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Properties of hopanoids and phosphatidylcholines containing ω-cyclohexane fatty acid in monolayer and liposome experiments

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1,2,3,4-Tetrahydroxypentane-29-hopane (THBH) and a glycolipid derived from it are associated with ω-cyclohexane fatty acids-containing lipids in the membrane of *Bacillus acidocaldarius*. In order to elucidate the function of these lipids we studied mixed monolayer films and compared these with cholesterol-containing films. The hopanoids are able to condense a liquid-expanded film of di-ω-cyclohexyldodecanoylphosphatidylcholine (DCDPC). The condensing effect of THBH is smaller than that of cholesterol. Hopane glycolipid in comparison shows only little condensation. In a more condensed film, at lower temperatures, THBH slightly decreases while hopane glycolipid increases the molecular area. In egg phosphatidylcholine liposomes, 22-hydroxyhopane (diplopterol) and hopane glycolipid reduce the glycerol permeability to a smaller extent than cholesterol. In DCDPC liposomes, the effect of 22-hydroxyhopane is similar to that of cholesterol, while the hopane glycolipid shows only a weak reduction of the permeability. The results demonstrate that hepanoids have a cholesterol-like function in membranes. This function is also discussed in the context of membrane adaptation of a thermoacidophilic bacterium.

Introduction

Hopanoids, a group of pentacyclic triterpenes (Fig. 1), are common in bacterial membranes [1,2]. Most of them contain an extended sidechain with hydroxygroups.

Hopanoids are amphiphilic molecules which form monolayers at the air/water interface. Hopanoids condense mixed monolayers of DPPC [3,4], of DIHPC and DAHPC [5] and abolish the phase transition [3–5]. In DPPC liposomes, tetrahydroxybacteriohopane diminishes the phase transition and lowers the transition enthalpy. All effects are very similar to those of cholesterol in various lipid membrane model systems; hence the suggested cholesterol-like function of hopanoids [2] seems to be established.

In thermoacidophilic bacilli, e.g., *Bacillus acidocaldarius,* hopanoids are accompanied by lipids containing ω-cyclohexane fatty acids [6,7].

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Abbreviations: PC, phosphatidylcholine; THBH, 1,2,3,4-tetrahydroxypentane-29-hopane; hopane glycolipid, 1-(*O*-β-*N*-acylglucosaminyl)-2,3,4-tetrahydroxypentane-29-hopane; diplopterol, 22-hydroxy-29-hopane; hopane monol, 29-(2-hydroxyethyl)hopane; DCDPC, 1,2-di-ω-cyclohexyldodecanoylphosphatidylcholine; DCDPA, 1,2-di-ω-cyclohexyldodecanoylphosphatidic acid; DPPC, 1,2-dipalmitoylphosphatidylcholine; DIHPC, 1,2-diisoheptadecanoylphosphatidylcholine; DAHPC, 1,2-dianteisoheptadecanoylphosphatidylcholine; egg PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; egg PA, 1,2-diacyl-*sn*-glycerophosphatidic acid.

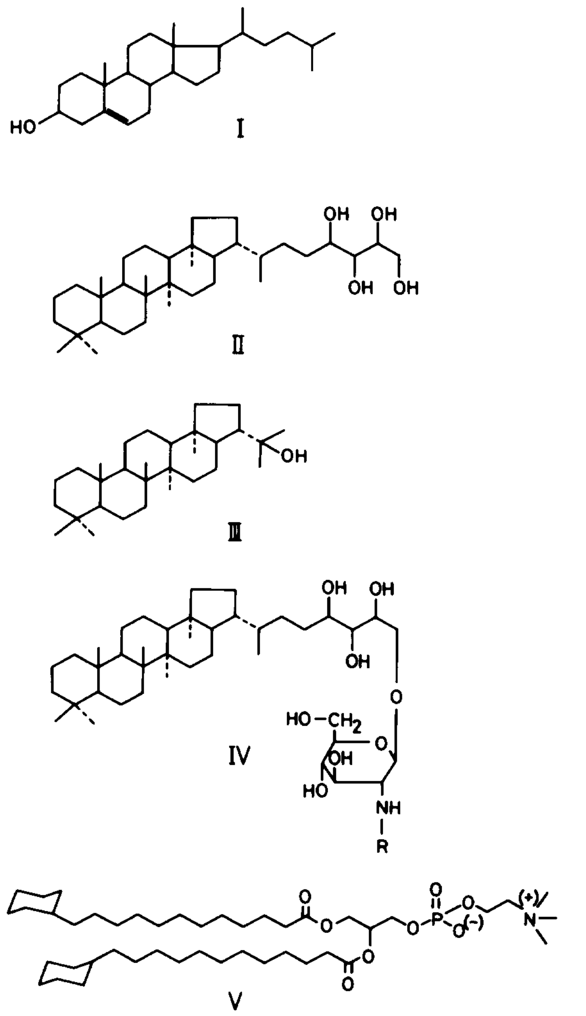

Fig. 1. Lipids used in this study: I, cholesterol; II, tetrahydroxybacteriohopane (THBH); III, 22-hydroxyhopane (diplopterol); IV, hopane glycolipid (R is a fatty acid residue consisting predominantly of ω-cyclohexylundecanoate and ω-cyclohexyltridecanoate); V, DCDPC.

In another group of thermoacidophilic bacilli, ω-cycloheptane fatty acids are abundant [8]. In *B. acidocaldarius,* the amount of hopanoids [9] and the amount of ω-cyclohexane fatty acids as well are dependent on temperature and pH [10,11]. Hence, the contribution of hopanoids and ω-cyclohexane fatty acids to membrane stability and integrity (under normally antagonistic conditions) are of special interest. Phospholipids with ω-cyclohexane fatty acids are in a more condensed state in comparison to phospholipids with linear saturated fatty acids of comparable chain-length at temperatures above the phase transition [12,13]. So far, no data are available on the interaction of hopanoids with lipids containing ω-cyclohexane fatty acids, though this is of special interest for the understanding of membrane adaptation in thermoacidophilic bacilli. For this reason, we studied mixed monolayers and liposomes of hopanoids with DCDPC and other lipids. For comparison, the effect of cholesterol on these lipids was examined.

Materials and Methods

The hopanoids THBH and hopane glycolipid were isolated from *B. acidocaldarius* as published elsewhere [3]. Diplopterol was isolated from *Methylobacterium organophilum* and characterized as described by Benz et al. [14]. Cells of *Methylobacterium* were grown on a synthetic medium from Patt and Hanson [15]. The diplopterol for the monolayer experiments was a gift from Dr. Rohmer, Ecole National Superieure de Chemie, Mulhouse/France.

12-Cyclohexyldodecanoic acid was synthesized in a five-step procedure starting from cyanundecanoic acid (Fluka, Neu-Ulm, F.R.G.). The basic step in the fatty acid synthesis was the introduction of the isocyclic moiety into the aliphatic chain. This was achieved by Friedel-Crafts-acylation of benzene with aluminium chloride using 11-cyanundecanoic acid chloride as electrophilic reagent. Thionyl chloride was used to prepare the reagent from cyanundecanoic acid in a precedent reaction. The 11-cyanodecylphenyl ketone, which had been obtained in 58% yield (m.p. 56°C), was hydrolysed into the acid in ethanolic sodium hydroxide solution. The white powder of 11-carboxydecylphenyl ketone (m.p. 89°C) was treated with hydrazine hydrate in triethyleneglycol and potassium hydroxide yielding 93% of white crystalline 12-phenyldodecanoic acid (m.p. 60°C) after Huang-Minlon reduction. The purified acid was finally hydrogenated over a polymer-supported catalyst (Ref. 16 and Blanz, N. and Geckeler, K., unpublished results) in order to obtain the cycloaliphatic group. Classical hydrogenation over nickel was not successful for this crucial step. The 12-cyclohexyldodecanoic acid was obtained in 75%

yield with a melting point of 60°C (Lit.: 61–62°C [17]). Another method is described in Ref. 14. Elemental analyses as well as infrared and NMR spectra of the intermediates corresponded to the formulae. The DCDPC used was synthesized from Dr. Eibl, Max-Planck-Institute for Biophysical Chemistry, Goettingen/F.R.G. DCDPC shows a broad phase transition at 13°C with a total enthalpy of 4.1 kcal/mol as measured by DSC. 1,2-Di-ω-cyclohexyldodecanoylphosphatidic acid was obtained out of DCDPC by treatment with phospholipase D in a procedure described by Eibl and Kovatchev [18]. Egg PC and egg PA were chromatographically pure products from Sigma Chemical Company. All other chemicals were of analytical grade.

Monolayer experiments were carried out according to Blume [19] with a commercial Langmuir film balance (Messgerätewerke Dr. Wobser, Lauda, F.R.G.). For permeability measurements, lipid dispersions were prepared in a modified procedure described by De Gier et al. [20]. 15 μmol lipid were pipetted into a 10-ml round-bottom flask. The solvent was removed under nitrogen followed by high evacuation for 2 h. PC solutions contained 4 mol% of the corresponding phosphatidic acid. The solvent for the PCs was a mixture of chloroform/methanol (3:2, v/v), for cholesterol chloroform, and for diplopterol and hopane glycolipid, chloroform/methanol (2:1, v/v). To the dry lipid films were added a glass bead and 1 ml of 50 mM KCl. The lipids were dispersed by agitation on a Vortex mixer twice for 30 s above the transition temperature of the lipids. In order to remove the lipid film completely from the glass wall, some samples had to be sonicated in a Branson bath ultrasonicator for 30 s between the Vortex runs. The liposomes were checked microscopically for approximately comparable size and morphology. In mixtures with high concentrations of triterpenoids, small crystals of pure triterpenoid were observed. Only samples which appeared uniform under the microscope were used. Swelling of liposomes in isotonic glycerol was measured at 436 nm in an Eppendorf photometer (Type 1101 M) connected to a Servogor (Type RE 551) recorder. The cylindrical glass cuvette was fitted into a metal block, the temperature of which was controlled. 2.5 ml isotonic solution were mixed with 30 μl samples of lipid dispersion by a fast-running stirrer in the cuvette in 1 s or less.

Results and Discussion

In Fig. 2, the monolayer behavior of hopanoids in comparison to cholesterol is shown and compared with that of cholesterol. Isobars were recorded at a lateral surface pressure of 25 dyn/cm with the exception of diplopterol, as the monolayer film of diplopterol was not stable at this surface pressure. The isobar for diplopterol was measured at a surface pressure of 18 dyn/cm. It was stable up to 21°C. At higher temperatures, the film slowly collapsed. The isotherms of diplopterol showed a peculiar behavior in so far as the second run was not identical to the first. The isotherm was shifted to smaller molecular areas and displayed a lower compressibility. The areas occupied by hopanoids were standardized.

As shown in Fig. 2, the monolayer films of hopanoids show low compressibilities and are almost temperature-invariant. Thus, they behave very similar to cholesterol. Hopanoid molecules seem to be oriented perpendicular to the air/water interface, although in an inverted orientation compared to cholesterol, as the hydroxy group(s) are in the sidechain (Fig. 1) and not in the ring structure.

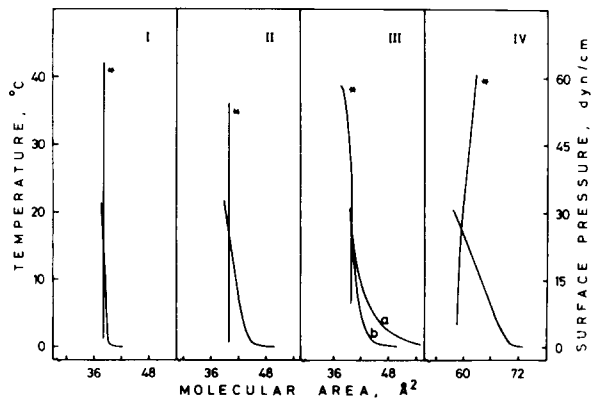

Fig. 2. Isobars (*) recorded at a surface pressure of 25 dyn/cm and isotherms recorded at a temperature of 22°C of cholesterol (I), THBH (II), diplopterol (III) and hopane glycolipid (IV); the diplopterol isotherm, a, represents the first measurement and, b, the following; the diplopterol isobar was recorded at 18 dyn/cm.

As it can be seen from Fig. 1, the alicyclic ring structure of hopanoids has perpendicularly oriented methyl groups on both sides of the pentacyclic ring plane. These methyl groups may disturb optimal molecular packing and may be the reason for the slightly higher compressibility of THBH and diplopterol compared to cholesterol. Diplopterol was unique in so far as its isotherms were dependent on its previous history. It can be shown that the hydroxy group of diplopterol is not oriented in the plane of the hopanoid structure but has an orientation almost perpendicular to the planar ring system. For optimal hydration, the molecules may therefore be tilted at low surface pressure and increasing pressure may reorientate them perpendicular to the water surface. This reorientation could show some hysteresis, as the perpendicular arrangement could be stabilized by hydrophobic interactions. This reorientation may also reduce the interactions of the hydroxy group with the water phase. Thus, molecules may be squeezed out of the monolayer at higher surface pressures and thereby explain the film instability at these pressures. In comparison to cholesterol, THBH and diplopterol, hopane glycolipid possesses a higher compressibility and its molecular area increases with higher temperature. These differences are presumably due to the fatty acid residue in the glycolipid structure (Fig. 1).

In Fig. 3, the molecular areas occupied by monolayers composed of varying proportions of DCDPC and either cholesterol, THBH or hopane glycolipid are presented. The lateral surface pressure was 25 dyn/cm. At this pressure and even at 35 dyn/cm, DCDPC showed no significant phase transition between 1 and 40°C in monolayer experiments [12]. At 40°C, cholesterol induces a pronounced condensation of the molecular area. THBH is less effective, while hopane glycolipid shows only a slight condensation. At 4°C, a temperature at which DCDPC is in a more condensed state [12], cholesterol is still able to induce tighter molecular packing, while THBH reduces the molecular area only slightly and hopane glycolipid expands the monolayer. Phospholipids containing an ω-cyclohexane fatty acid, e.g., DCDPC, show a higher degree of order and a lower fluidity in their liquid-crystalline state compared to lipids containing straight acyl chains [12–14,21]. This behavior is probably caused by the bulky cyclohexane endgroups, which may lead to a higher density in the core of the membrane and thus to a relatively high degree of order in the middle of the bilayer. Since the area occupied by the rigid cholesterol molecule is almost temperature-invariant, the area occupied by DCDPC is further markedly reduced compared to the molecular area occupied in the absence of cholesterol. One can speculate that cholesterol by its ability to restrict mainly the first ten carbon atoms of acyl chains [22] may strongly influence the linear parts of the acyl chains of DCDPC. Hopanoids, with the hydroxygroups in the sidechain, have an inverted orientation in mono- and bilayers compared to cholesterol. The ring system is therefore somewhat shifted towards the ends of the acyl chains. As the hydrophobic regions of cholesterol and hopanoids are of almost the same extent, the planar hopanoid ring system should extend to the acyl chain ends of DCDPC. Hence, the lower condensation of THBH could be due to a perturbation of the interactions of the cyclohexyl rings by the ring system of the hopanoids and by the insertion of the hopanoid ring system in a region of presumably decreasing acyl chain order [23]. The still weaker condensation caused by hopane glycolipid should be an effect of the additional fatty acid in the molecular structure, which will possibly further disturb hydrophobic interactions.

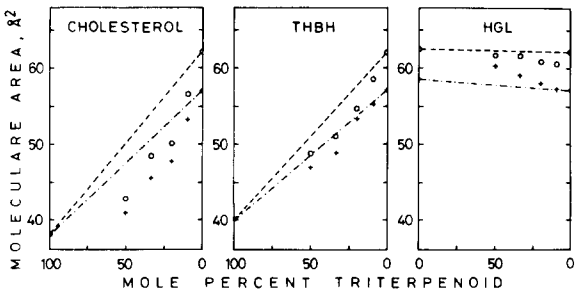

Fig. 3. Effect of cholesterol, THBH and hopane glycolipid (HGL) on the molecular areas of DCDPC at varying molecular ratios of triterpenoid to lipid at a surface pressure of 25 dyn/cm; ------, calculated areas at 40°C; ·–·–·, calculated areas at 4°C; O, measured areas at 40°C; +, measured areas at 4°C; the standard error margin is ±1 Å.

As already mentioned, the monolayer film of DCDPC at 4°C in a more condensed state can still

be further condensed by cholesterol. It seems likely that the rigid cholesterol ring system influences the straight acyl chain part, while the flexible sidechain [24] either does not extend to the cyclohexyl rings or is not able to perturb the packing of these rings. The explanation for the weak THBH influence at 4°C will probably be the same as that given for the effect on the expanded film. Hopane glycolipid, however, with its additional fatty acid, is extremely bulky in the hydrophobic core region and thus seems unable to integrate into the more ordered DCDPC film.

In various lipid systems, hopanoids are able to condense film packing and hence to increase molecular order [3–5]. Therefore, they are believed to be cholesterol-like molecules with an analogous function in prokaryotic membranes [3]. In general, the influence of THBH and hopane glycolipid on the DCDPC film verify this interpretation. However, in other phospholipid systems these hopanoids show more pronounced effects (Ref. 5, and unpublished data). Further investigations should clarify whether this is due to special molecular properties of DCDPC and if the physiological acyl chain-length of *B. acidocaldarius* lipids will optimize the molecular interactions with hopanoids.

On the basis of our monolayer results it is likely that hopanoids should influence the barrier properties of biological membranes. Therefore, we studied the influence of hopanoids on the glycerol permeability of liposomes. According to previous reports [20,25], liposomes of different lipid composition behave as practically ideal and comparable osmometers. This was shown with liposomes of different lipid composition. A change of liposomal volume caused by the penetration of a solute can be measured as a change in light-scattering or turbidity [25]. Hence, the initial change in absorption, i.e., the initial swelling rate of the liposomes, is a measure for the permeability of a solute [20]. For our permeability studies we used diplopterol instead of THBH. The hydrophobic part of the diplopterol molecule is 3.5 Å shorter than that of THBH. Nevertheless, both molecules have similar properties as shown by the monolayer results (Fig. 2). This was further confirmed by Benz et al. [14] who compared diplopterol with hopane monol. Hopane monol in its hydrophobic part is identical to THBH but has a 2-hydroxyethyl sidechain. As shown by the influence on the translocation rate of a cation-carrier complex in black lipid membranes of dioleoylphosphatidylcholine, both triterpenoids reduce the microviscosity of the lipid film, although diplopterol acts in a somewhat more pronounced manner.

In Fig. 4, the influence of cholesterol and hopanoids on the permeability of glycerol through egg PC bilayers is shown. In the temperature region studied, egg PC is in the liquid-crystalline state. Cholesterol strongly reduces glycerol permeation and decreases the temperature dependence of the permeability. These findings are in accordance with De Gier et al. [20]. Diplopterol shows the same effects as cholesterol, but to a lesser extent. Due to the crystallization of diplopterol mentioned above, the true amount of diplopterol incorporated into the bilayer may be lower than estimated. Thus, a quantitative comparison cannot be made. With hopane glycolipid, egg PC also shows reduced initial swelling rates, with a maximum effect at 20 mol%, while higher concentrations were less effective (data not shown). The reduced effect at higher hopane glycolipid

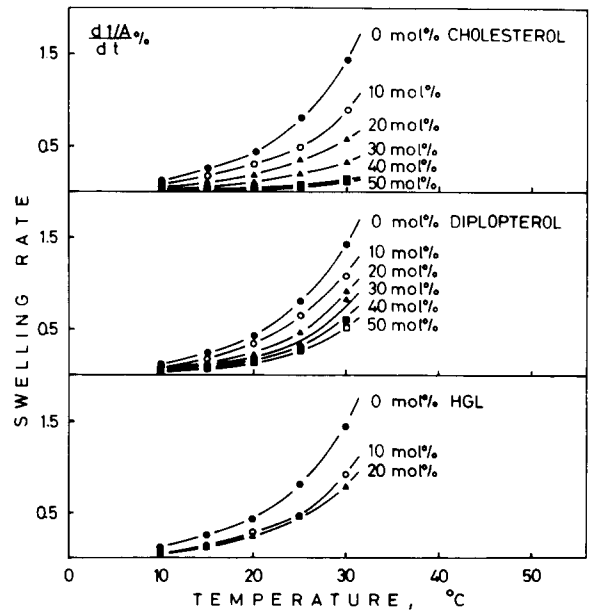

Fig. 4. Initial swelling rate of egg PC/triterpenoid liposomes as function of the temperature and the triterpenoid content. HGL, hopane glycolipid.

concentrations may also be due to crystallization processes in the lipid dispersion. However, up to 10 mol% hopane glycolipid reduces the permeability for glycerol in egg PC just as effectively as cholesterol. The somewhat lower effectiveness of diplopterol in reducing permeation should be due to the molecular structure of diplopterol (Fig. 1) and its hydrophobic region which is by 4.2 Å shorter than cholesterol. The striking reduction of glycerol penetration rates by small amounts of hopane glycolipid may be a consequence of an almost homogeneous distribution of hopane glycolipid in the bilayer structure. At higher concentrations, hopane glycolipid may separate by lateral diffusion due to a low solubility in egg PC or may not be incorporated into the bilayer as a consequence of microcrystallization during the liposome preparation.

These findings are confirmed by results obtained with black lipid membranes of dioleoylphosphatidylcholine [14]. According to this study, the influence of diplopterol on the microviscosity is slightly smaller than that of cholesterol, but at higher concentrations cholesterol is much more effective. However, low concentrations of hopane glycolipid reduced the microviscosity stronger than cholesterol, while higher concentration were less effective. A similar result was published by Bisseret et al. [26]. Thus, the permeability of water in dimyristoylphosphatidylcholine vesicles is significantly reduced by THBH, but again less effectively compared to cholesterol. In addition, monolayer condensation of THBH in egg PC was observed (data not shown), but again the reduction in the molecular area was smaller than the reduction caused by cholesterol [27,28].

In general, the results in DCDPC/triterpenoid-liposomes mimic those of egg PC, but they are shifted to higher temperatures. In contrast to the egg PC findings, high cholesterol concentrations reduce the glycerol permeability less effectively in DCDPC liposomes. Furthermore, diplopterol up to 20 mol% reduces glycerol permeabilities almost as well as cholesterol, but the effect of hopane glycolipid is weaker than in egg PC (Fig. 5). The low permeability of DCDPC [12,14] is further reduced by cholesterol incorporated into the bilayer structure, which should be due to the ability of cholesterol to condense the acyl chain region

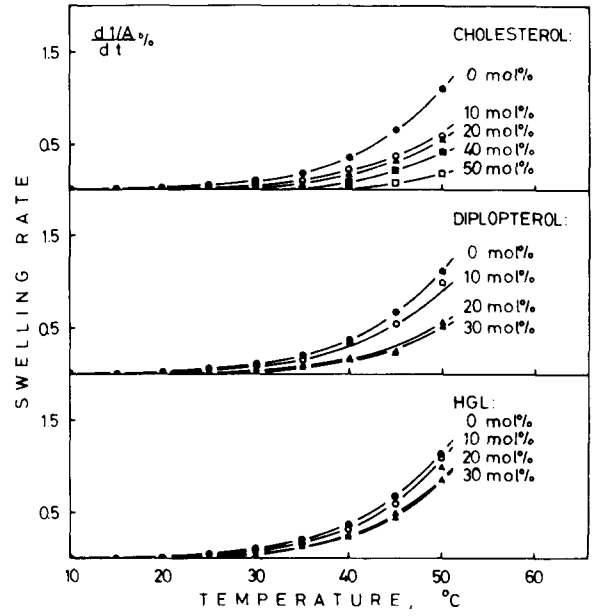

Fig. 5. Initial swelling rate of DCDPC/triterpenoid liposomes as functoin of the temperature and the triterpenoid content. HGL, hopane glycolipid.

located near the glycerol backbone [22]. Diplopterol shows a strong effect because of its shorter hydrophobic ring system, compared with hopane glycolipid/THBH. As a consequence, the molecule can fit well between the acyl chains without coming into contact with the bulky cyclohexane rings. Obviously, hopane glycolipid is unable to fit into the bilayer structure of DCDPC in an optimal way. This may be due to the bulkiness of the hopane glycolipid molecule and the length of its chain, which could extend down to the center of the bilayer and perturb the packing of the cyclohexyl residues.

Our monolayer data agree with the results from the permeability measurements. They are further confirmed by Benz et al. [14], who found a stronger reducing effect of diplopterol on the microviscosity of DCDPC membranes compared to hopane monol, which has the same extension of the hydrophobic region as hopane glycolipid.

There is a well-established relationship between membrane fluidity and the permeability for small non-electrolyte molecules [22]. Hence, the reduced permeabilities of egg PC and DCDPC liposomes

with incorporated hopanoids suggest that membrane fluidity and the degree of order in the liquid-crystalline state increase. This is directly shown by our monolayer results. Cholesterol stabilizes eukaryotic membranes and regulate their fluidity [22]. In general, prokaryotic membranes do not contain cholesterol, but there is a wide distribution of hopanoids in cytoplasmic membranes of cyanobacteria, rhodospirillaceae, methylotrophes and others [1,2]. Rohmer et al. [2] suggested that hopanoids are functional analogues and phylogenetic precursors of cholesterol. The functional analogy of hopanoids and cholesterol is further confirmed by our findings which show that THBH and hopane glycolipid condense DCDPC monolayers and that diplopterol and hopane glycolipid reduce the permeability of non-electrolyte molecules in egg PC and DCDPC.

As stated by Demel and De Kruyff [22], the structural requirement for sterols which are necessary for setting fluidity and permeability in eukaryotic membranes are a 3-β-hydroxy group, a planar configuration of the ring system and an intact sidechain. Our results with diplopterol, THBH and hopane glycolipid suggest that these requirements are less stringent. It may be sufficient to have a molecule with a hydrophilic head, a planar ring system and a hydrophobic region of almost 14 Å length.

To sum up, we can say that the role of hopanoids in model membranes as cholesterol-like molecules is further established. Together with lipids containing ω-cyclohexane fatty acids [12,14] they may be important for forming a biological membrane stable enough to withstand the extreme temperature and pH conditions to which thermoacidophilic bacteria have adapted themselves. Our growth experiments with *B. acidocaldarius,* which increases the hopanoid content in response to elevated temperatures at low pH [9], confirm this interpretation.

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