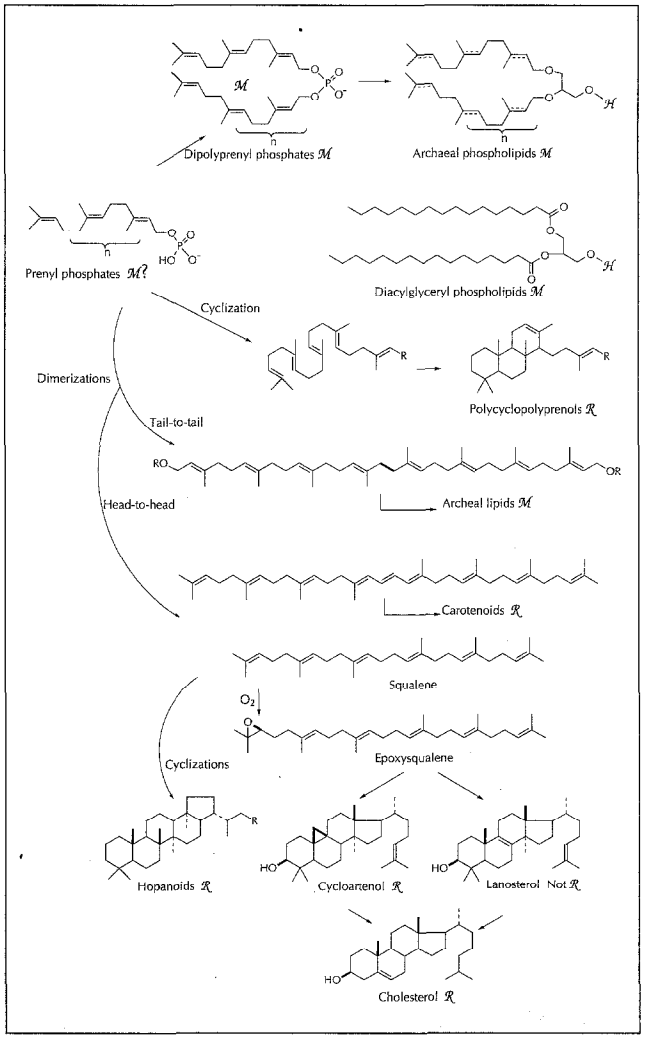

Fig. 10. Hypothetical evolution of membrane components and membrane reinforcers. The scheme presents a proposal for how several modern-day membrane components arose from prenyl phosphates (see text for details). Diacylglyceryl phospholipids are shown for comparison. $\mathcal{H}$, polar head; $\mathcal{M}$, membrane component; $\mathcal{R}$, reinforcer.

were present in the environment and able to phosphorylate alcohols even in an aqueous solution [50–53]. It is probably not a coincidence, but a reminiscence, that the modern biosynthesis of polyprenols involves phosphates or pyrophosphates at all stages. The importance of the properties of phosphates has been convincingly explained by Westheimer [54]. A plausible scheme for the phosphorylation and chain elongation of polyprenols is illustrated in Fig. 9, which shows a plausible scenario for the synthesis of these phosphates, on specific surfaces, with progressively longer chains — by C_5 increments — until they would peel off the surface to form vesicles, once critical length and concentration have been achieved. Phase separation would lead to vesicles made up of phosphates with polyprenyl chains of uniform lengths. This type of synthetic scheme has not yet been reproduced in the laboratory, however.

So far, we have only provided experimental evidence for vesicle formation from sodium di(polyprenyl) phosphates. It is likely, however, that the simpler sodium monopolyprenyl phosphates, or the corresponding pyrophosphates, could also form vesicles, perhaps when combined with suitable amounts of the free polyprenols: in much the same way, *n*-acyl carboxylates give vesicles when mixed with equimolar amounts of *n*-alcohols of the same chain length [55].

The phospholipidic head-groups

The next stage of elaboration from the polyprenyl phosphates could have been to change the head-group. Archeal membrane phospholipids, like eukaryotic phospholipids, can use glycerol as the head group, but archeal lipids can also use other complex alcohols, and can be modified by further conjugation with carbohydrates. Although such lipids often retain a phosphate group, the unsubstituted glycerol ethers are also often found [56]. There are several plausible ways in which the di-polyprenyl phosphates might have been converted, via the isomeric di-polyprenyl 1-glyceryl phosphates, to the corresponding ethers of glycerophosphoric acid, providing a transition to the types of lipids found in archaea (Fig. 10); these scenarios will have to be tested experimentally.

The hydrogenated lipidic chains

Most of the archaeal phospholipids isolated so far have been further modified by the reduction of the double bonds of the chains. This is a secondary reaction, as shown by the isolation of free geranylgeraniol, and that of squalene and its dihydro and tetrahydro derivatives, from halophilic [57], methanogenic or thermophilic archaea [58,59]. We predicted that non-hydrogenated phospholipids based for instance on geranylgeraniol, not on phytanol, would be found in some archaea [60], and this is indeed the case [61].

The head-to-head dimers

Another enzymatic reaction must be recruited to lead to the head-to-head duplication of the chains, and the formation of the cyclopentane ring along these chains, that is characteristic of many thermophilic archaea. The duplication leads to an ω/ω′ C-C bond between two methyl groups, and the cyclopentane ring formation to a C-C bond between a methyl and a methylene group. Analogous reactions are known in the anaerobes *Thermotoga* and *Butyrivibrio*, leading to (ω-1)/(ω′-1)

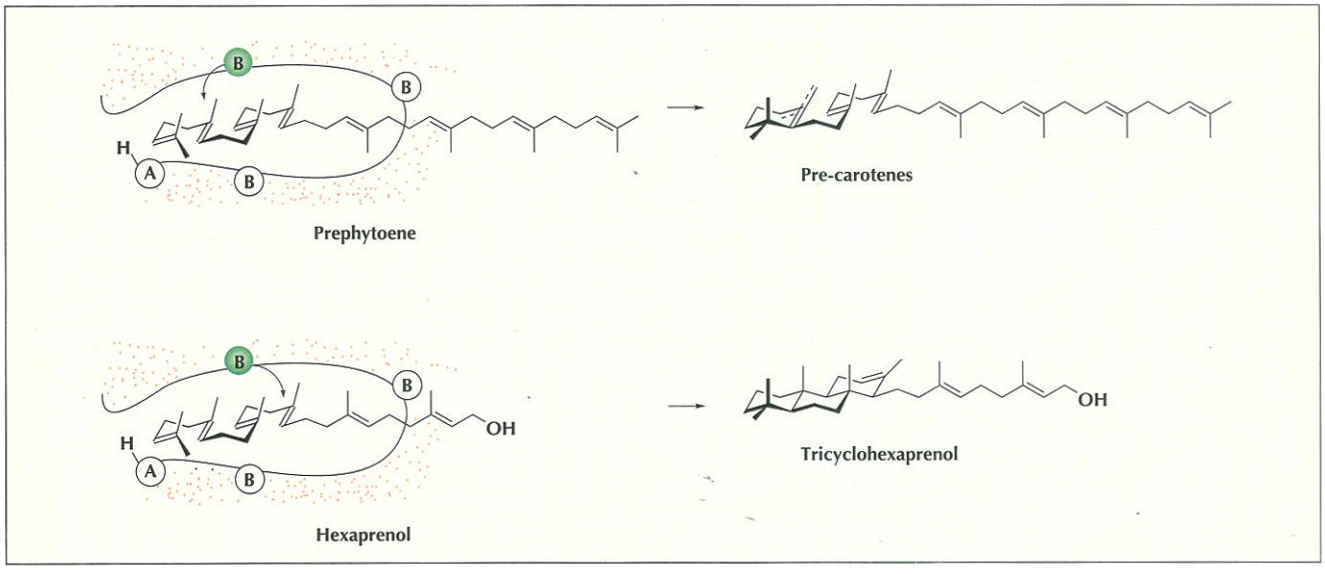

Fig. 11. Hypothetical enzymatic cyclization of prephytoene and hexaprenol. The reaction starts by addition of a proton from the acidic site A-H and is terminated by the removal of a proton by the nucleophilic site B at the other end. The positions of the two sites differ only slightly in the two cases. Pink shading designates the active site of the enzyme, which determines the conformation of the chain of the substrate. Mutations in the amino acid sequence of the enzyme may change the shape of the active site, and therefore change the reaction that is catalysed by the enzyme. The reaction steps involved are all of the type shown in scheme A, and follow Markovnikof's Rule (see Fig. 6); they involve only chair-like transition states (see Fig. 7, upper scheme). These reactions can be simulated in solution using superacids. A, acid; B, nucleophile or base. The green shading designates an active nucleophile; the others are inactive.

dimers, the phospholipids based on glyceryl esters of the diabolic acids [62]. Also, in other common bacteria, in particular some thermophilic ones, the ω-cyclohexyl or ω-cycloheptyl acyl chains are obviously formed similarly by an intramolecular methyl–methylene coupling [63]. So far, nothing has been published about the biochemistry of these very interesting reactions, for which there is no abiotic analogy.

Squalene, a tail-to-tail dimer; a prebiomimetic synthesis of squalene

The next enzymatic system enrolled leads to the tail-to-tail linking of the polyprenols, to yield squalene from farnesol (as the pyrophosphate as usual), or prephytoene from geranyl-geraniol pyrophosphate [64,65]. We have mentioned above that squalene is present in several archaea; dehydrosqualene is also found in *Staphylococcus*

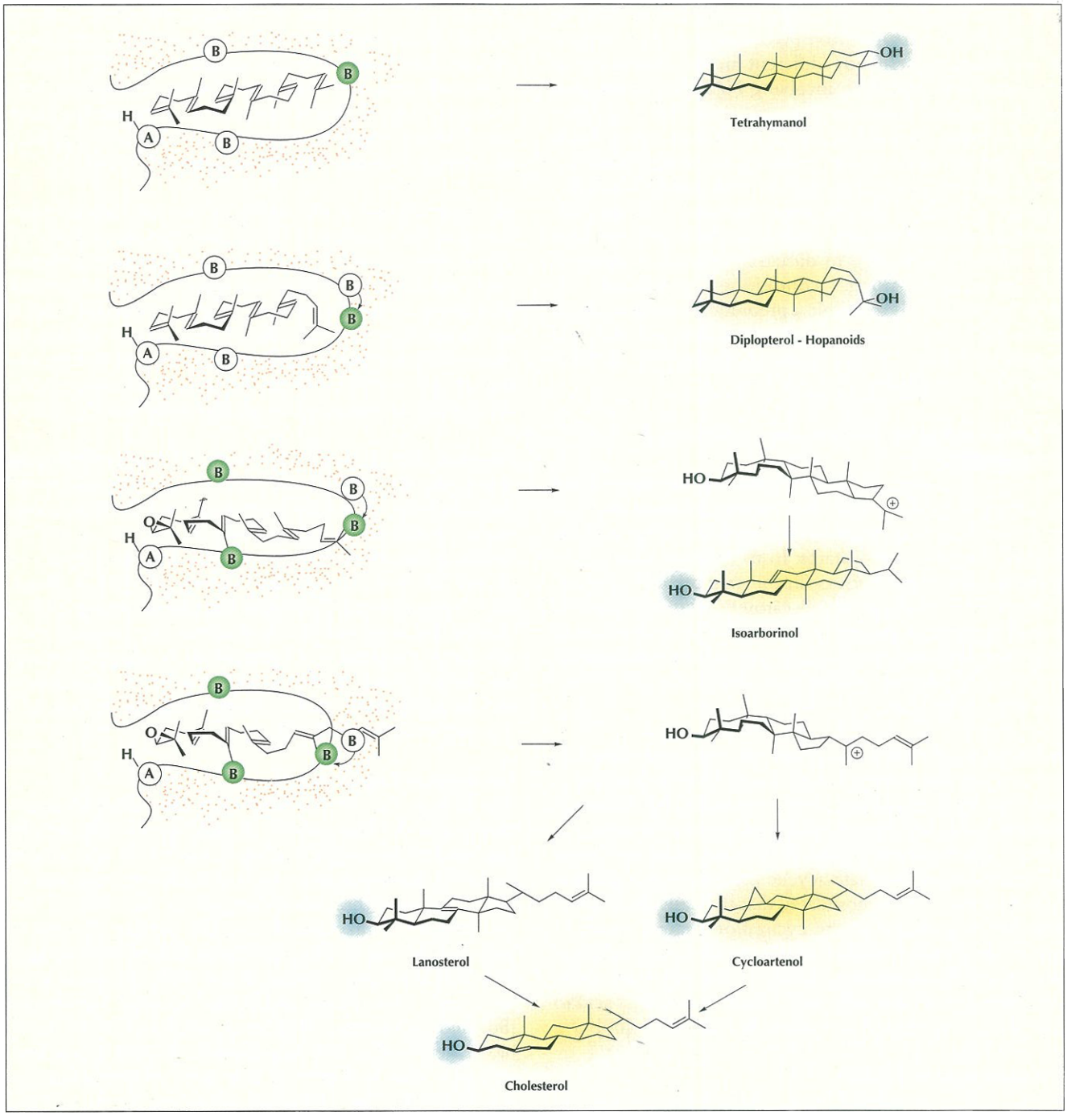

Fig. 12. The squalene/squalene epoxide cyclase family could have evolved by small shifts in the positions of the reactive bases (shaded green) in the active site of the protein, or by successive activation of one base by another (by mutations in the amino acid sequence). These changes, combined with different topological arrangements of the polyprenyl substrate, can give rise to a number of different cyclic products, some of which require anti-Markovnikof addition reactions (see Fig. 6) and/or boat-like transition states (see Fig. 7). These reactions cannot be simulated in solution using superacids, unlike the reactions shown in Fig. 11. Blue shading, polar head groups. Yellow, rigid reinforcer. A-H, acidic site. B, inactive or displaced nucleophilic or basic site. Shaded B, active nucleophilic or basic site.

aureus (C_{30} prephytoene, the precursor of C_{30} carotenoids) [66]. In other bacteria and in *Halobacterium*, prephytoene must be present as a precursor of carotenoids. The formation of this head-to-head linkage has no direct counterpart in *in vitro* chemistry, even though it can be regarded, from an electronic point of view, as akin to the Würtz alkane synthesis by sodium reduction of alkyl halides, or its variants involving Grignard reagents [67] or to the couplings of allylic alcohols with low-valent titanium [68], reactions which can be used in syntheses of squalene but which are very far from prebiomimetic. The biosynthetic reaction is slightly different in the formation of squalene (with reduction involving NADPH) and in that of prephytoene (without reduction), but the two enzymatic systems involved appear to be related; not only do they perform very similar reactions by similar (and unusual) pathways, but they are also not tightly substrate-specific, as each of the enzymatic systems can accept the alternate substrate, though less efficiently. Indeed, we have found that it is possible to dimerize farnesol to squalene by reduction using conditions postulated by Wächtershäuser [4] to mimic a prebiotic world, namely, an anaerobic medium containing Fe(II) sulfide and H_2S, and producing pyrite (D. Hasenbratl, M. Keller, Y.N., G. Teller and G.O., unpublished data).

The fully conjugated polyenes, and cyclizations of polyprenols

From prephytoene, dehydrogenation enzymes are needed to obtain the carotenoids. But another reaction that can often occur during carotenoid biosynthesis is the cyclization of the polyprenyl chains. In its simplest monocyclic version, carotene precursors (in this case prephytoene) are cyclized, eventually giving β-carotene, the precursor of retinal (present in halophilic archaea). This cyclization of polyprenyl chains is exactly analogous to the fundamental reaction A (shown above) run internally; it follows Markovnikof's Rule.

It would require only a small change, as suggested by Fig. 11, to apply the same reaction in a polycyclic manner, to yield the tricyclopolyprenols (for example, tricyclohexaprenol), again obeying Markovnikof's Rule. Similarly, the polycyclopolyprenols would be formed along the same lines from the appropriate acyclic precursors. For changes of this nature we have postulated that the basic site responsible for termination of the cyclization process could be moved progressively farther away from the acidic initiation site by point mutations. Similar substrates have been cyclized by Vlad with superacids like fluorosulfonic acid to give the tri- or tetracyclic systems in high yields [69].

The cyclization of squalene

With squalene available as well as polyprenyl-cyclases, the pentacyclization to the hopane skeleton might require only minor steps, provided the enzymes involved are not very sensitive to the nature of the substrate. In extant microorganisms this is, remarkably enough, the case. The squalene-hopene cyclases of *Acetobacter*, *Methylococcus* and *Tetrahymena* can cyclize not only squalene, their normal substrate, but also its 2,3(R) and 2,3(S)-epoxides [70]. They can also cyclize polyprenyl derivatives, such as penta- or hexaprenyl methyl ethers [71–73], or farnesol derivatives [74]; in these cases Markovnikof's Rule is obeyed. In *Methylococcus*, which contains not only hopane derivatives, but also 4α-methyl sterols derived from lanosterol, the epoxysqualene-lanosterol cyclase also accepts both enantiomers of epoxysqualene as the substrate, giving a mixture of lanosterol and its 3α-diastereomer as product [72]. This lack of substrate selectivity contrasts with the high selectivity of the eukaryotic epoxysqualene cyclases, which do not accept squalene or its (R) epoxide. One must note, however, that all these results have been obtained with cell-free systems, not with the pure cyclases, which have recently been purified [75–78] and sequenced by cloning from several microorganisms [79–81].

All these cyclases must surmount the anti-Markovnikof nature of the cyclizations of squalene that are necessary to obtain rings C and D (see box) of the hopane skeleton. Such reactions have large activation energies, and can probably only be achieved by using the energy of binding to the enzyme to reduce the entropy of the system, for example by imposing a high degree of order on the substrate in the active center, at the same time arranging the topography of the substrate appropriately (see Fig. 12).

Similarly, through small changes in the topography of the active site, one could impose on the epoxysqualene still more enthalpy-demanding pre-boat conformations, leading to cycloartenol and lanosterol, from which sterols can finally derive. Now that the primary structures of several of the cyclases involved are known [79–81] it should soon be possible to determine the precise topography of their active sites and check whether they indeed form a natural progression as predicted. A repetitive motif has already been noted in the sequences of squalene and epoxysqualene cyclases, supporting the notion of a stepwise evolution [82,83].

Lanosterol and cycloartenol

The finding that cycloartenol is the first tetracyclic intermediate in the biosynthesis of plant sterols [84], initially appeared to be simply a complication from the simpler route via lanosterol, demonstrated earlier in yeast and rat [85]. This view has been changed by the finding that cycloartenol, unlike lanosterol, can replace cholesterol both *in vitro* and *in vivo* [42]. Plants treated with antagonists that block the cleavage of the 9(11) bond can grow with only cycloartenol derivatives in their membranes [86], and cycloartenol derivatives are present in the membrane sterols of at least one amoeba [87,88]. It has been suggested that cycloartenol may have a bent conformation that 'hides' the 14-methyl group (see Fig. 4), explaining its capacity to replace cholesterol [42], and indeed we have been able to show directly that the more stable quasi-planar conformation of cycloartenol is in equilibrium with a bent one [89] which may be the conformation adopted by cycloartenol in membranes. Lanosterol, in contrast, has a rigid planar conformation in which the

methyl group protrudes unavoidably, and therefore can never substitute for cholesterol.

It therefore seems reasonable to propose that the cyclization of squalene 3S-epoxide to cycloartenol, directly producing a compound that reinforces membranes, was at one point the end of the biosynthetic pathway. Organisms containing only cycloartenol as membrane reinforcer could have survived, and some might still be extant. As the concentration of dioxygen in the atmosphere increased, however, the unavoidable metabolic degradation of cycloartenol would have led to sterols, which are at least equally good as membrane reinforcers. And once this catabolism had been established, the same steps could also have been used to degrade lanosterol, and to convert it to sterols.

Construction of a cell

We shall now return to the key question, that of the origin of the first cells. What we have suggested above is only that the first vesicles may have been formed by self-assembly of terpenoid amphiphilic molecules. A vesicle is not a cell, but, as Morowitz points out [2], the formation of a vesicle is by itself a far reaching event.

Firstly, the self-assembly of amphiphilic molecules leads in fact to the differentiation not of two, but of three compartments: the inside of the vesicle and the outside, both of which are aqueous, and the membrane itself, which is hydrophobic. The existence of a hydrophobic environment will lead the least polar molecules present (for instance squalene and the other large terpenoids, such as dolichols and ubiquinones) to concentrate in this small space [90]. More importantly, the self-assembly of identical molecules into an approximately spherical vesicle automatically distinguishes the outside and the inside halves of the double-layer. This is intuitively obvious, and has been confirmed by ^{31}P–NMR [91] : the slightly larger area of the outside layer leads to a slightly larger area available per head group (76 Å^2) than for the inner molecules (68 Å^2). This must lead to vectorial properties, and must of course also be the case for the polyprenyl phosphates. For instance, the pKa of the outer head groups (in these polyprenyl phosphate vesicles) must be slightly lower than that of the inner ones, but by how much? We do not know, and thus we do not know whether this difference can exert measurable effects. Nor do we know how much effect this difference would have on the preference of different phospholipids, with head groups of different sizes, for the inner or outer face (for instance, di-(polyprenyl) phosphates mixed with prenyl phosphates might prefer the inner layer, while prenyl pyrophosphates might prefer the outer layer). Not only the properties of the head-groups, but also the texture of the highly anisotropic lipidic part of the membrane must be altered by the tapering resulting from the formation of the vesicle. Again, we do not yet know whether this effect is so small as to be undetectable by available techniques. There are many ways in which this asymmetry might be important, for example by influencing the orientation of polypeptide chains in the membrane; the most basic parts of the polypeptide (and probably the amino-terminus) should be outside, near the most acidic head-groups. Such differences will further increase the difference between the inner compartment and the outside of the vesicle.

The vectorial properties of a membrane of course become more marked with increasing curvature, and therefore are stronger in smaller vesicles. These properties may be preserved in large vesicles, however, if small vesicles grow by fusion and if the fusion mechanisms preserve the asymmetry of the membrane. One might imagine that small vesicles could grow until they reach an optimal size range, then fragment again into smaller vesicles. In such a system, the asymmetry of the smallest vesicles would be transferred to the larger ones, where it would no longer be thermodynamically induced, but kinetically maintained.

Thus the simple self-organization of amphiphilic molecules into closed lipidic vesicles results in a number of changes in the properties of the system which are potentially important; this cascade of changes leads automatically to a complex system.

Conclusion

It is clear that terpenoids, such as sterols, hopanoids, some carotenoids, archeal lipids and cycloartenol, are involved in the formation or reinforcement of all known biological membranes. We have proposed here that the evolution of membrane constituents proceeded from polyprenyl phosphates by progressive recruitment of novel enzymatic systems. This hypothesis provides an attractive way of ordering the terpenoids; like all evolutionary theories, it cannot be tested directly. The ideas summarized here do, however, suggest a multitude of experiments having some bearing on the fundamental and fascinating question: how did the first cells appear? We hope to carry out some of them.

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References

1. Trân Thanh Vân, Mounolou, J.C., Schneider, J. & McKay, C. (eds) (1992). *Frontiers of Life.* Frontières, Gif-sur-Yvette.
2. Morowitz, H.J. (1992). *Beginnings of Cellular Life.* Yale University Press, New Haven.
3. Cairns-Smith, A.G. (1982). *Genetic Take-over and the Mineral Origin of Life.* Cambridge University Press, London.
4. Wächtershäuser, G. (1988). Before enzymes and templates: a theory of surface metabolism. *Microbiol. Rev.* **52**, 452–484.
5. Ourisson, G. & Rohmer, M. (1992). The biohopanoids. *Accounts Chem. Res.* **25**, 403–408.
6. De Rosa, M., Gambacorta, A. & Gliozzi, A. (1986). Structure, biosynthesis and physico-chemical properties of archaebacterial lipids. *Microbiol. Rev.* **50**, 70–80.
7. Ourisson, G. & Albrecht, P. (1992). The geohopanoids. *Accounts Chem. Res.* **25**, 398–402.
8. Peters, K.E. & Moldowan, J.M. (1993). *The Biomarker Guide. Interpreting Molecular Fossils in Petroleum and Ancient Sediments.* Prentice Hall, Englewood Cliffs.

9. Callot, H.J. (1991). Geochemistry of chlorophylls. In *Chlorophylls.* (Scheer, H.C., ed), pp. 339–364, CRC Press, Boca Raton.
10. Ourisson, G., Rohmer, M. & Poralla, K. (1987). Microbial lipids betrayed by their fossils. *Microbiol. Sci.* **4**, 52–57.
11. Albrecht, P. & Ourisson, G. (1969). Triterpene alcohol isolation from oil shale. *Science* **163,** 1192–1193.
12. Hauke, V., *et al.*, & Peakman, T.M. (1992). Novel triterpene-derived hydrocarbons of arborane/fernane series in sediments. *Tetrahedron* **48,** 3915–3924.
13. Ensminger, A., Joly, G. & Albrecht, P. (1978). Rearranged steranes in sediments and crude oils. *Tetrahedron Lett.* 1575–1578.
14. Rubinstein, I., Sieskind, O. & Albrecht, P. (1975). Rearranged sterenes in a shale: occurrence and simulated formation. *J. Chem. Soc., Perkin I* 1833–1836.
15. Henderson, W. & Steel, G. (1971). Isolation and characterization of a triterpenoid alcohol from the Green River shale. *Chem. Comm.* 1331–1332.
16. Michaelis, W. & Albrecht, P. (1979). Molecular fossils of archaebacteria in kerogen. *Naturwissenschaften* **66**, 420–421.
17. Chappe, B., Albrecht, P. & Michaelis, W. (1982). Polar lipids of archaebacteria in sediments and petroleums. *Science* **217,** 65–66.
18. Chappe, B. (Chap Sim), Michaelis, W., Albrecht, P. & Ourisson, G. (1979). Fossil evidence for a novel series of archaebacterial lipids. *Naturwissenschaften* **66,** 522–523.
19. Aquino Neto, F.R., Restlé, A., Connan, J., Albrecht, P. & Ourisson, G. (1982). Novel tricyclic terpanes (C19, C20) in sediments and petroleums. *Tetrahedron Lett.* 2027–2030.
20. Ekweozor, C.M. & Strausz, O.P. (1982). 18,19-Bisnor-13bH, 14aH-cheilanthane: a novel degraded tricyclic sesterterpenoid-type hydrocarbon form the Athabasca oil sands. *Tetrahedron Lett.* 2711–2714.
21. Corey, E.J. & Burk, R.M. (1987). Total synthesis of (±)-tricyclohexaprenol, a possible forerunner of sterols in the evolution of biomembranes. *Tetrahedron Lett.* **28,** 6413–6416.
22. Heissler, D., Ocampo, R., Albrecht, P., Riehl, J.J. & Ourisson, G. (1984) Identification of long chain tricyclic terpene hydrocarbons (C_{21}–C_{30}) in geological samples. *J. Chem. Soc., Chem. Commun.* 496–498.
23. Ladenburger, C. & Heissler, D. (1991). Synthesis of (Z,Z)-tricyclohexaprenol. *J. Chem. Research* 330.
24. Schaeffer, P., *et al.*, & Connan, J. (1994). Novel optically active hydrocarbons in sediments: evidence for an extensive biological cyclization of higher regular polyprenols. *Angew. Chem.*, in press.
25. Volkman, J.K., Farrington, J.W., Gagosian, R.B. & Wakeham, S.G. (1983). Lipid composition of coastal marine sediments from the Peru upwelling region. In *Advances in Organic Geochemistry 1981* (Bjoroy, M., ed), pp. 228–240, Wiley, Chichester .
26. Dany, F., Riolo, J., Trendel, J.M. & Albrecht, P. (1990). 3b-Carboxysteranes, a novel family of fossil steroids. *J. Chem. Soc., Chem. Commun.* 1228–1230.
27. Dahl, J., Moldowan, J.M., McCaffrey, M.A. & Lipton, P.A. (1992). A new class of natural products revealed by 3-alkylsteranes in petroleum. *Nature* **355,** 154–157.
28. Demel, R.A. & De Kruyff, B. (1976). The function of sterols in membranes. *Biochim. Biophys. Acta* **457,** 109–132.
29. Conner, R.L.M., Landrey, J.R., Burns, C.H. & Mallory, F.B. (1968). Cholesterol inhibition of pentacyclic triterpenoid biosynthesis in *Tetrahymena pyriformis. J. Protozool.* **15,** 600–605.
30. Raederstorff, D. & Rohmer, M. (1988). Polyterpenoids as cholesterol and tetrahymanol surrogates in the ciliate *Tetrahymena puriformis. Biochim. Biophys. Acta* **960,** 190–199.
31. Holler, S., Pfennig, N., Neunlist, S. & Rohmer, M. (1993). Effect of a non-methanogenic symbiont and exogenous stigmasterol on the viability and tetrahymanol content of the anaerobic ciliate *Trimyema compressum. Europ. J. Protistol.* **29,** 42–48.
32. Rottem, S. & Markowitz, O. (1979). Carotenoids act as reinforcers of the *Acholeplasma laidlawii* lipid bilayers. *J. Bacteriol.* **140,** 944–948.
33. Smith, P.F. & Langworthy, T.A. (1979). Existence of carotenoids in *Acholeplasma axanthum. J. Bacteriol.* **137,** 185–188.
34. Rohmer, M., Bouvier, P. & Ourisson, G. (1979). Molecular evolution of biomembranes. Structural equivalents and phylogenetic precursors of sterols. *Proc. Natl. Acad. Sci. USA* **76,** 847–851.
35. Takaichi, S., Furihata, K., Ishidsu, J. & Shimada, K. (1991). Carotenoid sulphates from the aerobic photosynthetic bacterium *Erythrobacter longus. Photochemistry* **30,** 3411–3415.
36. Nes, W.R. (1974). Role of sterols in membranes. *Lipids* **9,** 956–612.
37. Milon, A., Wolff, G., Ourisson, G. & Nakatani, Y. (1986). Organisation of carotenoid-phospholipid bilayer systems. Incorporation of zeaxanthin, astaxanthin, and their C50 homologues into dimyristoyl-phosphatidylcholine vesicles. *Helv. Chim. Acta* **69,** 12–24.
38. Luzzati, V., Gulik, A., De Rosa, M. & Gambacorta, A. (1987). Lipids from *Sulfolobus solfataricus*, life at high temperature and the structure of membranes. *Chem. Scripta* **27B**, 211–219.
39. Poralla, K., Kannenberg, E. & Blume, A. (1980). A glycolipid-containing hopane isolated from the acidophilic, thermophilic *Bacillus acidocaldarius* has a cholesterol-like function in membranes. *FEBS Lett.* **113**, 107–110.
40. Krajewski-Bertrand, M.A., *et al.*, & Ourisson, G. (1990). Tricyclohexaprenol and an octaprenediol, two of the 'primitive' amphiphilic lipids, do improve phospholipidic membranes. *Tetrahedron* **46**, 3143–3154.
41. Krajewski-Bertrand, M.A., Milon, A., Nakatani, Y. & Ourisson, G. (1992). The interaction of various cholesterol 'ancestors' with lipid membranes: a ^{2}H-NMR study on oriented bilayers. *Biochim. Biophys. Acta* **1105,** 213–220.
42. Bloch, K. (1983). Sterol structure and membrane function. *CRC Crit. Rev. Biochem.* **4,** 47–92.
43. Ourisson, G., Albrecht, P. & Rohmer, M. (1982). Predictive microbial biochemistry: from molecular fossils to procaryotic membranes. *Trends Biochem. Sci.* **7**, 236–239.
44. Florkin, M. (1944). *L'évolution biochimique.* Masson, Paris; (1949). *Biochemical Evolution.* Academic Press, New York.
45. Granick, S. (1950). Harvey Lectures **44**, 220–245.
46. Kasting, J.F. (1993). Earth's early atmosphere. *Science* **259,** 920–926.
47. Plobeck, N., Eifler, S., Brisson, A., Nakatani, Y. & Ourisson, G. (1992). Sodium di-polyprenyl phosphates form 'primitive' membranes. *Tetrahedron Lett.* **33**, 5249–5252.
48. Bloch, K. (1992). Sterol molecule: structure, biosynthesis and function. *Steroids* **57**, 378–383.
49. Rohmer, M., Knani, M., Simonin, P., Sutter, B. & Sahm, H. (1993). Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem. J.* **295,** 517–524.
50. Yamagata, Y., Watanabe, H., Saitoh, M. & Namba, T. (1991). Volcanic production of polyphosphate under primitive Earth conditions. *Nature* **353**, 516–519.
51. Miller, S.L. & Parris, M. (1964). Synthesis of pyrophosphates and its relevance to prebiotic evolution. *Nature* **204,** 1248–1250.
52. Balcheffsky, M. & Balcheffsky, H. (1992). Inorganic pyrophosphate and inorganic pyrophosphatases. In *New Comprehensive Biochemistry.* (Ernster, L., ed) pp. 331–348, Elsevier, Amsterdam.
53. Baba, Y., Tsuhako, M. & Yoza, N. (1990). Phosphorylation of biomolecules with inorganic phosphorylating agents. *Trends Org. Chem.* **1**, 53–75.
54. Westheimer, F.H. (1987). Why Nature chose phosphates? *Science* **235**, 1173–1178.
55. Hargreaves, W.R. & Deamer, D.W. (1978). Liposomes form ionic, single-chain amphiphiles. *Biochemistry* **17**, 3759–3768.
56. Kates, M. (1978). The phytanyl ether-linked polar lipids and isoprenoid neutral lipids of extremely halophilic bacteria. *Progr. Chem. Fats other Lipids* **15,** 301–342.
57. Kushwaha, S.C., Kramer, J.K.G. & Kates, M. (1975). Isolation and characterization of C_{50}-carotenoid pigments and other polar isoprenoids form *Halobacterium cutirubrum. Biochim. Biophys. Acta* **398,** 303–314.
58. Tornabene, T.G., Langworthy, T.A. & Hozer, G. (1979). Squalenes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic 'Archaebacteria'. *J. Mol. Evol.* **13**, 73–83.
59. Moldoveanu, N., Kates, M., Montero, C.G. & Ventosa, A. (1990). Polar lipids of non-alkaliphilic *Halococci. Biochim. Biophys. Acta* **1046**, 127–135.
60. Ourisson, G. (1989). The evolution of terpenes to sterols. *Pure & Appl. Chem.* **61**, 345–348.
61. Hafenbradl, D., Keller, M., Thiericke, R. & Stetter, K.O. (1993). *Systematic Appl. Microbiol.* **16**, 165–169.
62. Klein, R.A., Hazlewood, G.P., Kemp, P. & Dawson, R.M.C. (1979). A new series of long-chain dicarboxylic acids with vicinal dimethyl branching found as major components of the lipids of *Butyrivibrio* spp. *Biochem. J.* **183**, 691–700.
63. De Rosa, M., Gambacorta, A., Minale, L. & Bu'Lock, J.D. (1971). Bacterial triterpenes. *J. Chem. Soc. Chem. Commun.* 1334.
64. Julia, M.Y. (1991). Chemical studies on some early steps in the biosynthesis of squalene. *Chem. Soc. Rev.* **20,** 129–147
65. Spurgeon, S.L. & Porter, J.W. (1983). Biosynthesis of carotenoids. In *Biosynthesis of Isoprenoid Compounds.* (Porter, J.W. & Spurgeon, S.L., eds), pp.14–24, J.Wiley, New York.
66. Suzue, G., Tsukada, K. & Tanaka, S. (1968). Occurrence of dehydrosqualene (C_{30}-phytoene) in *Staphylococcus aureus. Biochim. Biophys. Acta* **164**, 88–93.
67. Yanagisawa, A., Nomura, N. & Yamamoto, H. (1994). Transition metal-catalyzed substitution reaction of allylic phosphates with Grignard reagents. *Tetrahedron* **50**, 6017–6028.

68. McMurry, J.E. & Silvestri, M. (1975). A simplified method for the titanium(II)-induced coupling of allylic and benzylic alcohols. *J. Org. Chem.* **40**, 2687–2688.
69. Vlad, P.F. (1993). Superacidic cyclization of terpenoids. *Pure & Appl. Chem.* **65**, 1329–1336.
70. Rohmer, M., Anding, C. & Ourisson, G. (1980). Nonspecific biosynthesis of hopane triterpenes by a cell-free system from *Acetobacter pasteurianum*. *Eur. J. Biochem.* **112**, 541–547.
71. Bouvier, P., Berger, Y., Rohmer, M. & Ourisson, G. (1980). Nonspecific biosynthesis of gammacerane derivatives by a cell-free system from *Tetrahymena pyriformis*. *Eur. J. Biochem.* **112**, 549–556.
72. Rohmer, M., Bouvier, P. & Ourisson, G. (1980). Nonspecific lanosterol and hopanoid biosynthesis by a cell-free system from the bacterium *Methylococcus capsulatus*. *Eur. J. Biochem.* **112**, 557– 560.
73. Renoux, J.M. & Rohmer, M. (1986). Enzymatic cyclization of all-trans pentaprenyl and hexaprenyl methyl ethers by a cell-free system from the protozoon *Tetrahymena pyriformis*. The biosynthesis of scalarane and polycyclohexaprenyl derivatives. *Eur. J. Biochem.* **155**, 125–132.
74 Neumann, S. & Simon, H. (1986). Purification, partial characterization and substrate specificity of a squalene cyclase from *Bacillus acidocaldarius*. *Biol. Chem. Hoppe Seyler* **367**, 723–729.
75 Abe, I., Ebizuka, Y., Seo, S. & Sankawa, U. (1989). Purification of squalene-2,3-epoxide cyclases from cell suspension cultures of *Rhabdosia japonica*., Hara. *FEBS Lett.* **249**, 100–104.
76. Ochs, D., Tappe, C.H., Gärtner, P., Kellner, R. & Poralla, K. (1990). Properties of purified squalene-hopene cyclase from *Bacillus acidocaldarius*. *Eur. J. Biochem.* **194**, 75–90.
77. Saar, J., Kader, J.-C., Poralla, K. & Ourisson, G. (1991). Purification and some properties of the squalene-tetrahymanol cyclase from *Tetrahymena thermophila*. *Biochim. Biophys. Acta* **1075**, 93–101.
78. Corey, E.J. & Matsuda, S.P.T. (1991). Purification of squalene-2,3-epoxide cyclase from *Saccharomyces cerevisiae*. *J. Am. Chem. Soc.* **113**, 8172–8174.
79. Ochs, D., Kaletta, C., Entian, K.-D., Beck-Sickinger, A. & Poralla, K. (1992). Cloning, expression and sequencing of squalene-hopene cyclase, a key enzyme in triterpenoid metabolism. *J. Bacteriol.* **174**, 298–302.
80. Buntel, C.J. & Griffin, J.H. (1992). Nucleotide and deduced amino acid sequences of the oxidosqualene cyclase from *Candida albicans*. *J. Am. Chem. Soc.* **114**, 9711–9713.
81. Hoshino, T., Williams, H.J., Chung, Y. & Scott, A.I. (1991). Partial purification and characterization of oxidosqualene-lanosterol cyclase from bakers' yeast. *Tetrahedron* **47**, 5925–5932.
82. Poralla, K., Hewelt, A., Prestwich, G.D., Abe, I., Reipen, I. & Sprenger, G. (1994). A specific amino acid repeat in squalene and oxidosqualene cyclases. *Trends Biochem. Sci.* **19**, 157–158.
83. Poralla, K. (1994). The possible role of a repetitive amino acid motif in evolution of triterpenoid cyclases. *Bioorg. Med. Chem. Lett.* **4**, 285–290.
84. Benveniste, P., Hirth, L. & Ourisson, G. (1966). La biosynthèse des stérols dans les tissus de tabac cultivés in vitro. II. Particularités de la biosynthèse des phytostérols des tissus de tabac cultivés *in vitro*. *Photochemistry* **5**, 45–58.
85. Mulheirn, L.J. & Ramm, P.J. (1972). The biosynthesis of sterols. *Chem. Soc. Rev.* **1**, 259–291.
86. Hartmann, M.A., Grandmaugin, A. & Benveniste, P. (1985). Modification of the sterol composition of the plasma membrane from maize roots by fenpropimorph. *C. R. Acad. Sci. [III]* **301**, 601–605.
87. Raederstorff, D. & Rohmer, M.(1985). Sterol biosynthesis de novo via cycloartenol by the soil amoeba *Acanthamoeba polyphaga*. *Biochem. J.* **231**, 609–615.
88. Raederstorff, D. & Rohmer, M. (1987). Sterol biosynthesis via cycloartenol and other biochemical features related to photosynthetic phyla in the amoebae *Naegleria lovaniensis* and *Naegleria gruberi*. *Eur. J. Biochem.* **164**, 427–434.
89. Milon, A., Nakatani, Y., Kintzinger, J.P. & Ourisson, G. (1989). The conformation of cycloartenol investigated by NMR and molecular mechanics. *Helv. Chim. Acta* **72**, 1–12.
90. Morowitz, H.J., Deamer, D.W. & Smith, T. (1991). Biogenesis as an evolutionary process. *J. Mol. Evol.* **33**, 207–208.
91. Chrzeszczyk, A., Wishnia, A. & Springer, C.S., Jr. (1977). The intrinsic structural asymmetry of highly curved phospholipid bilayer membranes. *Biochim. Biophys. Acta* **470**, 161–169.

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