SPECULATIONS ON THE EVOLUTION OF STEROL STRUCTURE AND FUNCTION

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The question is rarely asked, why is it that only certain chemical structures and not others are present in living systems? Or, put differently, can we rationalize the detailed design and architecture of molecules that evolution has selected from an essentially unlimited organic reservoir? As the starting point in our query and in the absence of other plausible hypotheses, we adopt the Oparin-Haldane hypothesis, postulating that extant cell constituents or their prototypes were created from inorganic molecules present in the primitive terrestrial atmosphere before life began. These prebiotic and hence chemical syntheses leading to a diversity of products must have been random or chance events limited only by thermodynamic stability. Thermodynamic stability can be invoked, for example, for the selection of glucose as the universal cellular fuel. Glucose, the most stable of the 16 isomeric hexoses with its bulky substituents in equatorial positions, had the greatest chance to accumulate in the primordial soup. However, the stability argument does not lead us very far. It certainly does not explain the universal selection of 20 invariant amino acids of varying stability for protein synthesis. Indeed, no one has ventured to rationalize, e.g., the choice of alanine over α-aminobutyric acid or of leucine and isoleucine over norleucine as protein constituents.

The structure of the sterol molecule, which I have chosen for discussion, seems even more perplexing and to defy rationalization at least at first sight. Presently, I will attempt to show that evolutionary pressures shaped the sterol structure as it now exists. However, first I wish to argue one fundamental point which follows from one of the corollaries of the Oparin-Haldane hypothesis. Granting the absence of oxygen in the prebiotic atmosphere, we can exclude the possibility that cholesterol and related sterols were among the numerous products of chemical evolution. Oxygen is essential for sterol formation on purely chemical grounds, and for this reason alone, one cannot envision the arrival of the sterol structure prior to the existence of aerobic cells.

We will assume that lipids along with other organic molecules were formed under prebiotic conditions. Early precursors of the sterol molecule may have been among them, but given the restriction of anaerobiosis, the acyclic squalene (arising perhaps from acetic acid via isoprene and polyprenols) is the most complex of the precursors that could have been formed prebiotically. From here on, biological evolution must have taken over to develop the sterol pathway.

In biological systems, sterol synthesis occurs in two stages; one, the anaerobic phase terminates with squalene, the acyclic sterol precursor; during the second, aerobic phase, squalene is oxygenated, cyclized, and the tetracyclic product lanosterol modified oxidatively to yield eventually cholesterol or related sterols.

Molecular oxygen provides the sterol hydroxyl function and is the essential electron acceptor during the oxidative removal of substituents at carbon atoms 4 and 14 of lanosterol. That the completion of the sterol pathway occurred only after aerobic cells came into being therefore follows from both chemical and evolutionary arguments.

The main object of this discussion is to rationalize on functional grounds the transformation of lanosterol, the "earliest" sterol biogenetically, to cholesterol, the end product. Lanosterol shares with cholesterol the stereochemistry of the A/B/C/D ring

system, the side chain, and also an equatorial hydroxyl group. It differs from cholesterol only by having three extra methyl groups at the sterol underside or α-face. Since in cell membranes, whether of plant or animal origin, the cholestane derived structures ordinarily predominate, it is tempting to postulate that the structural modifications occurring at the α-face were not chance events, but selective, rendering the sterol molecule more effective functionally.

First of all, evidence from the behavior of some in vivo systems shows that the lanosterol molecule is a metabolic intermediate rather than an end product, unable to replace cholesterol in sterol-requiring eucaryotic cells. Such evidence exists for anaerobic yeast, pupating insects, and certain mutant animal cell lines. (1) Yeast kept under strictly anaerobic conditions will not grow in the absence of oxygen without a sterol supplement because anaerobically the cyclization of squalene cannot proceed. This requirement for an exogenous sterol source is fully met by cholesterol, whereas lanosterol and a derivative which still retains one of the methyl groups at C_4 (4-α-methylcholesten-8-ol) sustain growth of such cells only marginally.[1] (2) Growth and development of most insects is sterol-dependent because the entire sterol pathway is deleted. Investigating the sterol specificity for pupation of the hide beetle *Dermestes vulpinus*, Clark and Bloch found that cholesterol fully supported the developmental process, but lanosterol and 4,4 dimethylcholesterol did not.[2] The most recent evidence showing the inadequacy of methylated cholesterol precursors for eukaryotic cells comes from a paper by Vagelos and collaborators.[3] A mutant of Chinese hamster ovary cells which is blocked in one or more of the demethylation steps requires sterol for growth. Whereas wild type cells produce large amounts of cholesterol, the mutant synthesizes only squalene and lanosterol. When no exogenous cholesterol is supplied, the mutant cells lyse very rapidly and die. As these examples show, lanosterol per se is functionally incompetent in eukaryotic cells, whether animal or microbial.

Artificial phospholipid membranes (liposomes or vesicles) have for some time been popular as model systems for studying a variety of membrane properties as a function of lipid composition, including the presence or absence of sterol. Using glucose permeability as a parameter, van Deenen and associates have been able to define various structural features in the sterol molecule that modulate the fluidity of such artificial membranes. Principally, these are a rigid *trans*-fused tetracyclic ring system, the presence of a branched aliphatic side chain, and an equatorial hydroxyl group.[4] The postulate that lanosterol may not be functionally equivalent to cholesterol in membranes has now been tested in the same model system. Whereas the incorporation of cholesterol (at 50 mol%) into lecithin vesicles reduces the release of entrapped glucose from 50 to 3 to 5% (in 1 hr), lanosterol has essentially no effect on glucose release even though it readily enters the membrane.[5] The behavior of partially demethylated lanosterol intermediates that still retain one or two alkyl substituents at C_4 is more cholesterol-like. They are only slightly less effective than cholesterol in reducing glucose release. These results point to the presence or absence of a 14-α-methyl group as the principal structural feature that is responsible for the disparate membrane effects of lanosterol and cholesterol.

Recent ^{13}C NMR experiments carried out in collaboration with Yeagle and Martin (University of Virginia)[6] lead to the same conclusion. In mixed vesicles containing cholesterol and egg lecithin, resonances attributable to sterol carbon atoms cannot be detected, evidence that the cholesterol molecule is completely immobilized in the phospholipid bilayer. However, when lanosterol replaces cholesterol in the lecithin vesicles, resonances appear which can be assigned to various sterol carbons. Hence lanosterol, the 4,4',14-trimethyl cholestane derivative is much more mobile in the membrane than cholesterol. The reason why membrane-imbedded cholesterol is immobilized and lan-

osterol is not is readily understood on inspection of models built with specified spatial relationships between sterol and phospholipid acyl chains in the membrane bilayer. I will elaborate on these models later on.

Next, we raise the question why, in the course of the evolution of cells, the lanosterol structure was metabolically modified in a highly specific manner, i.e., by removal of substituents at C_{14} and C_4 while other methyl groups, notably the angular methyl groups between rings A and B and C and D, invariably remained untouched in membrane-associated sterols. Why conservation of substituents at the β-face of the ring system and elimination at the α-face?

Models for complimentary interactions between membrane sterols and phospholipids date back to the 1950s[7] and appear to have been prompted in part by the 1:1 stoichiometry of cholesterol and phospholipidin the red cell membrane. In essence, the early models and their more recent versions[8] postulate noncovalent van der Waals interactions between the bulky and planar ring system of the sterol molecule and phospholipid acyl chains. If the sterol is inserted between the acyl chains of two phospholipid molecules, such that the hydroxyl group is situated at the membrane-water interface and the isooctyl chain points towards the bilayer interior, then the principal and effective point of contacts will be between the tetracyclic ring region and the proximal segment (near the carboxyl group) of the fatty acyl chains. The planarity of the cholesterol α-face is especially favorable for such interactions. While the β-face of the sterol molecule is slightly puckered, it also allows for van der Waals contacts. It is important to note that in the molecular model, the two angular methyl groups do not noticeably interfere sterically with acyl chain packing. Space-filling models built along the lines discussed make it clear at once why lanosterol, unlike cholesterol, fails to be immobilized in the membrane bilayer. The 14α-methyl group of lanosterol is axial, protruding from the otherwise planar α-face. Immobilizing van der Waals interactions of the sterol underside with fatty acyl chains are therefore very much less favorable.

We have no ready explanation as yet why the biological demethylation process starting with the 14α-methyl group continues and removes the methyl groups at C_4 as well. Judging from space-filling models, the latter methyl groups do not interfere sterically with the postulated α-face interactions; conceivably, one effect of these methyl groups at C_4 is to weaken the hydrogen bonding capacity of the neighboring hydroxyl group. It is widely assumed,[9] although not proven, that a free OH group of the sterol molecule is essential for hydrogen bonding to water or to either one of the carbonyl functions in the polar phospholipid head group. There is no obvious experimental approach for testing the hypothesis that alkyl substituents at C_4 interfere with this process. At this moment, therefore, we must content ourselves with a rationale for the demethylation of the lanosterol structure at the 14α-position as least as far as membrane function is concerned. It should be noted that this methyl substituent is the first to be removed in the contemporary sterol pathway.

As pointed out already, no gain in terms of improved van der Waals interactions is to be expected by eliminating the angular methyl groups at the sterol β-face. In fact, the opposite can be shown to be the case.[5] When the viscosity of sterol-containing lecithin vesicles is measured by fluorescence depolarization with the probe diphenylhexatriene, cholesterol shows the expected large viscosity increase. 19-Norcholestanol, a synthetic sterol that lacks a methyl group at the β-face, also raises membrane viscosity, but significantly less than cholesterol. The fact that demethylation of lanosterol is selective and confined to the sterol α-face can therefore be ascribed more explicitly to molecular streamlining directed at perfecting the sterol molecule for membrane function. It is worthy of note that no existing sterol, natural or synthetic, surpasses the effectiveness of cholesterol in modulating membrane properties that are ordinarily measured.

Many details of the general model for cholesterol-sterol interactions remain to be defined. For example, one of the unresolved details is whether or not hydrogen bonding of the hydroxyl group is essential for anchoring the sterol in the bilayer, and if so, whether the hydrogen bond involves some element of the polar phospholipid head group directly or by way of water. Another point of interest raised by Huang[10] is whether saturated and olefinic fatty acyl chains interact selectively with the α- or β-faces of the sterol ring system, respectively. Huang argues that the angular methyl groups in contact with an olefinic chain may induce a conformational change from all *trans* to *trans-gauche* e.g., at C_{11} of oleic-acid. The hydrophobic pocket generated by the *trans-gauche* kink would be a highly favorable accomodation site for the β-face angular methyl groups. A preferential packing of olefinic chains to one of the sterol faces and of saturated chains to the other may have bearing on one problem that has long intrigued lipid biochemists. In the majority of membrane phospholipids, the C_2 glyceride position is esterified predominately with olefinic acids and the C_1 position with saturated fatty acids. Perhaps this nonrandom distribution can be rationalized by refining the model with respect to preferential sterol-phospholipid interaction sites.

Until recently, the sterol molecule was believed to be an invention of eukaryotic life. In fact, it was only a few years ago that a sterol containing prokaryotic organism was identified, and the case has remained unique. The organism in question, the obligate methanotroph *Methylococcus capsulatus* (Bird et al.[11]), first of all contains sterols in amounts comparable to those found in eukaryotic cells. Secondly, and bearing more importantly on sterol evolution, the sterols found in this organism are partially demethylated lanosterol derivatives, i.e., 4,4′-dimethyl- and 4-monomethyl cholestanols. The fully demethylated structures characteristic of eukaryotic cells appear to be absent. It is therefore tempting to argue that the sterol pathway, although it began in some prokaryotic cells, was fully developed only by eukaryotes. The properties of *Methylococcus* membranes and the role that the more "primitive" methyl-cholesterol derivatives play in this organism remain to be investigated.

Procaryotic sterol producers other than *Methylococcus* have not yet been found. Equally rare are examples of prokaryotic organisms that require an exogenous sterol source, but one exists. For many strains of *Mycoplasma*, bacteria without cell walls, a sterol such as cholesterol is an essential growth factor.[12] These organisms have proven uniquely useful for studying sterol structure-function relationships in a prokaryotic membrane. According to earlier work, sterols which like cholesterol raise the viscosity of artificial membranes also support the growth of *Mycoplasma* strains.[12] Data we have obtained so far with *Mycoplasma capricolum* show that for this organism at least, the sterol specificity is very much broader. The results were puzzling at first, but ultimately gratifying because they provide further support for the hypothesis of a gradual or stepwise evolutionary improvement of the sterol structure.[13]

First of all, lanosterol and its partially demethylated derivatives support *Mycoplasma* growth nearly as well as cholesterol. By contrast, as we have seen, lanosterol is without effect on the fluidity of artificial membranes, and it appears unable to replace cholesterol in eukaryotic systems in vivo. It is also intriguing that alkyl-substituted sterols of the type synthesized in one procaryote (*Methylococcus*) are adequate as a sterol source for another that requires sterol (*Mycoplasma*). Included in our screen were two (synthetic) sterol derivatives which unexpectedly supported *Mycoplasma* growth. They were, 3-α-methylcholestanol, a sterol that has a nonplanar α-face and should therefore be relatively mobile in a membrane, and secondly, cholesteryl methylether, a sterol that lacks the free OH group which is generally considered essential for membrane function.

It is important to note that without exception the sterols which support mycoplasma

growth are utilized per se and recovered unchanged from the bacteria. At least some of the unusual structures of the sterols that support mycoplasma growth are such as to suggest that they are relatively mobile in membranes, judging from their behavior in phospholipid vesicles. Since sterols are generally believed to function by raising membrane viscosity and since this effect appears to be correlated with sterol immobilization, our results would seem to suggest an unconventional role for sterols in *Mycoplasma* membranes. One such role may be to effect the separation of the phospholipid head groups which necessarily occurs when the bulky sterol molecule enters the bilayer. NMR studies show that lanosterol as well as cholesterol cause head group separation in phospholipid vesicles. However, only cholesterol effects membrane fluidity.[6] It seems possible that a variety of squalene-derived cyclization products, all bulky and rigid, but differing in structural detail are capable of causing head group separation and that this represents a more primitive membrane function of the sterol molecule and perhaps of other squalene-derived polycyclic structures.

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