# STEROL STRUCTURE AND MEMBRANE FUNCTION

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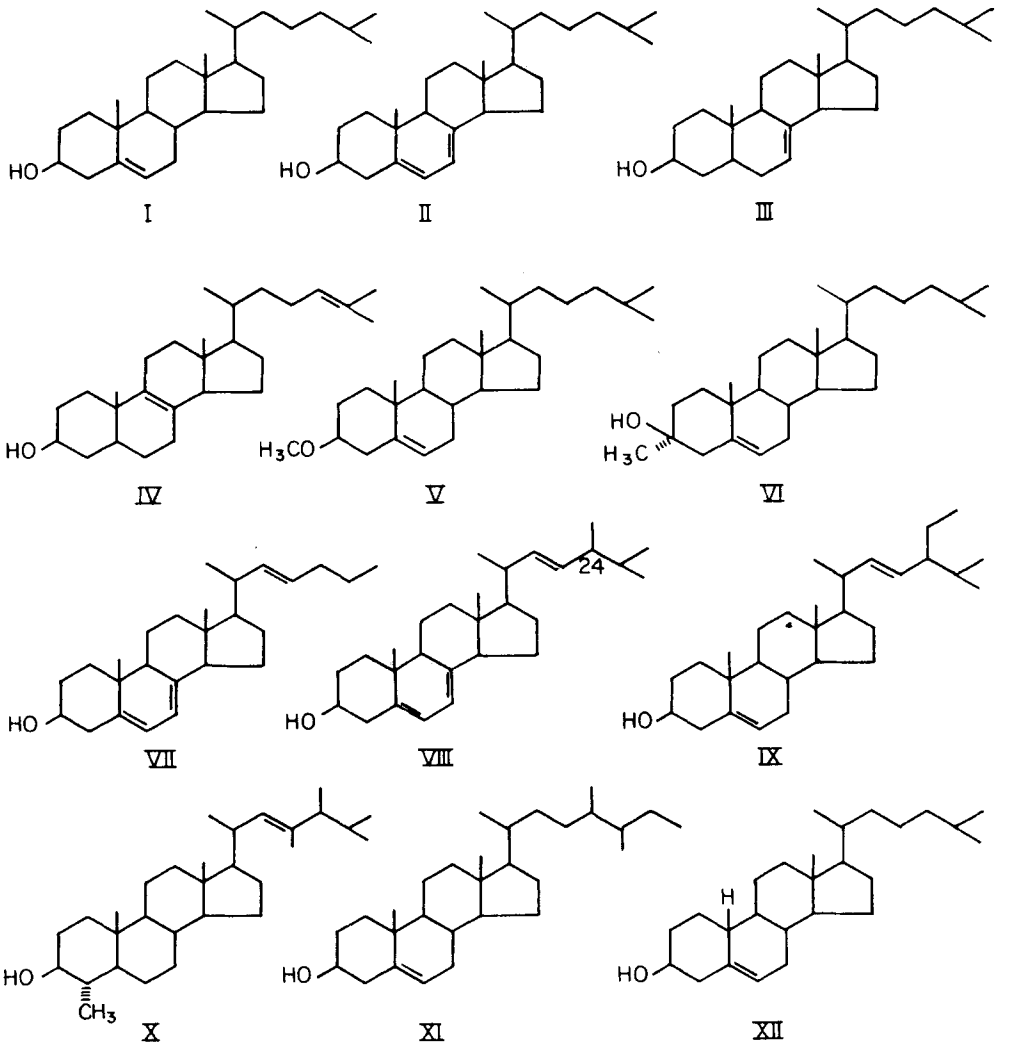
## I. INTRODUCTION

This review deals with sterol structure-function relationships in natural and artificial membranes covering for the most part research carried out in this laboratory during the last 4 years. It is assumed, in the absence of any evidence to the contrary, that whatever role the sterol molecule plays as a membrane bilayer component, only noncovalent interactions are involved. Measurable membrane effects are due to the sterol itself, cholesterol primarily in animals, or side-chain alkylated sterols in fungi and plants; nor are the intramembrane interactions associated with chemical modifications of the sterol structure. Structural effects, manifestations of which are seen wherever the sterol molecule is present in bulk (20 to 50 mol %) are clearly distinct from the regulatory effects of steroids, the sterol-derived hormones, or vitamin D derivatives and perhaps other oxygenated sterols all of which serve as metabolic signals in minute amounts. There is no evidence for a structural membrane role of steroids nor, conversely, for hormonal function of the sterol molecule per se.

In a largely speculative essay, antedating the experimental work to be described,[16] the cholesterol molecule was scrutinized with the object of rationalizing the contribution of over-all architecture, geometry, and presence or absence of nuclear methyl substituents to membrane function. The principal conclusions reached included:

1. Sterols, unlike most major cell constituents were generated in the course of biological evolution with the essential aid of oxygen.
2. Prebiotic processes, operating in an anaerobic environment might have produced squalene, the acyclic sterol precursor, but could not proceed further along the route to the sterol structure. On purely chemical grounds, an anaerobic pathway to sterols similar to those found in contemporary cells cannot be visualized.
3. Following aerobic cyclization of squalene to lanosterol (XIII — The structural formulae of the sterols mentioned in this review are shown in Figures 1 and 2 and are indicated by roman numerals when first mentioned in text), selective demethylations occur at the sterol $\alpha$-face streamlining the sterol structure. Successive methyl group removal imposed by selection pressures rendered the sterol molecule progressively more and, ultimately, optimally competent for membrane function.
4. The sterol pathway terminates with cholesterol, a molecule designed to optimize attractive van der Waal's interaction with phospholipid acyl chains in the membrane bilayer.

In essence, the question raised was whether one can discern in the contemporary sterol pathway and in the temporal sequence of modifying events a directed evolutionary process operating on a small molecule and, if so, whether each step of the sequence




| | | | |
|---|---|---|---|
| I | Cholesterol | VII | 5, 7, 22-Cholestatrienol |
| II | 7-Dehydrocholesterol | VIII | Ergosterol |
| III | $\Delta^7$-Cholestenol | IX | Stigmasterol |
| IV | Zymosterol | X | Dinosterol |
| V | Cholesteryl-methyl ether | XI | Aplysterol |
| VI | 3$\alpha$-methylcholesterol | XII | 19-Norcholesterol |

FIGURE 1. Cholesterol and cholestane derivatives.

produces a molecule functionally superior to its precursor. To provide the essential background for this examination, some of the relevant biochemical events will first be recapitulated.

### A. The Lanosterol-Cholesterol Conversion

In animal tissues and yeast, lanosterol demethylation (for reviews see Bloch,[15] Clayton,[34] Nes and McKean[65]) occurs by way of the principal intermediates shown in Figure 3: lanosterol → 4,4 dimethyl-$\Delta^8$-cholestenol → 4$\alpha$-methyl-$\Delta^7$-cholestenol

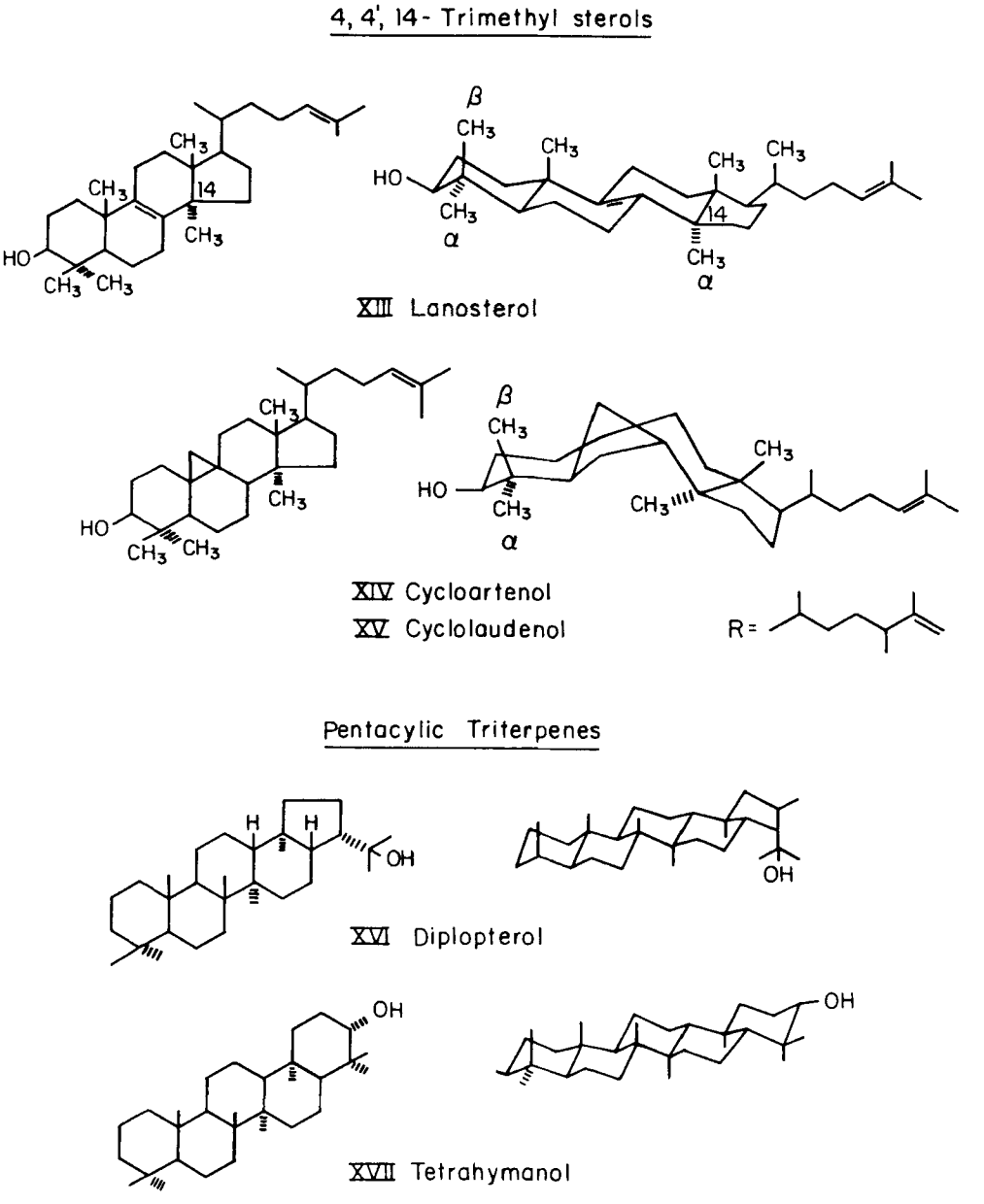



FIGURE 2. Trimethylsterol products of 2,3 squalene-epoxide cyclization and pentacyclic triterpenes, formed by direct squalene cyclization.

(lophenol) → cholesterol. The number of participating enzymes, all microsome-associated, can be estimated but it is immaterial for the purposes of the present discussion. Also, the identity of the various oxygenated intermediates formed prior to removal of the methyl carbon $C_{32}$ at $C_{14}$ as formate and of the two $C_4$ ($C_{30}$, $C_{31}$) methyl groups as $CO_2$ need not be considered here. Crucial, however, for the arguments to be presented is that the methyl group at $C_{14}$ which is axial and projects from the sterol $\alpha$-face, is the first of the three to be eliminated. In the case of lanosterol metabolism, removal of the gem-demethyl group always follows, never precedes demethylation at $C_{14}$. This involves, initially, oxidative removal of the $\alpha$-oriented alkyl substituent.

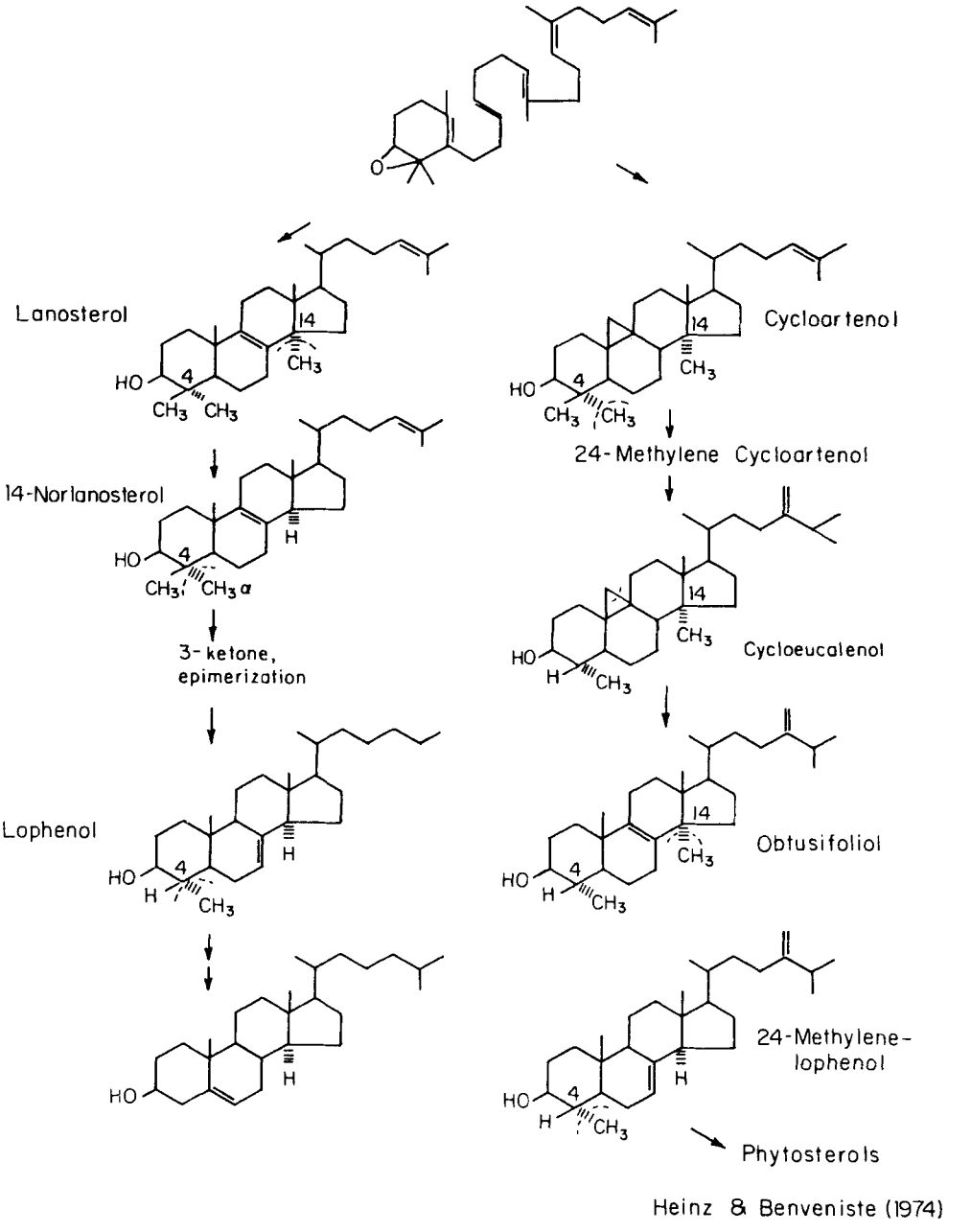



FIGURE 3. Sequence of sterol $\alpha$-face demethylations, lanosterol, left; cycloartenol, right.

Unexpectedly, the methyl group that remains is also $\alpha$-oriented. The explanation for this apparent paradox is as follows. Early evidence indicated a conversion of the 3-OH group to a ketone function during 4,4′-demethylation.[75] In more definitive experiments with stereospecifically labeled 4,4′-dimethyl cholestenols, several steps were shown to intervene. Following removal of the $4\alpha$-$CH_3$ substituent from the 4,4′-dimethylstenol, the remaining, originally $\beta$-oriented alkyl group epimerizes to the $4\alpha$-monomethyl compound by way of 3-keto-4-methylcholestenone.[106,130] Two explanations, one invoking enzyme specificity and the other evolutionary arguments, can be offered for this

circuitous route. A single α-specific 4-demethylase system brings about removal of both $C_{30}$ and $C_{31}$. This would necessitate inversion of the second, originally β-oriented methyl substituent prior to oxidation. Alternatively, formation of a discrete 4β-monomethyl intermediate may have been rejected as unproductive on the grounds that this epimer is not functionally superior to its 4,4′-dimethyl precursor. In support of this evolutionary argument, 4β-monomethyl derivatives have not been found in nature nor are they demethylated in any biological system. Only the 4α-monomethyl epimer has been isolated, occasionally in substantial amounts (lophenol, dinosterol, X). The notion of a single, α-specific demethylase is not ruled out but if valid would require that the enzyme — not available in pure form — metabolizes the 4,4′-dimethyl compound more effectively than the 4-monomethyl derivative.

Plant systems cyclize squalene epoxide to cycloartenol (XIV). Arguments attempting to explain why cycloartenol demethylation (Figure 3) takes a different course will be presented in Section III.

### B. Lanosterol Demethylation Improves Membrane Function

The above analysis of the sequential events in trimethyl-sterol metabolism implies that it is not chemical or thermodynamic facility which determines the order of methyl group removal but that the order is imposed by evolutionary pressures. "Incompetent" structures ultimately are transformed into a molecule that functions optimally in membranes. Beginning with lanosterol, each demethylation step produces an intermediate superior to its predecessor.

Some early studies already noted the inadequacy of lanosterol for sterol-requiring organisms. Yeast turns into a sterol auxotroph when grown under strictly anaerobic conditions.[3] Cholesterol and ergosterol (VIII) satisfy this requirement but lanosterol does not.[80,86,87] In most if not all of the Insecta, the entire sterol pathway is deleted[32] and here as well, lanosterol fails to substitute for cholesterol.[29-31] Most convincingly, demethylase mutants of Chinese hamster ovary cells which accumulate squalene and lanosterol are not viable but can be maintained on cholesterol.[28] While it appears that in all of these instances the failure to transform lanosterol to cholesterol causes cell death, membrane incompetence of lanosterol may not be the only cause. In some animal cells failure to produce essential cholesterol-derived hormones (e.g., ecdyson in insects) may be a growth-inhibiting factor. Finally, in animal tissues at least, intracellular lanosterol cannot be found in amounts sufficient to make a significant contribution to bulk membrane function.[119]

### C. Choice of Criteria for Membrane Fitness

Sterol-induced changes in membrane fluidity or solute permeability are widely viewed as meaningful indices of sterol function in vivo. Much experimental evidence in support of this notion exists but it should be kept in mind that the commonly used methods monitor only bulk fluidity. There may be localized membrane regions or domains of substantially lower or higher sterol content differing in fluidity from the bulk phase. Such domains of low cholesterol content have been recognized by freeze-fracture electron microscopy of fibroblasts.[82] They are thought to correspond to the coated pits found in invaginating regions that serve as specific LDL-binding sites.[96] Since receptor-bound LDL is subsequently internalized, a low cholesterol content and presumably high fluidity may be characteristic for membrane regions concerned with endocytosis of external ligands.

The phenomenon of sterol synergism to be discussed later (Section IX.) also points to sterol heterogeneity in membrane bilayers and, moreover, suggests that bulk fluidity, a structural parameter, is not the only sterol-controlled membrane property. In some

systems at least, sterols may function metabolically as well as structurally by regulating the biosynthesis of membrane phospholipid.

While these qualifications should be kept in mind, bulk fluidity measurements have provided a wealth of useful information. In many though not all instances, they have shown remarkably close correlations with physiological parameters, especially the growth response of sterol auxotrophs.

## II. STEROL EFFECTS ON ARTIFICIAL MEMBRANES

Sterol-induced changes in membrane fluidity can be conveniently monitored by determining either some transport function, e.g., solute permeability, by following changes in the spectral properties of some appropriate probe (spin-labeled fatty acids, fluorescent probes) or by $^{13}$C-NMR.

In the first systematic study of sterol effects on artificial membranes, Demel et al.[42] measured the rate of efflux of liposome-entrapped glucose as a function of sterol structure and concentration. Cholesterol at 50 mol % completely arrests this process.[42] The absence of a sterol double bond or shifting it to the $\Delta^7$-position only slightly diminishes the sterol effect. This effect is however totally abolished by changing the ring A/B geometry from *trans* to *cis*, as in coprostanol, by inverting the equatorial to an axial 3$\alpha$-OH group, or replacing it by carbonyl and finally by deletion of the isooctyl side-chain. These more drastically modified structures render the vesicles as permeable to glucose as they are in the absence of sterols. Of all the compounds examined cholesterol was the most effective in modulating the passive movement of solute across the bilayer.[43]

Membrane effects of various alkyl-substituted sterol derivatives were initially compared with those of cholesterol by measuring the exit rate of glucose entrapped in phospholipid liposomes (multilamellar) or vesicles (unilamellar).[72] The results (Table 1) will be discussed only briefly because the method is less precise and sensitive than others, e.g., fluorescence depolarization. In essence, at 50 mol %, cholesterol reduced glucose permeation from 50% (sterol-free liposomes) to 5%/hr at 40°. Lanosterol at the same molarity lowered the control value only slightly, from 50 to 43%. Sterol uptake is not limiting; lanosterol enters the vesicles as readily as cholesterol. In this system the partially demethylated intermediates, 4,4′-dimethylcholesterol, 4$\alpha$-methylcholesten-7-ol (lophenol) and 4$\beta$-methylcholesterol reduced glucose exit only somewhat less effectively than cholesterol. These early results probably understate the differences between cholesterol and the dimethyl- or monomethyl derivatives. However, it probably is significant that increasing the size of one or both alkyl groups at $C_4$, from methyl to ethyl, renders the sterol less efficient in lowering glucose permeability.

Of the compounds tested, lanosterol and 3$\alpha$-methylcholestanol (VI) have the least, nearly imperceptible effect on glucose permeability; presumably they fail to restrain solute permeation because they do not solidify the membrane significantly. As seen in space-filling models both compounds contain axial methyl substituents at positions which will impair sterol $\alpha$-face interactions with phospholipid acyl chains. Removal of the 14$\alpha$-methyl group from the 4,4′, 14 trimethyl cholestane system renders the molecule distinctly more "cholesterol"-like though not as effective as cholesterol itself. Why the 4,4′-dimethyl derivative is inferior to cholesterol is not apparent from model building. Neither the 4$\alpha$- (equatorial) nor the 4$\beta$-(axial) methyl substituents protrude into the sterol $\alpha$-plane. There are no steric reasons why they should disturb acyl chain packing. That bulk may be the more important factor is shown by the observation that the (unnatural) 4,4′-diethyl analogue or the epimeric 4,4′-methyl, ethyl derivatives decrease glucose permeability even less than the 4,4′-dimethyl sterol. A rationalization of these results will be given below.

**Table 1**
**EFFECT OF 4,4 CHOLESTANE-3β-OL DERIVATIVES ON GLUCOSE RELEASE FROM LECITHIN LIPOSOMES**

| Sterol | Glucose release (%) in 1 hr at 40° C |
|---|---|
| None | 46—50 |
| Cholesterol | 5 |
| Lanosterol | 43 |
| 4α-Methylcholest-7-enol | 8 |
| 4β-Methylcholestanol | 11 |
| 4,4′-Dimethylcholesterol | 11 |
| 4,4′-Diethylcholestanol[a,b] | 29 |
| 4α-Ethyl, 4β-methylcholestanol[a,b] | 17 |
| 4α-Methyl, 4β-ethylcholestanol[a,b] | 14 |
| 3α-Methylcholestanol[b] | 50 |

[a] Samples provided by F.T. Nelson and T. Spencer.
[b] Unnatural sterols.

Adapted from Reference 72.

### A. Microviscosity Measurements

Membrane viscosity of phosphatidylcholine vesicles as measured by fluorescence depolarization of the probe diphenylhexatriene rises as a function of cholesterol content.[125] This sensitive and deservedly popular technique permits a valid appraisal of membrane fluidity, provided comparisons are restricted to relative rather than absolute values.

The results of microviscosity ($\bar{\eta}$) measurements performed with unilamellar phosphatidylcholine vesicles (egg yolk) and diphenyl hexatriene as the fluorescent probe are shown in Figure 4.[38] The test sterols were chosen to allow assessment of the effects of single structural changes, either methyl substitution, methyl group orientation, or unsaturation. In accord with the glucose permeability data, cholesterol and cholestonal raised microviscosities to a much greater extent than any of the methyl-substituted sterols or stanols. Lanosterol and dihydrolanosterol gave $\bar{\eta}$ values, only slightly higher than observed with sterol-free control liposomes. Deletion of the 14α-methyl group (4,4′-dimethylcholestanol and $\Delta^5$-stenol) causes a substantial increase in microviscosity.* This structural change — which is also the first to occur in the sequential enzymatic demethylation — affects membrane fluidity more profoundly than dealkylations at $C_4$.*

Only one of the two 4-monomethylcholestanes, the 4α-(equatorial) epimer causes a further rise in microviscosity in the direction of the values obtained with the completely α-face demethylated sterols. Notably, the $\bar{\eta}$ values for 4β-monomethylcholestanol (axial) containing vesicles is the same as for vesicles containing 4,4′-dimethylstanol, i.e., lower than for the 4α-epimer. Clearly, orientation of the methyl substituent as well as bulk determine sterol competence as expressed by a fluidity parameter.

Bulk sterol components of natural membranes contain at least one nuclear double bond situated in ring B, usually in the 5,6 position. In all instances so far examined the

* 14α-methylcholestanol which does not occur in nature has been similarly tested. In concentrations up to 30 mol % it has no greater effect on $\bar{\eta}$ values than lanosterol. This sterol, a gift from C. Djerassi, was not available in sufficient quantity for duplicate experiments.

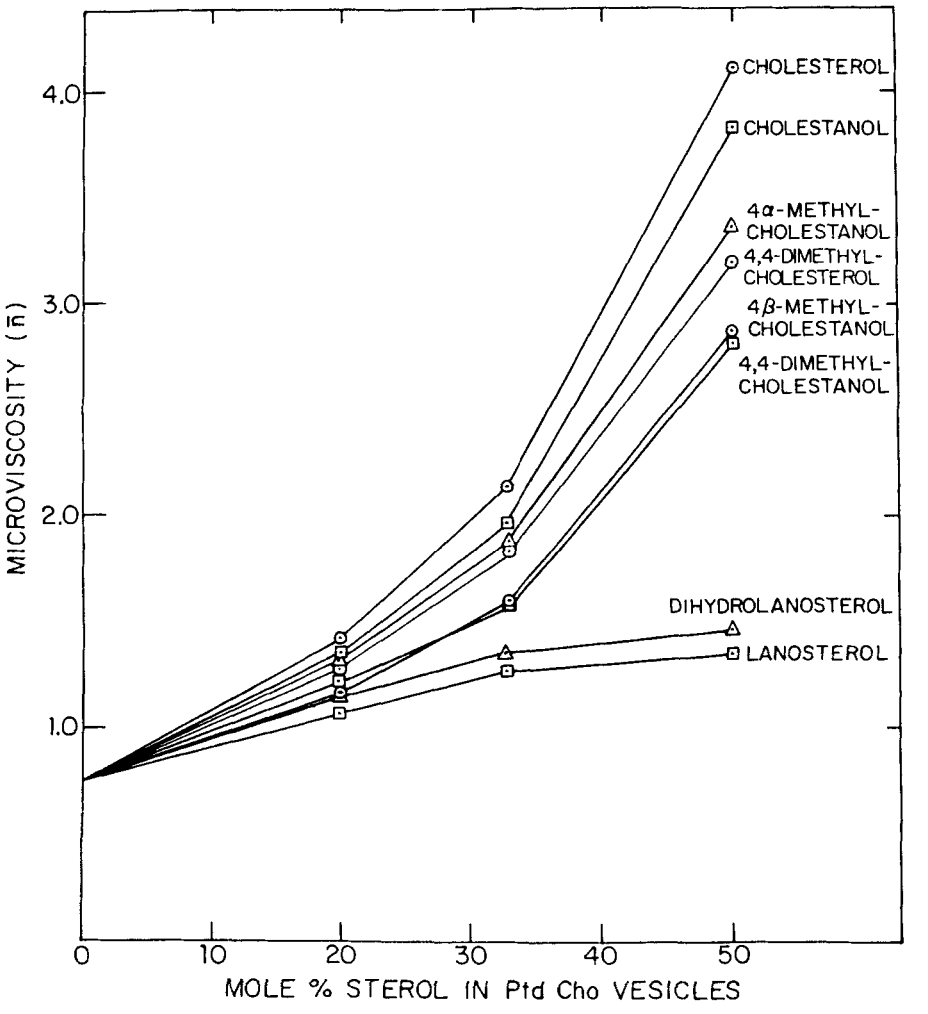



FIGURE 4. Effect of sterol alkyl groups on the microviscosity ($\bar{\eta}$) of phosphatidylcholine vesicles at 25°. (From Dahl, C., Dahl, J., and Bloch, K., *Biochemistry*, 19, 1462, 1980. With permission.)

stenols are somewhat superior to stanols in raising microviscosity.[38] This implies stronger packing with contiguous phospholipid acyl chains even though space-filling models do not reveal why this should be the case. For whatever reason, the naturally occurring stenols show greater membrane competence than their unphysiological stanol counterparts.

Models for sterol-phospholipid associations in membranes currently in favor[51,94,114] predict a weakening of these interactions by bulky groups protruding from the sterol α-face in the lipid-lipid contact regions. In line with this prediction, an exposed 14α-methyl group as in lanosterol or 14α-methylcholestanol interferes markedly with hydrophobic interactions allowing greater motional freedom of fatty acyl chains. The potential contact region in these instances probably is near central fatty acid carbons as ESR measurements suggest (see Section IV.). However, the totally inert membrane behavior of 3α-methylcholestanol (Table 1) suggests that van der Waals contacts will be diminished if not eliminated by any alkyl group projecting from the sterol α-face, i.e., at $C_5$ and $C_7$ as well as at $C_3$ and $C_{14}$. Since the positioning of the sterol molecule does not

appear to be altered when the $\alpha$-hydrogen at $C_3$ is replaced by methyl[37] and since sterol carbon $C_3$ is probably near fatty acyl $C_1$ or $C_2$ it appears that the increase in motional freedom is not restricted to the immediate contact site but transmitted to nearby and perhaps even more remote segments of the fatty acyl chain.

**B. The Sterol $\alpha$-Face**

We have attached evolutionary significance to the selectivity of biological lanosterol demethylation, emphasizing the fact that with rare exceptions membrane sterols retain the bridgehead or $\beta$-face methyl groups of the cholestane ring system.[16] If the argument is correct that no selective pressures existed for demethylation at the $\beta$-face then sterols lacking either methyl group $C_{18}$ or $C_{19}$ should be functionally equivalent or inferior to cholesterol. Support for this contention may be seen in the membrane behavior of one of these norsterols. Phospholipid vesicles containing 19-nor-cholesterol (XII) are far more fluid ($\bar{\eta} = 2.6$ at 40 mol %) than those containing cholesterol ($\bar{\eta} = 5.5$).[72] In the space filling model, three $\beta$-face cholesterol methyl groups ($C_{18}$, $C_{19}$, and $C_{21}$) are seen to lie in a plane* though they are laterally displaced from one another. Because they provide a planar contact region, their presence is likely to be important for sterol $\beta$-face interactions with fatty acyl chains, perhaps at higher sterol concentrations in the membrane. In experiments with sterol auxotrophs (see Section VII.). 19-norcholesterol supports growth somewhat less effectively than cholesterol itself. Clearly, at least one of the $\beta$-face-linked methyl groups contributes significantly to the immobilization of cholesterol in the membrane bilayer.

The fact that $\beta$-face methyl groups contribute positively to viscosity and that they are retained during sterol biosynthesis, unlike methyl groups at the $\alpha$-face, supports the evolutionary argument. Structural modifications that do not confer a selective advantage are avoided or may have been tried but abandoned. As indicated above, there are a few exceptions to this generalization. Sponges[81] (Minale and Sodano, 1974) and Gorgonians[102] contain sizable amounts of 19-norsterols derived from cholesterol demethylation.[45] Whether they are bulk constituents in the membranes of these primitive marine invertebrates or they are involved in a more specialized function is not known. At any rate it seems conceivable that this "over-demethylation" was not adopted or conserved during the evolution of higher animal cells (see Section XIII.).

The importance of the intact sterol $\beta$-face for optimal membrane function may have some bearing on the temporal sequence and stereo-chemistry of the demethylation process at $C_4$. As already noted, the equatorial 4$\alpha$-methyl group lies in the plane of the ring system and therefore should, as it does, interfere only minimally with acyl-chain packing. Its membrane behavior is closest to that of cholesterol. On the other hand, the axially oriented 4$\beta$-methyl group, though attached to an $\alpha$-face carbon is spatially close to the $C_{19}$ methyl group and therefore affects the contour of the sterol $\beta$-face. We postulate that *syn*-diaxial interactions between these two methyl groups may disturb contacts with fatty acyl chains.[38] For that reason the event of higher priority in $C_4$ demethylation is the (de facto) removal of the 4$\beta$- rather than of the 4$\alpha$-methyl group. We see further support for this argument in the fact that whenever 4-monomethyl sterol derivatives are encountered (animal tissues, dinoflagellates, and *Methylococcus capsulatus)* the orientation of the methyl group is always $\alpha$.

* This is true only if the aliphatic side chain bearing $C_{21}$ is attached in *trans*-orientation to $C_{17}$ of the ring system. Nes et al.[87] have shown that only this natural conformer satisfies the sterol requirement of anaerobic yeast.

### C. $^{13}$C-NMR

$^{13}$C-NMR spectroscopy has furnished independent evidence for the contrasting behavior of cholesterol and lanosterol in membrane bilayers. This technique directly measures the motional state of the test molecule. In principle, therefore, NMR is to be preferred to techniques that rely on signals from a probe (spin label or the fluorescent parinaric acids or diphenylhexatriene). It had been shown earlier that lecithin vesicles containing phospholipid and cholesterol in a molar ratio of 2:1 are devoid of resonances that can be assigned to carbon atoms of cholesterol because the molecule is strongly immobilized by interaction with phospholipid.[71] Immobilization produces line-widths too broad to be observed. However when lanosterol replaces cholesterol, distinct resonances not attributable to phospholipid appear in the spectrum.[147] These sterol-derived resonances can be assigned by comparison with the $^{13}$C-NMR spectrum of lanosterol in $CDCL_3$. Distinct resonances arise from $C_8$ and $C_9$ of the nuclear lanosterol double bond, two broad resonances from $C_{13}$ and $C_{14}$, both carrying methyl substituents, and others arising from $C_{18}$, $C_{19}$, $C_{21}$, and $C_{30}$. These are all quaternary or methyl carbons likely to be least broadened by immobilization. Significantly, the $^{13}$C-NMR spectrum of vesicles containing, 4,4′-dimethyl-cholesterol, like that of cholesterol, failed to show any distinct sterol resonances. The main conclusion emerging from this study is that lanosterol is considerably more mobile in the bilayer than cholesterol, in essential agreement with microviscosity data.[38] The absence of sterol resonances in 4,4′-dimethylcholesterol-containing vesicles suggests that this norsterol derivative is more strongly immbolized relative to lanosterol but, as the microviscosity data indicate, not necessarily as tightly as cholesterol. At any rate, the $^{13}$C-NMR data lend greater confidence to the notion that the 14α-methyl group is responsible for preventing immobilizing lanosterol-phospholipid contacts.

Studying the $^{31}$P $^{1}$H nuclear Overhauser effect, Yeagle et al.[145,146] have observed that the intermolecular interactions between positively and negatively charged phospholipid head group moieties are largely abolished by cholesterol. The explanation offered is that introduction of the bulky sterol ring system necessarily increases the distance between neighboring phospholipid molecules. Lanosterol causes the same phenomenon even though it does not interact strongly with fatty acyl chains in the bilayer. The ability of lanosterol to cause separation of phospholipid head groups, a property it shares with cholesterol, points to a separate function of sterols in membranes, unrelated to and distinguishable from fluidity control. Multiple or alternate sterol effects will be considered in Section IX.

## III. LANOSTEROL — CYCLOARTENOL, ISOMERIC TRIMETHYL STEROLS

For reasons still unknown, the pathway to sterols branches at the squalene epoxide stage yielding lanosterol in animal tissues and yeast, and cycloartenol in higher and lower plants.[10,49,109] Both analytical and enzymatic evidence supports this generalization.[85] Lanosterol and cycloartenol are isomeric 4,4′, 14-trimethylsterols differing only in the disposition of the bridgehead carbon $C_{19}$ at the juncture of rings A and B. In cycloartenol, $C_{19}$ is linked to nuclear $C_{10}$ and to $C_9$ as well; a cyclopropane ring takes the place of the free $C_{19}$ methyl group in lanosterol. Presumably the concerted events leading from squalene epoxide to the isomeric trimethyl sterols differ only in the final stage. Proton abstraction from $C_9$ produces the 8,9 double bond of lanosterol while abstraction of H from the $C_{19}$ methyl generates the 9,19 cyclopropane ring of cycloartenol. In cell-free systems also, squalene-epoxide cyclases are highly specific, those from animal tissues and yeast affording exclusively lanosterol while the plant enzymes invariably produce

cycloartenol. It should be noted that thermodynamically the cyclosterol is the less stable isomer. Acid readily isomerizes it to lanosterol and lanosterol double bond isomers.[11]

The inadequacy of drawing projection structures, i.e., without regard to conformation, is nowhere more striking than in the cases of lanosterol and cycloartenol (Figure 2). Without appreciating their conformational differences we expected the two isomeric sterols to affect membranes similarly since they share the obstructing methyl groups at the sterol $\alpha$-face. When we first examined the nutritional requirements for the sterol auxotroph *Mycoplasma capricolum,* cycloartenol proved to be much more effective in supporting growth than lanosterol.[91] This unexpected result was recorded without the comments that it deserved, at least in retrospect. Similar differences came to light when the effects of the two isomers on the microviscosity of artificial membranes were compared.[39] As already noted,[72] lanosterol affects this parameter only slightly but cycloartenol raised the $\bar{\eta}$ values substantially, indicating a much more "cholesterol-like" behavior. As stereomodels reveal, the cyclosterol has certain unique conformational features. Restraints imposed by the 9,19-cyclopropyl group on the $\beta$-face force sterol ring B into the boat conformation; the tetracyclic nucleus assumes a bent rather than a planar structure. Therefore, in view of the earlier emphasis on ring planarity for membrane competence, one might argue that cycloartenol should be as inefficient as lanosterol, if not more so, in condensing bilayer fatty acyl chains. Since this is not the case, we attribute the relative competence of cycloartenol to a conformation in which the 14$\alpha$-methyl group is more favorably disposed in space. In lanosterol, with rings A, B, and C in the chair form, the axial 14$\alpha$-methyl group projects from the otherwise planar $\alpha$-face. This minimizes van der Waals contacts and causes lanosterol to be rotationally mobile in the bilayer. In cycloartenol (A-chair, B-boat, C-chair) the same axial hydrogens which cause the $\alpha$-sterol face of an all-chair (cholestane) system to be planar, lie instead in an arc or belt. As a result the axial 14$\alpha$-methyl group does not protrude but rather becomes embedded in the $\alpha$-surface as indicated schematically in Figure 5. Thus the adverse effect of the 14$\alpha$-methyl group can be relieved not only by physical removal but also by changing its angular orientation. The effect of the bulky substituent on hydrophobic interactions becomes either neutral or perhaps enhanced.

As postulated earlier, the removal of the 14$\alpha$-$CH_3$ group during lanosterol metabolism occurs early because it interferes severely with lipid-lipid interactions in the bilayer. If, as in cycloartenol, the same substituent is buried rather than exposed, it can be argued that removing it ceases to be the high priority event — in terms of selective advantage — that it is in lanosterol demethylation. Indeed, in photosynthetic cells, cycloartenol initially loses the 4$\beta$- rather than the 14$\alpha$-methyl group to form the 4,14$\alpha$ dimethyl derivative cycloeucalenol.[61] It is eminently reasonable, as Heintz and Benveniste[61] propose, that in the course of cycloartenol demethylation (Figure 3) the axial 4$\beta$-methyl departs first because it interferes sterically with opening of the cyclopropane ring. Ring opening relieves the conformational restraints. The resulting 4$\alpha$, 14$\alpha$-dimethyl derivative (obtusifoliol) assumes the "normal" planar conformation (Ring B-boat) exposing the 14$\alpha$-$CH_3$ group as in lanosterol. $\alpha$-Face demethylation now proceeds in the order expected: (1) 14$\alpha$-$CH_3$, (2) 4$\alpha$-methyl, to produce intermediates on the way to phytosterols, e.g., stigmasterol (IX).

The rationale given for the sequential steps in lanosterol demethylation is well supported by studies with artificial and biological membranes as described above. For the cycloartenol-plant sterol pathway, the necessary information does not exist and in order to obtain it one would be faced, *inter alia,* with the complications of seemingly multiple and independent pathways. Cycloartenol metabolism may commence either with side-chain methylation to 24-methylene cycloartenol or to the isomeric cyclolaudenol (XV) (pathway A).[85] Only the latter compound has been subjected to the tests we are

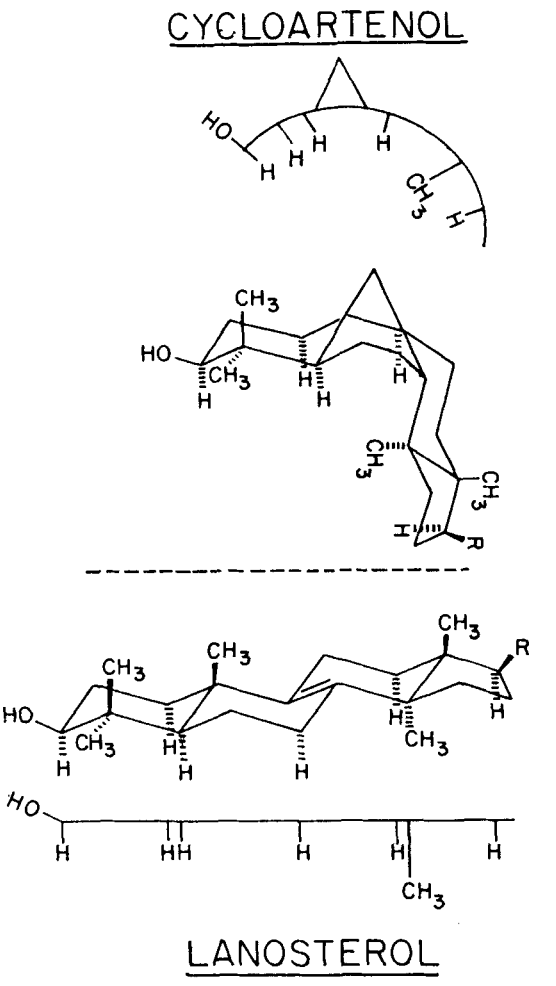



FIGURE 5. Disposition of the 14α-methyl group in cycloartenol and lanosterol.

employing for assessing membrane competence. When incorporated into phosphatidylcholine vesicles, cyclolaudenol changes microviscosity but little[39] unlike cycloartenol. Apparently the side chain methylene group dominates and overrides the condensing effects exerted by the sterol ring system as it does in ergosterol.[121] In an alternate branch, cycloartenol demethylation to 31-norcycloartenol (elimination of the 4α-methyl group) and possibly to 31-norlanosterol precedes side chain alkylation. This branch joins pathway (a) either at the stage of cycloeucalenol or obtusifoliol (Figure 3). Unfortunately, it is not yet clear whether in a given plant system the cycloartenol demethylation path is invariant, i.e., whether side-chain methylation precedes or follows opening of the cyclopropane ring and at what stage the α-face methyl group departs. Therefore, a rationalization of the demethylation sequence along the lines given for lanosterol remains a futile exercise so far. What appears well established however is that 14α-demethylation is not the critical event in cycloartenol metabolism. As an alternative to the explanation given above (evolutionary pressure), steric constraints may be responsible for the delayed removal of the 14α-$CH_3$ group. As long as this substituent is

buried in a hydrogen belt it may be inaccessible to the $C_{14}$-$CH_3$-specific oxygenase. Only after opening of the cyclopropane ring and flattening of the ring system will the 14$\alpha$-$CH_3$ group become sufficiently exposed to be oxidatively attacked.

The unexpected ability of cycloartenol to condense fatty acyl chains rather effectively — compared to lanosterol — leads to another conclusion which appears at variance with current views. Since the $\alpha$-face of the cyclosterol is curved rather than plane-linear the apposite fatty acyl carbons must be conformationally compatible for interaction with cycloartenol. They cannot be in the fully extended, all-*trans* conformation as generally assumed for the proximal ($C_1$ to $C_{12}$) segment of the fatty acyl chains.[120] Lateral displacement of acyl chains may however be tolerated and energetically favorable provided surface contacts with the sterol $\alpha$-face are sterically allowed. This postulate can be tested by $^2$H-NMR spectroscopy of appropriately $^2$H-labeled fatty acids.

## IV. SPIN-LABELED FATTY ACIDS AND $^2$H-NMR STUDIES

Cholesterol, lanosterol, and cycloartenol enter phospholipid vesicles with equal facility.[38,39] However, the studies described so far have not dealt with the respective sterol orientations in the bilayer nor have the contact points or regions between sterol and fatty acyl chains been specified. For clarifying the first of these topological aspects, the $C_3$-$\alpha$d derivatives of cholesterol, lanosterol and cycloartenol were prepared for $^2$H-NMR experiments.[37] The respective quadrupolar splitting values and their temperature dependence were determined. Assuming a rigid nucleus in all three molecules, a parallel alignment of the sterol molecule with extended fatty acyl chains, a fixed internal orientation of the $C_3$-$\alpha$d bond, and proximity of the polar sterol hydroxyl group to the water-membrane interface, the $C_3$-$\alpha$d bond will form an angle of about 90° both with the long acyl chain axis and the sterol nucleus. Based on this orientation, quadrupolar splitting values for cholesterol have been calculated and confirmed experimentally.[53,95] The data obtained in this laboratory for cholesterol[37] agree with those reported in the literature. They show, moreover, identical values for lanosterol in spite of the greater bulk and mobility of the trimethylcholestane derivative.

For cycloartenol the corresponding values were somewhat lower but the temperature dependence was the same. According to the stereomodel, the nonplanar conformation displaces the angle of tilt of the $C_3$-$\alpha$d bond in cycloartenol by 2 to 3° compared to cholesterol, a change which may account for the slightly lower quadrupolar splitting value. In any event, judging from the $^2$H-NMR data, the average orientation of all three sterol molecules relative to the phospholipid acyl chains is similar, regardless of the strength of van der Waal's contacts between the two lipid components. The same data make it likely that the three structurally diverse sterols penetrate the bilayer to approximately the same depth.

Nitroxide spin-labeled fatty acids (NS) carrying the paramagnetic reporter group at different positions along the chain provide more precise information on the contact points or regions for the interacting lipids. The order parameters for cholesterol and cycloartenol (Figure 6) are identical at all sterol concentrations. Significantly this is true regardless of the spin-label position along the fatty acyl chains. Moreover, for these two sterols the slope of the ordering effect is similar with 5-NS, 7-NS, and 12-NS. Thus, the topology of the interactions with fatty acyl chains and also their strength at the various contact points are the same for cholesterol and cycloartenol in spite of the nonplanarity of the latter. These results are in qualitative agreement with the microviscosity data presented earlier which revealed the relatively cholesterol-like behavior of the cyclosterol in phospholipid bilayers.[39]

As anticipated, lanosterol has substantially smaller ordering effects at all NS acyl-

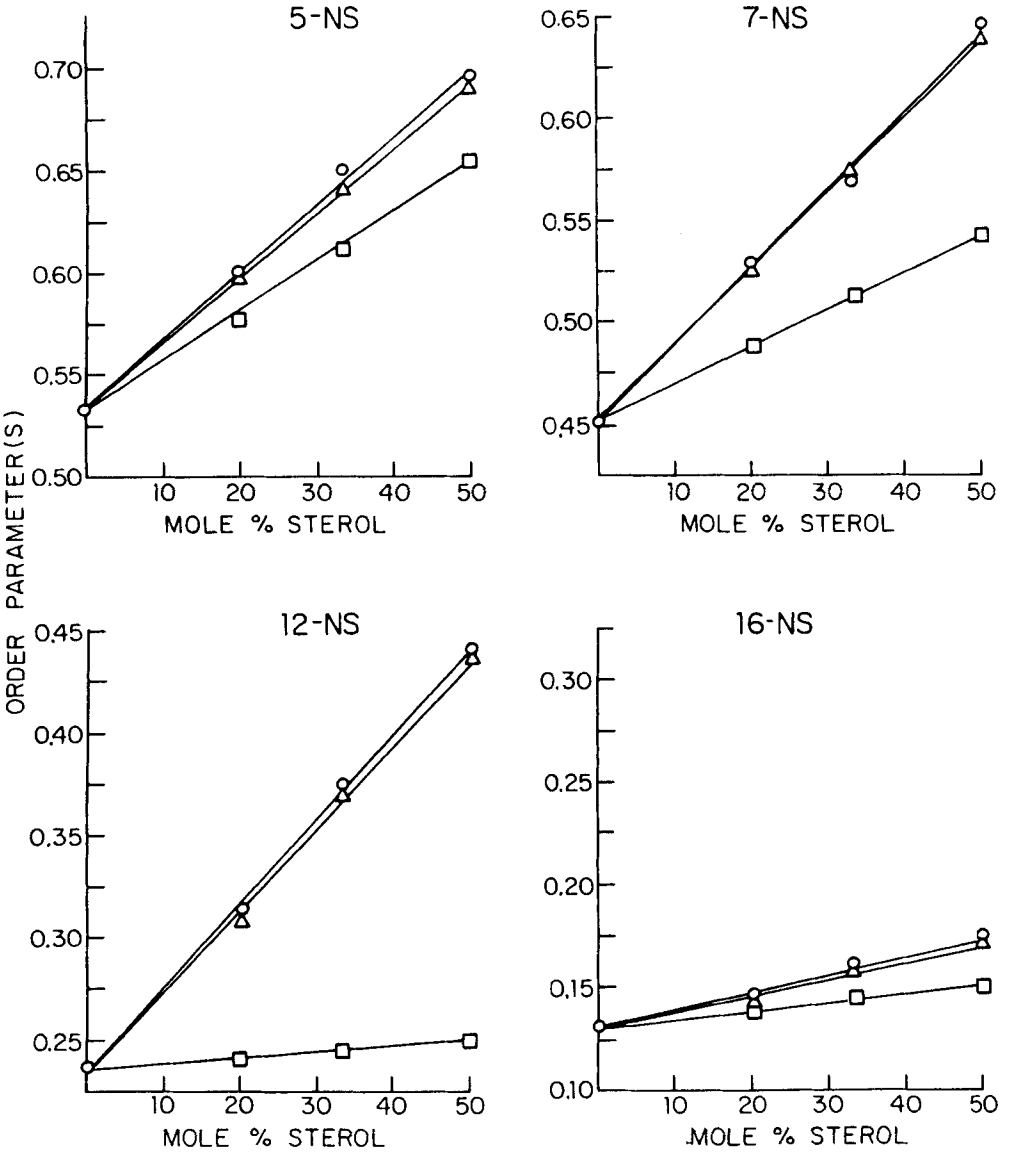



FIGURE 6. Effect of sterol structure on the order parameter (S) at 25° of 5-, 7-, 12-, or 16-nitroxyl stearic acid in phosphatidylcholine vesicles containing cholesterol (0), cycloartenol (Δ), or lanosterol (□). (From Dahl, C., *Biochemistry*, 20, 7158, 1981. With permission.)

chain positions (Figure 6). Relative to cholesterol and cycloartenol, the differences are least at C5, greater at C7, and most pronounced at C12, the position at which the ordering effect due to lanosterol is insignificant. Alignment of sterol and fatty acyl chains in the manner proposed by Rothman and Engleman[114] will place the ring D edge of the tetracyclic nucleus opposite fatty acid C12 and the interfering 14α-methyl group of lanosterol closer to spin label at C12 than to the spin labels at C5, C7, and C16. The minimal chain ordering observed with 12-NS in lanosterol containing membranes is perhaps the most direct and convincing result so far in support of the thesis which ascribes the adverse membrane effects of lanosterol primarily to the 14α-methyl group. It is noteworthy that the same methyl group not only abolishes contacts with nearby fatty acyl carbons (C12) but also weakens interactions at acyl chain carbons (C5 and C7) that

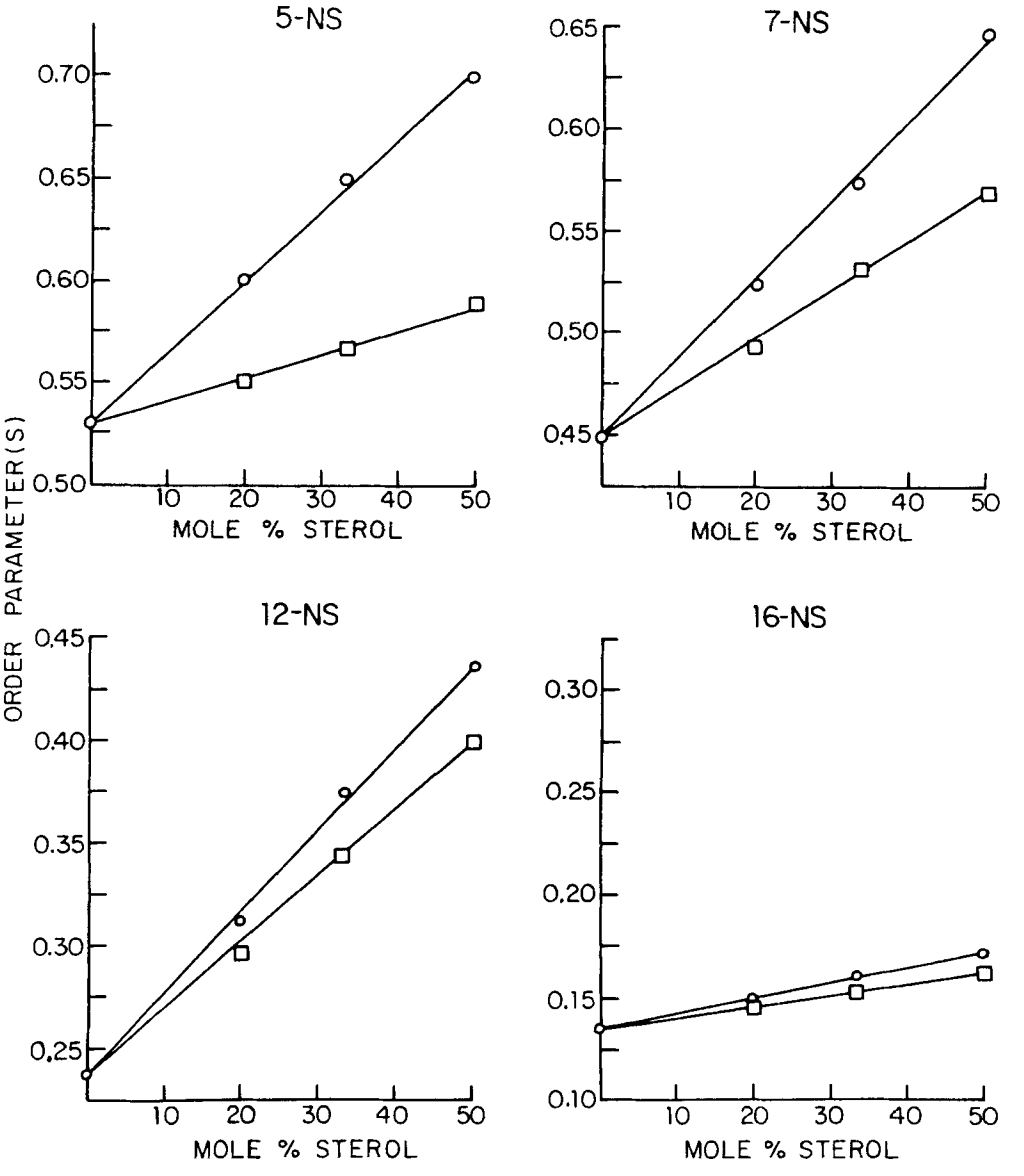



FIGURE 7. Effect of sterol structure on the order parameter (S) at 25° of 5-, 7-, 12-, or 16-nitroxyl stearic acid in phosphatidylcholine vesicles containing cholesterol (0) or 3α-methylcholesterol (□). (From Dahl, C., *Biochemistry*, 20, 7158, 1981. With permission.)

are more distant. Transmission of this effect is perhaps to be expected from the rigidity of the tetracyclic ring system. Whether the 4,4'-dimethyl substituents of lanosterol makes a contribution to the slight ordering effect seen with 5-NS remains to be tested.

Chain-ordering is lowest by far for all sterols when 16-NS is the reporting spin label. Since C16 is spatially close to the flexible isooctyl side chain but remote from sterol contact regions, no van der Waals contacts are to be expected in this instance.

How severely a single, bulky substituent at the cholesterol α-face can interfere with chain ordering is shown by the various spin label responses to 3α-methylcholesterol (Figure 7). In this instance the order parameter value is smallest for C5, the probe in closest proximity to the alkyl substituent, and significantly greater for C7. At C12 the

order parameter is only slightly lower than the corresponding value for cholesterol. Thus with increasing distance from the projecting 3α-methyl group, van der Waal's contacts become more effective, but remain weaker than with cholesterol, even at C12. As will be recalled, microviscosity values were equally low for vesicles containing lanosterol or 3α-methylcholesterol (Figure 4). It should be noted that the viscosity parameter measures only overall or bulk fluidity, but does not identify the specific contact regions. Appropriate spin labels on the other hand can probe and localize the effected contacts with considerable precision. These are clearly different for lanosterol and 3α-methylcholesterol (Figures 6 and 7). This ability to discriminate is of considerable value provided it is realized that the spin-label itself is bulky and potentially interferes with van der Waal's interactions.

## V. FUNCTIONAL SIGNIFICANCE OF VARIOUS STRUCTURAL FEATURES

### A. The Sterol Hydroxyl Group

A free, secondary hydroxyl group is the ubiquitous structural trait of all sterols found in natural membranes, regardless of origin. If exceptions exist, they have not been recorded in the literature. This fact and the inability of steroid hydrocarbons, steroid ketones, or sterol esters to modulate the physical state of artificial membranes have led to the view that an unsubstituted sterol hydroxyl is mandatory rather than incidental for sterol-function, and more specifically for sterol-phospholipid interactions in membranes. It may be asked whether this is indeed the case, or simply a consequence of the fact that the free sterol alcohol is the biosynthetic end product. The polar hydroxyl group renders the sterol molecule amphipathic. To locate this group near the membrane-water interface as all membrane models do, seems eminently reasonable. The polar function serves to anchor and orient the sterol in the membrane bilayer. Whether it contributes more specifically to membrane stability and structure remains unresolved. Over the years, direct as well as water-mediated hydrogen-bonded interactions between sterol-hydroxyl and potential H bond-accepting groups of the polar phospholipid moieties (nitrogenous base, phosphoryl anion, or ester carbonyl) have been proposed[20,146] but none have been experimentally verified.

In the course of screening a variety of sterols and sterol derivatives as promoters of mycoplasma growth we found cholesterylmethyl ether to be nearly as effective as free cholesterol.[73] The bacterial cells contained no free sterol, only the unchanged ether derivative and therefore the growth-supporting property of the methyl ether is intrinsic, not dependent on demethylation to the free alcohol. With cholesteryl acetate as sterol supplement, the results were similar; the bacteria grew well and only unchanged ester was recovered from the mycoplasma lipids. Since membrane-active sterols are commonly thought to condense the lipid phase by interacting with phospholipid acyl chains the physical state of the membrane lipids was examined. The microviscosities of membranes isolated from cholesterylmethyl ether-enriched cells were the same as those shown by cholesterol-containing mycoplasma membranes ($\bar{n}$ = 3.77 compared to 3.87 for cholesterol), a somewhat surprising result in view of the greatly reduced polarity of the methyl ether derivative compared to the free alcohol. Presumably the methyl ether is similarly oriented and penetrates the bilayer to the same extent as cholesterol, leaving the lipid-condensing effects of the sterol ring system unimpaired. The adequacy of cholesteryl methyl ether per se for mycoplasma further suggests that esterification or perhaps other modifications of the hydroxyl group do not affect the sterol utility for this bacterium in a major way. In line with this conclusion, esterifying enzymes or hydroxyl dehydrogeneases have not been encountered in mycoplasma. In fact all sterols that promote mycoplasma growth are recovered unchanged from the cells.

The yeast mutant GL7, defective in 2,3-oxidosqualene cyclase and heme biosynthesis[57] also accepts sterol methyl ether as an effective growth supplement. Cultures of the yeast mutant dealkylate the derivative to the free alcohol to some extent (10 to 30% of cholesteryl methyl ether and 3 to 6% of ergosteryl methyl ether) depending on the degree of aeration.[73] In the complete absence of oxygen cholesteryl methyl ether supports growth of the mutant nearly as well as cholesterol, in this case with no detectable conversion of the methyl ether to the free sterol alcohol.

It follows from our results that blocking the sterol hydroxyl groups only partially impairs sterol function at least in the instances of a bacterial sterol auxotroph and in a mutant strain of an unicellular eukaryote. By the same token, esterification or other modifications of the hydroxyl group are not essential for sterol utilization or function in these systems. Whether this is true also for wild-type yeast is not clear since in the presence of oxygen, GL7 converts some of the derivative to free sterol alcohol providing substrate for esterification. Such esterification does indeed take place readily in aerobic GL7 cells provided with free cholesterol. Wild-type yeast has been reported to accumulate steryl esters as they approach the stationary phase.[105,132] Also, a role for steryl ester in the genesis of yeast mitochondria during aerobic adaption has been suggested.[99] Our finding that anaerobic GL7 grows well on methyl ether without modifying it fits the observation that strictly fermentative yeast does not accumulate steryl ester.[105]

Cholesteryl ethyl ether supports the aerobic growth of GL7 somewhat less efficiently than the methyl ether analogue but only negligible growth is observed with the propyl ether analogue (Jones, S., unpublished). It appears that the chain-length specificity of the dealkylating enzyme rather than cellular uptake determines the growth response. In line with this conclusion, cholesteryl propionate is equivalent to free cholesterol as a growth supplement, presumably owing to hydrolysis by a membrane-bound esterase.

Demethylations of hydroaromatic O-ethers have, to our knowledge, not been described previously. A remarkable feature of this conversion as observed in aerobic GL7 is that although the process is oxidative it does not appear to require a cytochrome-linked electron transport chain. The yeast mutant GL7 is, *inter alia,* heme deficient.[57]

While steryl alkyl ethers are foreign substrates, the conclusions we have derived from their growth-promoting properties are nevertheless pertinent to the question of sterol structure-function relationships. Hydrogen-bonding of sterol-OH to polar phospholipid moieties either directly or by way of a water bridge is not essential. Nevertheless it seems likely that for membrane orientation the amphiphatic character of the sterol molecule has to be preserved to some degree. For this purpose an ether or ester oxygen seems to be sufficient. If the methoxyl or ester group — like the free OH function — is positioned near the water-membrane water interface (as the microviscosity data indicate) methyl ether oxygen could still be engaged in hydrogen bonding, but only with solvent.

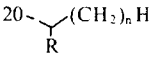
Cholesteryl methyl ether has been reported to enter multilamellar phosphatidylcholine vesicles only poorly,[93] a result we have confirmed.[73] Nevertheless, the ether derivatives are nearly as competent as free sterol in the two systems we have described. Their entry into biological membranes does not appear to be limiting. In this instance, at least, the behavior of a test molecule in an artificial membrane is at variance with a biological response. In this context it is to the point that ergosterol, while readily incorporated into a variety of cells, enters artificial membranes only to a limited extent.[121]

## VI. THE STEROL SIDE CHAIN

Squalene-epoxide cyclizations to either lanosterol or cycloartenol generate tetracyclic systems, leaving eight polyprenol carbon atoms in the form of a flexible isooctyl side chain. All eukaryotic membrane sterols (for exceptions see Sections XIV. and XV.) share this structural duality, a rigid and bulky tetracyclic ring system to which a mobile

aliphatic tail is appended. It seems virtually certain from X-ray and neutron diffraction studies[51] that the ring system is contiguous with the proximal segment of the phospholipid acyl chains, while the side chain extends deeply into the bilayer interior. Differential van der Waal's interactions resulting in a fluidity gradient along the sterol-phospholipid contact regions are therefore to be expected. $^2$H-NMR studies with model membranes containing deuterated acyl chains as well as ESR studies are consistent with this arrangement.[120]

The vast majority of eukaryotic membrane sterols retain an unmodified isooctyl carbon chain except for reduction of the 24,25 double bond. In artificial systems at least, side chain shortening or extension appears to impair membrane function, rationalizing the conservation of this feature. Synthetic cholesterol analogues of the general structure



bearing straight rather than terminally branched alkyl chains are well incorporated into phosphatidylcholine vesicles in concentrations up to 40 to 50 mol %. A variety of techniques (ion permeability,[83] osmotic shrinking,[131] and ESR measurements[131]) have been used to assess side-chain length effects. The results generally agree; the native cholesterol side-chain causes maximum ordering. When sterol effects were examined by fluorescence depolarization of the probe diphenylhexatriene,[25] all analogues were inferior to cholesterol in raising membrane microviscosity. The impairment was least for $n = 6$ (8 carbon straight chain) and $n = 5$ (7 carbon straight chain), intermediate for $n = 3$, 4, and 7 and most pronounced with the shortest ($n = 2$) and longest ($n = 9$) analogues. Moreover, replacing the normal terminally branched isooctyl moiety by an unbranched $C_8$ chain ($n = 6$) slightly but distinctly impaired membrane effectiveness as judged by the microviscosity parameter.

Whether the length and the branching of the 1,5 dimethylhexyl moiety of natural sterols confers optimal properties for function in natural membranes as well appears likely but is unproven. Attempts to examine the efficiency of side-chain analogues as growth supplements for the sterol-requiring yeast mutant GL7 have failed because cellular (in contrast to liposomal) sterol uptake is highly selective.[25] Analogues with shorter or longer side chains are more poorly incorporated than cholesterol and comparative growth rates are therefore not interpretable. The preferential uptake by intact cells of a sterol bearing a side-chain of "normal" length is an interesting phenomenon by itself. It brings to mind the highly specific structure-dependent absorption of sterols by the gastrointestinal tract of higher animals. Lipophilicity per se — which presumably differs only slightly between sterol side chain analogues — is clearly not a major determinant for the absorption process, whatever its mechanism.

Side chain conformation is equally crucial for sterol fitness either in promoting growth[87] or as a substrate for biological transformations, e.g., in *Tetrahymena pyriformis*.[86] In naturally occurring sterols (17$\beta$, 20R) the preferred conformer has a "right-handed" structure. With respect to the C/D ring system, $C_{22}$ of the side chain is *trans*-oriented providing for minimal nonbonded interactions with the angular methyl carbon $C_{18}$ and hence optimizing the potential for sterol-phospholipid interactions. As Nes et al.[87] have shown only the *trans* conformer but not the unnatural *cis* conformer with *cis* side-chain orientation supports the growth of anaerobic yeast. This is consistent with the Rothman-Engleman[114] model for cholesterol-sterol interactions that envisions a fully extended sterol structure bearing the *trans*-oriented side chain. According to the same model, a *cis*-oriented sterol side-chain would create considerable membrane disorder, *inter alia*, by obstructing contacts between the $\beta$-face of the sterol ring system and fatty acyl chains.

While nature has apparently seen fit not to shorten or linearly extend the isooctyl side chain, reactions increasing its bulk by "postsynthetic" methylations occur commonly. The $C_{24}$-methyl or $C_{24}$-ethyl derivatives are in fact the prototypic sterols of fungi and plants respectively. A rationalization of these alkylations will be attempted in later sections (X. and XI.). More recently, analyses of the complex sterol mixtures found in marine invertebrates (corals, sponges) have disclosed a remarkable spectrum of unconventional side-chain structures (Section XIII.).

## VII. STEROL SPECIFICITIES FOR STEROL AUXOTROPHS

While model membranes have furnished valuable background information on sterol-phospholipid interactions in chemically defined systems, an ultimate understanding of sterol function can come only from studies with natural membranes. These are necessarily much more complex not only because they contain protein but also because the bilayer phospholipids never consist of a single component. The identity of the phospholipid head group, the length, degree of unsaturation, and branching of the fatty acyl moieties all effect membrane properties profoundly. This latter aspect of structure-function relationships in natural membranes is almost totally unexplored.

Whether and to what degree a given sterol is functionally competent overall can be ascertained by nutritional studies with cells or organisms that require an external source of sterol for growth. There exist only a few "natural" sterol auxotrophs to choose from.

1. The Insecta, the first class of constitutive sterol mutants to be recognized[63] appear to lack all enzymes of the sterol pathway.[29] Cholesterol satisfies the sterol requirement of all insect species examined[33,76] suggesting that in invertebrates as well as vertebrates cholesterol functions as the universal membrane sterol. An interesting example of nutritional adaptation came to light in comparative nutritional studies of representative carnivorous *(Dermestes vulpinus)* and herbivorous (or omnivorous) insects *(Blatella germanica).* For the former, only cholesterol and some isomeric cholestenols were satisfactory sterol sources.[30] Plant-feeding insects, however, grow and develop equally well on sterols from nonanimal sources (ergosterol, $\beta$-sitosterol, stigmasterol[12]). The fact that insects in this category (e.g., *Blatella*) modify side-chain substituted sterols such as ergosterol by dealkylation to 22-dehydrocholesterol[31] support the generalization that only sterols of the cholesterol type can perform the functions, presumably several, for which sterols are needed. *Blatella* and other omnivorous or plant-feeding insects evidently elaborate enzymes for side-chain dealkylation at $C_{24}$ while obligate carnivores such as *Dermestes vulpinus* do not. Their absence is certainly one, if not necessarily the only, metabolic deficiency responsible for the carnivorous style of life.

   As the comparison of carivorous and herbivorous insects shows, the absence of metabolic sterol modifications in a test organism must be rigorously established if valid conclusions about structure-function relationships are to be drawn. Few organisms, certainly not normal animal cells, satisfy this essential criterion.
2. Yeasts and fungi typically synthesize ergosterol *de novo* and can, of course, be raised on fully synthetic, sterol-free media. In one of the landmark papers in the field of sterol biochemistry and physiology Andreassen and Stier[3] reported that *Saccharomyces,* when totally deprived of oxygen, fails to grow unless supplied with a sterol and an unsaturated fatty acid. Since sterols are essentially stable metabolically in the absence of oxygen, anaerobic yeast is, at least in principle, an ideal organism for establishing structure-function relationships in an eukaryotic microbe. The technical difficulty of maintaining strictly anaerobic culture conditions which is essential for the purpose, is the major factor which restricts use of this

technique. Numerous yeast mutants deficient in sterol biosynthesis have been described. They are useful, within limits for defining some aspects of sterol structure-function relationships (Sections X. and XI.).

3. *Tetrahymena pyriformis* uniquely among eukaryotes neither synthesizes sterol nor requires an exogenous sterol for growth. Instead the protozoan cyclizes squalene to tetrahymanol (XVII) a pentacyclic triterpene.[6,27,77,138] Exogenous cholesterol suppresses tetrahymanol synthesis without inhibition of growth,[77] a finding which points to the functional equivalence of cholesterol and tetrahymanol in *T. pyriformis*. Potentially, this phenomenon might be exploited to examine the sterol specificity for negative feedback control of tetrahymanol biosynthesis. Section XV. deals with this subject in greater detail.
4. Sterols and Prokaryotes — Microbial eukaryotes such as yeasts, fungi, and algae usually contain as much sterol as some animal membranes but, with one exception, prokaryotes either lack sterols altogether or contain them in quantities (0.01% or less) probably insufficient for affecting bulk membrane fluidity. The exceptional prokaryote is *Methylococcus capsulatus* which produces sizable amounts of lanosterol metabolites[14] but probably not the fully demethylated cholestane derivatives found in eukaryotic cells.[19] Sterol auxotrophy in prokaryotes is equally rare. It is encountered only in the saprophytic or parasitic mycoplasmas.[48,107]

## VIII. STEROL NUTRITION OF *MYCOPLASMA CAPRICOLUM*

For investigating sterol structure-function relationships mycoplasma strains have come to be the organisms of choice,[4,116] first of all because the sterol requirement for growth is absolute and, equally important, none of the sterols tested so far are metabolically modified. Moreover, the organism has a single, easily isolated membrane and, finally, mycoplasmas are auxotrophic for both saturated and unsaturated fatty acids.[115] The lipid composition of the mycoplasma cell membrane is therefore amenable to manipulation both qualitatively and quantitatively.

As mentioned at the outset, the experimental work described here was undertaken to obtain evidence for the hypothesis of a directed evolution of the sterol molecule that resulted in improved membrane function. The bulk of the evidence in support of the hypothesis has come from studies with *Mycoplasma capricolum* a strain that can be cultured in serum-free media.[108]

*M. capricolum* requires a relatively complex medium for growth though serum is not an essential component. Nevertheless, care must be taken to exclude inadvertent contaminants, especially lipids, to assure reproducible growth rates and maximum absorbances.* In extension of initial observations that lanosterol and some partially demethylated intermediates support moderate mycoplasma growth,[91] carefully controlled experiments were carried out in order to establish the relative efficiencies of the sterol supplements.[38] In Figure 8 the mass doubling times and maximum absorbances for cholesterol and the various trimethyl-, dimethyl-, and monomethyl derivatives are shown. These growth data show a remarkably close correlation with the relative ability of the same set of sterols to raise the microviscosity of artificial membranes (Figure 4). A given structural modification changes both parameters in the same direction. This correlation holds also for $\Delta^5$-unsaturated as compared to saturated sterols although the differences between a given sterol and stanol are slight.

* Bovine serum albumin is an essential component of the mycoplasma culture medium ("Modified Edward" medium.[108]) In our experience, various batches of BSA, even from the same supplier, vary substantially in cholesterol and fatty acid content.

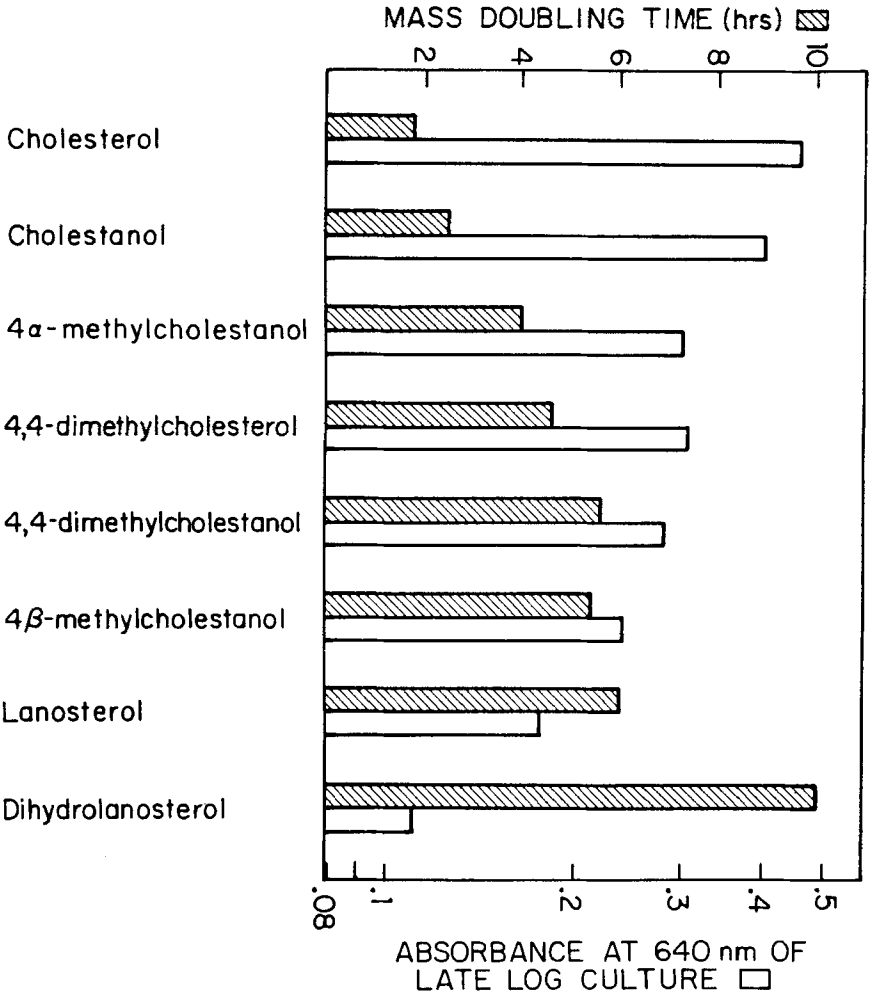



FIGURE 8. Effect of various methylated cholestane derivatives on the growth rate and cell yield of *M. capricolum*. (Adapted from Reference 38.)

While lanosterol is the least effective of the series, it nevertheless affords substantial growth of mycoplasma cells. This result is somewhat unexpected since lanosterol affects the physical properties of artificial membranes only minimally.[38,72] If, as is conceivable, uptake from the external medium is a limiting factor, conclusions about relative efficiences will, of course, not be valid. This issue is not entirely settled. The sterol content of the isolated mycoplasma membranes, ranging from 22 to 33 mol %, tended to be higher for the more effective sterols but there was no clear correlation between sterol content per se and the rate of growth.[40] Structure rather than uptake appears to determine the efficiency of a given sterol.

If growth under the chosen conditions is indeed a function of the physical state of the membrane, then growth rates should correlate with the microviscosities of membranes isolated from the various cells. The values shown in Table 2 bear out this prediction for all but one of the sterols tested (see below). The more efficient a sterol is as a bacterial growth factor, the greater the microviscosity of the isolated cell membranes. Thus, qualitatively at least, artificial and natural membranes show the same sterol responses; the direction of change is not affected by the presence or absence of proteins or by the nature of the

**Table 2**
**EFFECT OF VARIOUS METHYLATED CHOLESTANE DERIVATIVES ON THE STEROL CONTENT, FATTY ACID COMPOSITION, AND MICROVISCOSITY OF *M. CAPRICOLUM* MEMBRANES**

| Sterol added to growth media (10 μg/mℓ) | Mol % sterol of total lipid | Fatty acid composition of membrane lipids (wt % distribution) | | | Saturated/unsaturated fatty acid ratio | $\bar{\eta}$ |
|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 18:1 | | |
| Cholesterol | 30.6 | 44.7 | 2.2 | 52.9 | 0.88 | 5.05 |
| Cholestanol | 32.0 | 43.2 | 1.9 | 53.8 | 0.81 | 4.72 |
| 4α-Methylcholestanol | 27.5 | 43.2 | 1.5 | 55.0 | 0.81 | 4.53 |
| 4,4′-Dimethylcholesterol | 28.0 | 39.4 | 2.3 | 57.1 | 0.72 | 4.31 |
| 4,4′-Dimethylcholestanol | 33.0 | 44.5 | 1.6 | 53.5 | 0.85 | 4.09 |
| 4β-Methylcholestanol | 21.8 | 42.6 | 1.3 | 55.7 | 0.78 | 3.69 |
| Lanosterol | 24.9 | 47.6 | 1.5 | 50.8 | 0.97 | 3.17 |
| 3α-Methylcholesterol | 37.1 | 44.8 | 1.6 | 53.5 | 0.86 | 3.69 |

From Dahl, C., Dahl, J., and Bloch, K., *Biochemistry*, 19, 1462, 1980. With permission.

bilayer lipid, phosphatidylcholine in artificial vesicles, and largely phosphatidylglycerol in the mycoplasma membranes.[128]

Reliance on a physical parameter as a dominant factor for cell viability may nevertheless lead to pitfalls. In at least one instance, mycoplasma cells have been found to grow at a rapid, near optimal rate even though the bulk membrane fluidity was high at the time of harvest. The one "aberrant" sterol is 3α-methylcholesterol.[37] The data for this unnatural sterol are included in Table 2.

For regulating membrane fluidity in response to environmental changes, bacteria commonly resort to the device of adjusting the ratios of saturated and unsaturated fatty acids they incorporate into membrane phospholipids.[36] *M. capricolum* relies entirely on the external medium as a source of fatty acid yet the ability of this organism to alter the proportional uptake of saturated and unsaturated fatty acids, e. g., of palmitate and elaidate, appears to be limited. When presented with external sterol which produces relatively fluid membranes, the cellular fatty acid composition changes only slightly.[40] Thus the SFA-UFA ratios for lanosterol-grown cells was 0.96, only somewhat higher than for cells grown on cholesterol — 0.81 (Table 2).

By choosing *M. capricolum* as a test organism we have been able to reinforce and extend to a biological system the structure-function relationships deduced from artificial membranes. A rationale is provided for the almost universal selection of cholesterol or side-chain substituted cholestane derivatives for optimal function in biological membranes. Cholesterol appears to owe this superiority to several features which are, in order of importance, (1) planarity of the sterol α-face at the C-D ring junction, (2) absence of methyl groups at $C_{14}$ and $C_4$, and (3) the presence of a B ring double bond. Steric and conformational arguments rationalizing why each of the biosynthetic steps during the lanosterol-conversion produces a functionally more competent membrane sterol and why for this reason nature may have chosen the temporal order in which these steps occur have already been given. We conclude that cholesterol, a small molecule, is the end product of directed evolutionary pressures, not of chance mutations.*

* As D. E. Koshland has pointed out (*Fed. Proc.*, 35, 2104, 1976), "the evolution of function tends to occupy a back seat in contemporary analyses of evolution . . . . function must be the driving force of evolution, the phenomenon which provides the selective advantage".

For technical reasons — largely more complex nutritional requirements — the above analysis has not yet been extended to other mycoplasma strains, all of which require sterol for growth.[107]

## IX. STEROL SYNERGISM

The studies described in the preceeding section dealt *inter alia* with the relationship of membrane physical state to the growth rate of mycoplasma. Certain inconsistencies remained. In particular, the ability of lanosterol to support growth of this organism, albeit moderately, presented a problem because this molecule is essentially without effect on the various physical parameters (microviscosity or glucose permeability) of model membranes. One source of concern was the lack of assurance that the culture medium, though rigidly delipidated, was indeed entirely cholesterol-free. Even traces of contaminating cholesterol might invalidate the growth data obtained with lanosterol.

A more detailed comparison of the mycoplasma responses to cholesterol and lanosterol resolved this issue at least partially.[40] Over a 10-fold concentration range (10 to 1 $\mu$g/m$\ell$) of medium cholesterol, the initial growth rates remained essentially constant, but absorbances of late log cultures, a measure of cell yield, fell by approximately one half. In the same series, mycoplasma membrane viscosities declined from 4.53 to 3.34 along with a drop of cellular cholesterol content from 28 to 14%. These results are unremarkable and in line with expectation. On the other hand, over the same range of sterol concentration, lanosterol afforded three times lower growth rates in all cases, but essentially the same late log absorbances and there was no change in $\overline{\eta}$ values (3.10 to 3.16) of the isolated membranes. Clearly, cholesterol effects membrane fluidity as a function of cellular concentration while lanosterol which does not condense phospholipid acyl chains fail to modulate the fluidity parameter. Granting this to be the case — i.e., failure of lanosterol to affect the ordering of fatty acyl chains — it seemed worth exploring whether for lanosterol-grown cells the fatty acid requirements (chain-length, unsaturation) were stricter than they are for cells supplied with cholesterol. This turned out to be the case.[40] As shown in Table 3, cholesterol-supplied cells grew at essentially the same optimal rates either with elaidate alone or with 1:1 mixtures of saturated acids ranging from $C_{14}$ to $C_{20}$, and either $18{:}1_t$, $18{:}1_c$, or $18{:}2_{c,c}$ as unsaturated fatty acid supplements. With lanosterol as the sterol source only certain fatty acid combinations supported growth, e.g., C16:0 or C18:0 along with $C18{:}1_t$ or $C18{:}1_c$, but not with elaidate alone, myristate-elaidate, myristate-oleate, or palmitate-linoleate. This restricted range of fatty acid suitability for growth on lanosterol and the unchanged competence of cholesterol when presented with a wide range of fatty acids may be explained as follows. When membranes are rich in cholesterol, phospholipid-acyl chain-sterol interactions determine the bulk physical state of the membrane. On the other hand, when lanosterol is the membrane sterol, fatty acyl chain-sterol contacts are minimal because of the interfering bulky substituents at the sterol $\alpha$-face. Instead, mutual fatty acyl-chain interactions will predominate and these in turn depend critically on the chain length and degree of unsaturation of the fatty acid supplied. Indeed when the more liquid oleate replaces elaidate, lanosterol-grown cells increase their cellular SFA/UFA ratio from 0.96 to 1.70, an adjustment which leads to enhanced van der Waal's interactions. By contrast, replacement of oleate by elaidate in cholesterol-grown cells leaves the cellular SFA/UFA ratio unchanged.* Clearly, in mycoplasma more than one mechanism can adjust the bulk

* It was stated above that *M. capricolum* does not respond to a limited sterol supply by changing the SFA/UFA ratio. This appears to be the case when cholesterol is the sterol source but such control clearly does not go into effect when the sterol is relatively "incompetent" (lanosterol).

**Table 3**
**GROWTH OF *M. CAPRICOLUM* IN MEDIUM CONTAINING CHOLESTEROL OR LANOSTEROL AND VARIOUS FATTY ACIDS**

| Sterol added to growth medium | SFA added to growth medium | $A_{640}$ of late log culture | | |
|---|---|---|---|---|
| | | UFA added to growth medium | | |
| | | 18:1$_t$ | 18:1$_c$ | 18:2$_{c,c}$ |
| Cholesterol | none | 0.43 | b | b |
| | 14:0 | 0.38 | 0.38 | b |
| | 16:0 | 0.42 | 0.48 | 0.43 |
| | 18:0 | 0.48 | 0.47 | b |
| | 20:0 | 0.42 | b | b |
| Lanosterol | none | <0.07 | b | b |
| | 14:0 | <0.07 | <0.05 | b |
| | 16:0 | 0.25 | 0.20 | <0.07 |
| | 18:0 | 0.16 | 0.18 | b |
| | 20:0 | 0.26 | b | b |

[a] Lipid-depleted modified Edward media were supplemented with 10 μg/mℓ sterol with 5 μg/mℓ SFA and 6.5 μg/mℓ UFA or 11.5 μg/mℓ elaidate alone.
[b] Not tested.

From Dahl, J., Dahl, C., and Bloch, K., *Biochemistry*, 19, 1467, 1980. With permission.

physical state of the membrane. To mycoplasma cells growing in the rich, natural habitat provided by animal tissues, ready access to cholesterol insures viability in spite of a wide-ranging fatty acid milieu. For the reasons stated, availability of lanosterol would scarely be of survival value because, in this instance, the fatty acid requirement becomes much more stringent. This phenomenon illustrates rather strikingly how functional improvement may drive the structural evolution of biological molecules.

### A. Synergistic Sterol Effects on Growth

From the foregoing discussion, lanosterol emerges as a molecule of marginal competence capable of supporting growth only in a narrow and probably artificial range of nutrients. As mentioned repeatedly, lanosterol might be a poor substitute for cholesterol because it condenses fatty acyl chains only weakly. Alternatively, if cells require sterol for more than one function, then lanosterol in contrast to cholesterol may satisfy one but not the other. The later possibility became attractive when it was realized that mycoplasma growth media, even after exhaustive delipidation, contained small amounts of cholesterol (about 0.02 μg/mℓ). Moreover, cells grown on various sterols invariably contained 1 to 2% of the total cellular sterol in the form of cholesterol. Evidence that these small amounts of cholesterol might be essential for growth when some other, less effective sterol is supplied in bulk is shown in Figure 9.[40] First to be noted, the growth curves for cholesterol-supplemented cells could be extrapolated to the low initial absorbance of $8 \times 10^{-4}$. For lanosterol-supplemented cells (with no added cholesterol), if the growth curve is extrapolated along a line corresponding to exponential growth, the initial absorbance intersects with the X-axis at the very much higher value of $3 \times 10^{-2}$. Evidently, initial growth on lanosterol is faster than the exponential rate measured at later time points. This suggests a synergistic effect between

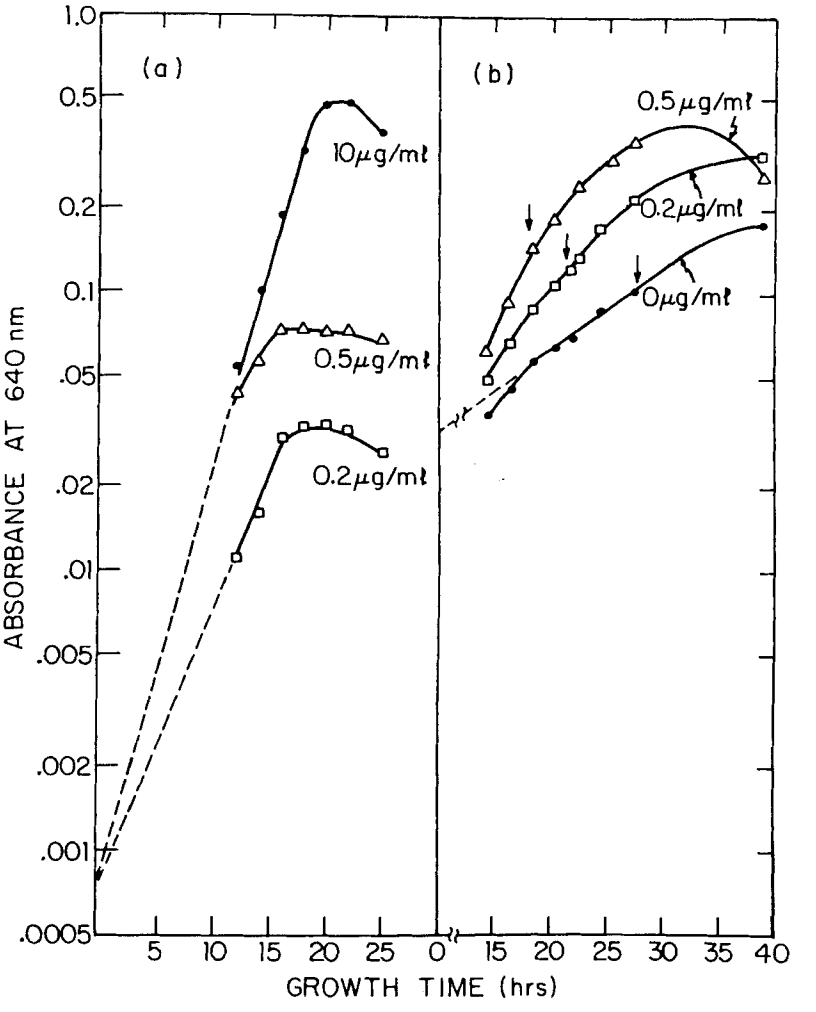



FIGURE 9. Growth curves of *M. capricolum* cultured at 37° on media containing (a) 5 μg/mℓ palmitate, 6.5 μg/mℓ elaidate and the amounts of cholesterol indicated; or (b) the same quantities of fatty acids as in (a), 10 μg/mℓ of lanosterol and the amounts of cholesterol indicated from Dahl et al.[40]

lanosterol and some adventitous component derived from the medium, most likely cholesterol. In support of this suspicion, it was found that low levels of added cholesterol (1/20 the amount of lanosterol) raise the growth rate on lanosterol and increase the absorbance of late log cultures far above the levels seen with a corresponding low dosage of cholesterol alone. The cholesterol effects are synergistic, not additive. Equally notable, membranes of cells grown under "synergistic" conditions remained fluid ($\bar{\eta} = 3.31$). Inclusion of small amounts of cholesterol in the growth medium did not raise their microviscosities above the "lanosterol levels" ($\bar{\eta} = 3.34$). Evidently the cellular content of cholesterol was too low (0.6 to 3% of total lipid) to have a measurable effect on the bulk phase microviscosity parameter. The significance of this result is that mycoplasma can grow vigorously whether the sterol supplement induces a high or low bulk microviscosity. The circumstances and observations leading to the discovery of sterol synergism have been dealt with in detail because they provide the first direct evidence for a multiple role of sterols in a biomembrane.

There is little reason to doubt that modulation of bulk membrane fluidity or other measures of physical state is one important attribute of the sterol molecule. Such effects have been abundantly demonstrated but only at relatively high sterol concentrations, e.g. above 10 mol %.[43] Less attention has been paid to the possibility of another, possibly more primitive sterol function. This might be separation of phospholipid head groups, resulting necessarily from insertion of the bulky and rigid tetracyclic sterol ring system into the bilayer. NMR evidence exists that head group separation can be affected by lanosterol as well as cholesterol in model membranes.[145,147] The consequences of phospholipid head group separation by sterol have not been explored extensively. In phosphatidylcholine bilayers, the predictable result is weakening or disruption of electrostatic interactions between a positively charged base and neighboring phosphoryl anions. In membranes containing predominantly anionic phospholipids (e.g., phosphatidylglycerol) such as mycoplasma, the increased head group distance should diminish charge density. In either case binding of cations and perhaps also ion transport might be effected.

### B. Cholesterol and Lipid Synthesis

The question remained whether, in addition to purely structural effects, sterols may play some other role unrelated to the control of a physical membrane parameter. From what has been said before it follows that this second function should be cholesterol-specific and expressed by cholesterol levels too small to alter bulk physical properties. The working hypothesis was that the cholesterol-specific role might be metabolic.

In a search for this process, cells were grown under synergistic conditions, i.e., by addition of small amounts of cholesterol to lanosterol-rich media as well as on cholesterol or lanosterol alone. As expected, the rates of macromolecular synthesis and palmitate incorporation into lipids were essentially synchronous with growth; rapid in cholesterol-media and slow in media containing lanosterol.[41] The effects of supplementing lanosterol-rich media with small amounts of cholesterol were striking. Protein and lipid synthesis as well as the growth rate rose to the rates seen in cultures growing on nonlimiting amounts of cholesterol.

Both RNA and lipid synthesis are known to slow down in amino acid-starved, "stringent" *Escherichia coli* strains.[103] It could be reasoned by analogy that lanosterol-rich mycoplasma cells, unlike those adequately supplied with cholesterol, grow more slowly because eventually an essential nutrient becomes limiting. If cholesterol facilitates the uptake and utilization of this nutrient as the synergistic sterol effect on growth suggests, then supplementation of lanosterol-rich media with small amounts of cholesterol should render this process no longer rate-limiting. In the search for the growth-limiting nutrient, palmitate and elaidate concentrations proved to be critical for growth when lanosterol was the sole sterol source. Raising the concentrations of both fatty acids 2- to 4-fold increased the growth rate by a factor of 10, i.e., to levels seen with cells supplied with nonlimiting cholesterol and "normal" amounts of fatty acids.[41] Cells receiving larger amounts of cholesterol or cholesterol-lanosterol in a ratio of 1:20 did not show this response. They already grow optimally on media containing low fatty acid concentrations. It should be noted that fatty acid-stimulated growth — tantamount to sparing cholesterol — is seen only when a relatively ineffective bulk sterol (lanosterol) is provided. Growth in cultures containing limiting cholesterol alone is not stimulated by raising fatty concentrations in the medium. The sterol requirement persists under all circumstances but it can be met adequately by lanosterol provided the cells have access to high fatty acid concentrations.

In essence then, either high concentrations of fatty acids or small cholesterol supplements are of equal benefit for lanosterol-grown cells, suggesting strongly that

cholesterol controls in some manner fatty acid influx or some phase of fatty acid utilization for phospholipid synthesis.* The basis of sterol synergism therefore appears to be control of a metabolic event or at any rate a process distinct from membrane fluidity control. As already mentioned, mycoplasma membranes derived from either lanosterol-rich or lanosterol + cholesterol (20:1) grown cells have the same low microviscosity.

In more direct experiments to test for a metabolic role, cholesterol could be shown to have a profound effect on the cellular uptake of certain fatty acids from the growth medium. In resting mycoplasma cells fatty acid uptake exhibits saturation kinetics characteristic of a facilitated process. Three types of cells with different nutritional histories were examined, (a) growth on nonlimiting cholesterol, (b) on a 1:20 mixture of cholesterol and lanosterol, and (c) on lanosterol alone. For palmitate uptake the $K_m$ values were independent of the sterol originally supplied to the cells ($K_m$ 1.3 to 1.6 $\mu M$), but they were not for unsaturated fatty acids. For cholesterol-rich cells the apparent $K_m$ for oleate was 2 to 3 $\mu M$ and equally low for cells raised on cholesterol-lanosterol 1:20. Lanosterol-grown cells, however, took up oleate much less efficiently; the $K_m$ value in this instance was 15 to 16 $\mu M$. Cellular cholesterol, whether present in large or small concentrations, therefore facilitates utilization of a phospholipid precursor. Virtually all of the incorporated labeled fatty acid, whether palmitate or oleate, becomes rapidly esterified and appears in phosphatidylglycerol (Dahl, J., unpublished).

Whether cholesterol specifically promotes fatty acid transport per se, fatty acid activation or a later step in phosphatidylglycerol synthesis has not been ascertained yet. As for the underlying mechanism, the phenomenon described can be demonstrated not only with cells grown with the synergistic cholesterol-lanosterol combination but also with cells raised on lanosterol alone and supplied with cholesterol 20 hr later. Under these conditions the cholesterol effect on fatty acid uptake is not seen immediately but only about 1 hr after cholesterol exposure (Dahl, J., unpublished).

Whatever the mechanism of control over phospholipid synthesis, our evidence indicates that it is unrelated to changes in the bulk physical state of the membrane bilayer. Membranes of cells raised on the synergistic sterol mixture have the same low microviscosities as cells raised on lanosterol alone, as pointed out. However, when first obtained, these data by no means excluded the existence of less-fluid, cholesterol-rich domains in membranes whose bulk sterol is lanosterol. Since mycoplasma cells are very small, such low fluidity regions would be difficult to detect. We are inclined, however, to conclude thay they do not exist from the following observation. 3$\alpha$-Methylcholesterol, a very effective growth-supporting sterol, does not raise the microviscosity of mycoplasma membranes but produces the same synergistic effect on growth when supplied in small amounts along with a 20-fold excess of lanosterol.[41] In this case neither component of the sterol combination raises membrane microviscosity as cholesterol does. The conclusion is that sterol-stimulated phsopholipid biosynthesis involves neither bulk or regional effects on the physical state of the membrane bilayer.

### C. Other Examples of Sterol Synergism

The idea and, in fact, evidence that sterols per se are needed for both metabolic** and structural, i.e., dual purposes, is not new. Larvae of the hide beetle *Dermestes vulpinus,* an obligate carnivore, when reared on synthetic diets, grow and pupate when provided with either cholesterol or pairs of sterols which individually will not meet the insect's sterol requirement.[30] One such pair is $\beta$-sitosterol and cholesterol (10:1) and another,

* Fatty acid oxidation does not occur in mycoplasma.[128]

** Excluding, of course, conversion to steroid hormones, bile acids and vitamin D.

cholestanol and cholesterol. In nonlimiting amounts and when given alone, only cholesterol satisfies the sterol requirement; β-sitosterol and cholestanol do not. By analogy with the mycoplasma results, it appears that the "foreign" sterol can satisfy the requirement for a bulk sterol, which is structural. Only cholesterol is competent as the metabolic component as well. In insects the basis for the latter may be, in part, the transformation to ecdyson, the cholesterol-derived molting hormone.

More recently, sterol synergism has been observed also in cultures of a mouse fibroblast sterol auxotroph.[117] The sterol requirement was met either by nonlimiting cholesterol alone or by β-sitosterol supplemented with trace amounts of cholesterol. The plant sterol alone failed to support growth. These results are especially interesting because they show that plant sterols can be taken up and utilized by some animal cells. Normally, some barrier in the gastrointestinal tract prevents plant sterols from entering the circulation. Whether only the brush border membranes reject side-chain alkylated sterols has not been investigated. Anaerobic and therefore sterol-requiring yeast also responds to pairs of sterols which individually are inadequate for supporting growth. In this instance, lanosterol can serve as the bulk sterol and traces of ergosterol, the sterol yeast normally produces, will evoke the synergistic response.[129] A brief report by Nes et al.[88] also suggests a synergistic response to sterols by anaerobically growing yeast.

## X. STEROL NUTRITION AND METABOLISM OF YEAST

For studying the sterol requirement of an eucaryotic cell *Saccharomyces cerevisiae* has been the most widely used organism. The utility of yeast stems from the fact that, cultured under rigidly anaerobic conditions, yeast becomes an absolute sterol auxotroph.[3] Expectedly, ergosterol, the sterol yeast normally synthesizes, satisfies this requirement best. Under the conditions described by Nes et al.,[87] other 24β-methyl sterols, for example 24-methylcholesterol and brassicasterol, substituted well, but cholesterol or other $C_{27}$ sterols only poorly for ergosterol. No detectable growth occurred with lanosterol or cycloartenol (Table 4).

The sterol responses of the yeast mutant GL7 which is deficient in squalene epoxide cyclase and also in heme biosynthesis[57] bear little resemblance to the sterol specificities described for anaerobic wild-type yeast except that both cell types grow well on ergosterol (Table 4). Clearly the mutant is much less fastidious than the wild type from which it is derived. Whether the responses of either cell type are representative of the sterol specificities of normal yeast, let alone other eukaryotes, seems dubious. Thus, a strictly anaerobic environment is certainly unphysiological for yeast, an organism more properly classified as microaerophilic. As for GL7, apart from the deficiencies in sterol and heme biosynthesis,[57] the mutant not only lacks mitochondria[21] but also promitochondria, the structures which have been identified in anaerobic wild-type yeast.[118] Perhaps it is for this reason, i.e., the absence of respiratory organelles, that the sterol specificities of GL7 are less stringent. As mentioned earlier, ergosterol has been implicated in aerobic yeast adaptation, i.e., the promitochondria-mitochondria transformation.[98] It seems unlikely that in the heme deficient GL7 strain this process is operative. At any rate, the different sterol specificities of wild-type cells and GL7 may once again point to a dual function of sterols. Compounding the complexities, the mutant GL7 responds differently to various sterols when grown under anaerobic and aerobic conditions (Table 4). Thus, judging from growth responses alone the sterol requirement of GL7 is quite readily met by lanosterol, dihydrolanosterol, and cycloartenol in anaerobic but not in aerobic cultures. While these results suggest a broader sterol specificity for anaerobic GL7 and perhaps a more restricted metabolic pattern, some of the numerical data are probably misleading. Competence for a given

**Table 4**
**STEROLS AS GROWTH FACTORS FOR ANAEROBIC *SACCHAROMYCES CEREVISIAE* AND STRAIN GL7**

| | *S. Cerevisiae*[a] wild-type | GL7[b] | |
|---|---|---|---|
| | | Anaerobic | Aerobic |
| Ergosterol | 100 | 100 | 100 |
| Cholesterol | 23 | 100 | 100 |
| Cholestanol | 23 | | 100 |
| Lathosterol ($\Delta^7$-cholestenol) | 38 | — | 100 |
| 7-Dehydrocholesterol | 23 | — | 100 |
| Lanosterol | <1 | 69 | <1, 90[c] |
| Dihydrolanosterol | <1 | 124 | 95[c] |
| Cycloartenol | <1 | 90 | 45 |

[a] Data from Reference 87.
[b] Data from References 23 and 24.
[c] After adaptation by serial transfer.

cell can be judged only if the sterol molecule remains intact as supplied. This is true for anaerobic GL7 but not when the mutant is grown in air. Originally it was observed that cultures derived from cells raised on ergosterol fail to grow on lanosterol or dihydrolanosterol. However, by a serial transfer of cells which diluted the ergosterol content of successive generations, GL7 eventually fared well on the two trimethyl sterols.[24] The basis of this adaptation is metabolic modification. Dihydrolanosterol adapted cells contained, apart from the administered sterol (68%), 4,14-dimethyl-$\Delta^8$-cholestenol (18%) and 14$\alpha$-methyl-$\Delta^8$-cholestenol (14%). The retention of the 14$\alpha$-methyl group in these metabolites is expected since the heme-deficient GL7 must also lack cytochrome $P_{450}$, the electron acceptor known to be required for demethylation at $C_{14}$.[55] Adapted cells were even more active in metabolizing 14-desmethyl-4-methyl sterols, e.g., 4,4′-dimethyl-$\Delta^7$-cholestenol to $\Delta^7$-cholestenol and 7-dehydrocholesterol (>95%), and 4$\alpha$-methylcholestanol to cholestanol (80%). The unnatural 4$\beta$-monomethyl epimer was recovered unchanged. While these metabolic modifications complicate any interpretations as far as relative sterol efficiencies are concerned, a reasonably clear picture emerges if growth responses to adapted and unadapted cells are compared (Table 5). Aerobically, it appears, lanosterol or dihydrolanosterol per se do not support the growth of GL7. Growth on these substrates after adaptation is the result of $C_4$ demethylation to 4,14′-dimethyl- and 4-desmethyl-14-methyl derivatives. 4,4′-Dimethylsterols and 4-monomethyl stenols afford good growth of both unadapted and adapted cells ostensibly because 4-dealkylation of substrates lacking the 14$\alpha$-methyl group proceeds readily even without adaptation. Because of these interfering events, it is not clear whether 4-mono- and 4,4′-dimethyl-14-desmethyl derivatives as such, fully or partially replace $C_{27}$ sterols for purposes of growth. It seems likely, however, that they do not — or only very poorly — on the basis of the following circumstantial evidence. Anaerobic wild-type yeast will not grow at all on 4$\alpha$-methyl-$\Delta^7$-cholestenol (lophenol) or 4,4′-dimethyl-$\Delta^7$-cholestenol (Sobus and Bloch, unpublished); oxygen is necessary for demethylation, regardless of the position of the alkyl substituent. When supplied to GL7, 4$\beta$-methyl-$\Delta^7$-cholestenol causes only slight growth of aerobic cultures; the molecule is recovered intact from the cells because the yeast 4-demethylase is $\alpha$-specific. This has also

**Table 5**
**AEROBIC GROWTH RESPONSES OF UNADAPTED AND ADAPTED (DIHYDROLANOSTEROL) GL7**

| | Unadapted | Adapted |
|---|---|---|
| Cholestanol | 100 | 100 |
| Dihydrolanosterol | <1 | 90 |
| 4,4'-dimethyl-$\Delta^7$-cholestenol | 50 | 100 |
| 4$\alpha$-methyl-$\Delta^7$-cholestenol | 100 | (100) |
| 4$\beta$-methyl-$\Delta^7$-cholestenol | 9 | — |
| 14$\alpha$-methyl-$\Delta^7$-cholestenol | 90 | |
| 3$\alpha$-methylcholesterol | 10 | |

From Buttke, T. and Bloch, K., *Biochemistry*, 20, 3267, 1981. With permission.

been reported for rat liver.[123] In sum, yeast seems to require sterols unsubstituted at $C_4$ or perhaps at other positions near the $C_3$-hydroxyl group. The fact that 3$\alpha$-methyl cholesterol is a very poor sterol source for yeast strain GL7 reinforces this conclusion (Table 5). By contrast, *M. capricolum* grows reasonably well with $C_4$- and $C_3$-alkylated sterols without modifying these molecules.[38]

As for the influence of the 14$\alpha$-methyl substituent, this exposed methyl group appears to have no adverse effect on sterol competence in yeast, contrary to expectation. Thus the ability of GL7 to adapt to growth on dihydrolanosterol was attributed to $C_4$-dealkylation, yielding products still containing the 14$\alpha$-methyl group.[24] In a more direct experiment, 14$\alpha$-methyl-$\Delta^7$-cholestenol itself proved to be nearly as efficient for GL7 as the dealkylation product $\Delta^7$-cholestenol (Table 5). These results, while consistent with the ability of other yeast mutants defective in $C_{14}$ demethylation to grow without receiving exogneous sterol,[101,137] are nevertheless anomalous or seemingly incompatible with one of the principal points made throughout this review: the importance of a planar sterol $\alpha$-face, unobstructed by axial methyl groups, for acyl chain ordering. Perhaps this generalization — based both on a great variety of experimental findings and model building — needs to be modified or refined. We see no reason to abandon the postulate that a totally dealkylated sterol $\alpha$-face optimizes van der Waal's interactions and membrane order. However, the retention or artificial introduction of a single axial methyl group either at $C_{14}$ or near the OH function in ring A, need not totally impair sterol competence as long as fatty acyl segments of sufficient length remain for productive interactions with regions in the sterol ring system more remote from the obstructing $\alpha$-face substituent. Comparing the patterns for *M.capricolum* and yeast (either GL7 or wild type) we suggest that these minimal segments need not be the same for the two sterol auxotrophs. For mycoplasma growing well on 3$\alpha$-, or 4$\alpha$-methyl-cholestenol, but poorly on 14$\alpha$-methylcholestenol, the critical contact regions may be in the vicinity of rings B, C, and D, (i.e., fatty acyl carbons distant from $C_1$). Conversely for yeast, growing well on 14$\alpha$-methylcholestenol but not on 3$\alpha$-methyl or 4$\alpha$-methylstenols, the essential interactions may involve ring A and a more proximal segment of the acyl chains. Some support for this hypothesis is found in the ESR data showing differential ordering effects depending on methyl group position and/or orientation (Section IV.).[37] The above proposal neglects, of course, sterol interactions with membrane constituents other than phospholipid acyl chains such as membrane proteins.

We have recently determined the minimum amount of sterol required by the yeast mutant GL7 and found it to be much less than expected, about 0.5 $\mu$g/m$\ell$. This is much lower than the sterol concentrations used routinely in earlier comparative studies (~10 $\mu$g/m$\ell$). The question therefore arises whether in mutant cells receiving a minimal supply of ergosterol the cellular sterol concentrations or sterol-phospholipid ratio ever reach a level sufficient for significantly influencing the physical state of the membrane.

With the qualifications mentioned, mutant GL7 has been useful for examining and defining the sterol requirements of a simple eukaryotic cell. It should be kept in mind that the mutant is impaired both enzymatically and structurally. In addition, the heme deficiency of GL7 has helped to characterize several enzymes involved in the demethylation of lanosterol and in some other sterol transformations. As pointed out, 4-mono- and 4,4'-dealkyl-sterols readily undergo oxidative demethylation in unadapted cultures and dihydrolanosterol is similarly metabolized after suitable adaptation. These events occur in spite of the heme deficiency. Demethylation at $C_4$ by yeast enzymes, a process already known not to require $P_{450}$,[92] therefore is mediated by an electron transfer chain devoid of cytochromes. In other, more thoroughly studied instances, the initial attack on terminal or branched methyl groups ($\omega$-oxidation of fatty acids, lanosterol demethylation at $C_{14}$) $P_{450}$ serves as the electron acceptor and link to oxygen (the oxidative demethylation of cholesteryl methyl ether in the mutant GL7 has already been noted — see Section V.).

Strain GL7 readily introduces a $\Delta^5$ bond into $\Delta^7$-cholestenol. Hence the enzyme responsible for establishing the 5,7 diene system, formally by hydrogen abstraction, requires oxygen, but evidently not heme-protein. Oxygenated intermediates have not been encountered in this reaction. Ring B saturated sterols are not dehydrogenated by GL7 suggesting assistance by the $\Delta^7$ double bond.

Yeast and rat liver sterol demethylases share certain properties but not others. Yeast demethylates $C_4$ alkyl sterols whether or not the $C_{14}$ methyl groups has already been removed.[24,137] For $C_4$ demethylation by rat liver microsomes, prior dealkylation at $C_{14}$ appears to be essential.[85,122] While the GL7 4-demethylase is clearly less specific, the "conventional" demethylation sequence appears to operate in wild-type *Saccharomyces,* i.e., the $C_{14}$ methyl substituent is the first to be removed from lanosterol. 4-Desmethyl-14 methyl sterols have been isolated only from mutants but not from wild-type yeast.

The invariant order of lanosterol demethylation ($C_{14}$ methyl → 4$\beta$-methyl → 4$\alpha$-methyl) has been attributed to evolutionary pressures, the selection of steps which successively improve the functional competence of the sterol in membranes. Inspection of the yeast data just discussed, showing that 14$\alpha$-methylcholestenol is an excellent sterol source for GL7 but that 4-methyl sterols are not, appears to be at variance with the evolutionary hypothesis. Should the yeast demethylation sequence not begin at $C_4$($\beta$-$CH_3$) rather than at $C_{14}$? One can escape from this dilemma by postulating that yeast adopted and retained the conventional pathway that already existed in more primitive predecessor cells. If $C_{14}$ demethylation of lanosterol is sufficiently rapid, i.e., not rate limiting, then no advantage was to be gained by changing the sequence. While GL7 has the potential of demethylating at $C_4$ without prior removal of the 14$\alpha$-methyl group, the 4$\alpha$-demethylase which acts on 32-nor 4-methyl sterols may not be the same enzyme that catalyzes the same process with lanosterol as the substrate. This possibility is raised by the observation that GL7 needs to be adapted in order to grow on lanosterol but not for growth on 32-norsterols, even though growth is the result of $C_4$-demethylation in both instances. As already noted, for the corresponding liver systems removal of methyl at $C_{14}$ is mandatory prior to demethylation at $C_4$. Either the evolutionarily later mammalian enzyme has a higher degree of substrate specificity than the enzyme of yeast or, alternatively, yeast elaborates two independent $C_4$ demethylases, one more and the other

less dependent on the presence of the 14-methyl group; the latter enzyme may have been lost during evolution.

Another mechanistic aspect of $C_4$ demethylation in yeast came to light during a comparison of various 4-mono- and 4,4′-dimethylcholestane derivatives.[24] The test sterols differed in the orientation of the 4-monomethyl substituent, some were saturated and others contained a B-ring double bond in different positions. With all 4$\beta$-monomethyl derivatives (of cholestanol, $\Delta^7$-cholestenol, or cholesterol) GL7 growth was poor and ceased at about 1/10 the optimal density obtained with the corresponding 4-desmethyl compounds. In no instance was dealkylation observed. The high degree of steric $\alpha$-specificity for 4-demethylases, from whatever source, has already been noted. By contrast growth as well as dealkylation was rapid with 4$\alpha$-monomethyl derivatives of either $\Delta^7$-cholestenol or cholestanol. However the corresponding cholesterol derivative, $\Delta^5$-4$\alpha$-methylstenol supported only marginal growth. It may be inferred that it was not metabolized since the cells grew well on cholesterol. A closely analogous pattern was seen with the corresponding 4,4′-dimethyl compounds.

Demethylation and hence growth occurred only with $\Delta^7$-stenol or stanol but not with the corresponding $\Delta^5$-stenol. The finding that B-ring unsaturation is not required for $C_4$ dealkylation, that the $\Delta^7$-stenol is completely demethylated while the $\Delta^5$-isomer is not, parallels the specificity of the corresponding liver 4-demethylases. Mechanistically, this discrimination is not readily explained. While B-ring unsaturation clearly does not assist in oxidative demethylation, there are no steric or electronic reasons why a 5,6 double bond should impair or abolish this process. Introduction of this bond does not perceptibly alter the B-ring conformation or the orientation of the oxidizable 4$\alpha$-methyl group. While more subtle mechanistic factors may explain these double bond effects they bear significantly on the sequence of biosynthetic events. In the normal pathway, the $\Delta^7$ bond arises from the 8,(9) olefin by isomerization[85] and remains in this position throughout the demethylation events that terminate with the formation of $\Delta^7$-cholestenol. Only then is the 5,6 position dehydrogenated. Earlier desaturation, e.g., at the 4,4′-dimethyl stage, would block methyl group removal at $C_4$ as discussed above. At the same time the retained $\Delta^7$ double bond — which does not interfere with demethylation — facilitates the later $\Delta^5$ desaturation. The resulting 5,7-diene system survives in ergosterol but animal tissues reduce it to form $\Delta^5$-stenol. Earlier reductive elimination of the $\Delta^7$ double bond, i.e., formation of a saturated B-ring, would not interfere with $C_4$ demethylation but would block $\Delta^5$-desaturation. Once fully reduced, the sterol B ring is not desaturated by the yeast mutant $\Delta^5$-dehydrogenase.[24] The specificities of the sterol demethylases, dehydrogenases, and reductases are therefore fully compatible with the sequential events the biosynthetic pathways has chosen.

We wish to raise here a relevant, not hitherto considered aspect of comparative sterol biochemistry. Yeast, whether wild type or mutant, retains the 5,7-diene system in ring B, while animal tissues do not except for the specialized purpose of vitamin D formation. The 5,7-diene system is notoriously sensitive to autoxidation. Is it possible that animal cells, exposed necessarily to oxygen, have chosen to reduce the 5,7-diene system to protect their membrane sterols against harmful autoxidation? Yeasts exist in a relatively more anaerobic environment and therefore may not face the problem of oxygen toxicity. Alternatively, yeasts may have special mechanisms for protecting the 5,7-diene system against autoxidation.

## XI. THE ROLE OF ERGOSTEROL IN YEAST

Traditionally, ergosterol and related minor side-chain alkylated sterols are regarded as the typical membrane sterols of yeasts and fungi with the tacit but by no means proven assumption that their function is similar to that of cholesterol in animal tissues.

Ergosterol differs from cholesterol by having an extra methyl group at $C_{24}$ of the side chain and two additional sites of unsaturation. Both structural features appear relatively late during ergosterol biosynthesis by modification of zymosterol (IV), a $\Delta^{8,24}$ analogue of cholesterol.[85] In a sense therefore, side-chain alkylation and the introduction of additional double bonds may be viewed as "postsynthetic", presumably beneficial events. They may have evolved to satisfy some specialized needs related to yeast physiology. In efforts to extend the analysis of structure-function relationships to ergosterol, our focus has been on the rationale of side-chain methylation, in the expectation that methyl addition can be shown to be beneficial and why.[23]

Much as model membrane studies have clarified cholesterol structure-function relationships, for ergosterol they have been much less informative. When introduced into lecithin vesicles, ergosterol increases membrane order only at low concentrations (up to 8 mol %). At higher levels the condensing effects are reversed, indicating weakening instead of strengthening of van der Waal's interactions.[121] Since the molar sterol/phospholipid ratios in the plasma membranes of yeast are only sightly lower than they are in hepatic plasma membranes,[111] one would expect the former to be relatively disordered. Indeed mutants of *S.cerevisiae,* that accumulate $C_{27}$ sterols (zymosterol and cholesta-tri- and tetraenols,[7]) contain more ordered membranes than wild-type cells.[74] The disordering effect of ergosterol can therefore be reasonably attributed to the bulky methyl substituent at $C_{24}$. Why a relatively disordered membrane, i.e., greater mobility of acyl chains, is advantageous for yeast physiology and apparently not for animal cells may be related to the fact that Saccharomyces grows optimally at 30° rather than at 37°.

In wild-type yeast grown under strictly anaerobic conditions and therefore auxotrophic for sterol, cholesterol, or 7-dehydro-cholesterol meet the sterol requirement to some extent but clearly not as well as ergosterol.[87] A beneficial effect of the $C_{24}$ methyl group is therefore apparent.

In our experience, sterol efficiencies can be more reproducibly compared with yeast strain GL7, the mutant lacking squalene epoxide-lanosterol cyclase. Moreover, since GL7 is pleiotropic, deficient also in heme synthesis, growth media must be supplemented with an unsaturated fatty acid as well.[57] This provides a handle for controlling and varying both the fatty acid milieu and the sterol composition of the mutant. When supplied with a mixture of oleate and palmitoleate, the unsaturated fatty acids normally synthesized by wild-type yeast, cells grow as well on cholesterol or 7-dehydrocholesterol as on ergosterol.[26] When oleate alone is the external fatty acid source, growth on ergosterol remains optimal but is significantly poorer with cholesterol or 7-dehydrocholesterol. Single fatty acids which afford essentially equal growth rates on ergosterol and nonalkylated sterol are all relatively low-melting: linoleate and the isomeric $\alpha$- and $\gamma$-linolenates. By contrast, petroselenate, the relatively high-melting (30°) $\Delta^6$-isomer of oleate (m.p. 12°) allowed optimal growth on ergosterol but only marginal growth on 7-dehydrocholesterol. The choice of unsaturated fatty acids available to the cell for phospholipid synthesis therefore determines whether or not the yeast cell profits from the presence of an extra methyl group in the sterol side chain. In the absence of this substituent membrane disorder (or high fluidity) is achieved by a supply of low-melting fatty acids. When ergosterol is available, the degree of fatty acid unsaturation matters less or not at all. Presumably in this case the side-chain methyl group, i.e., the noncyclic sterol moiety, creates the membrane disorder that is apparently beneficial.

In parallel studies,[26] the effects of replacing one sterol by another on cellular phosphilipid composition were examined. All cultures received palmitoleate-oleate (1:4) as fatty acid supplements. With ergosterol as the sterol source, the percentages of saturated fatty acids ($C_{16}$ and $C_{18}$) were invariably high (40 to 45%) in both major phospholipids (phosphatidylethanolamine and phosphatidylcholine) about twice those

found in cells supplied with 7-dehydrocholesterol. Thus sterol replacement (presence or absence of side-chain alkyl groups) controls the fatty acid composition of the yeast membranes to a considerable degree. The direction of the changes again fits the notion that relative disorder is the state yeast membranes prefer. In normal yeast it is achieved by sterol side-chain alkylation. In $C_{24}$ methylase mutants or in the absence of sterol synthesis, cholesterol or 7-dehydrocholesterol will replace ergosterol but their condensing effects will be compensated or counteracted by the formation of more unsaturated phospholipids.

It should be feasible to verify this interpretation by comparing bulk fluidities of yeast membranes derived from GL7 cells growing at the same rate either on ergosterol or on cholesterol (or 7-dehydrocholesterol) and supplemented with one of the more unsaturated fatty acids. At any rate, yeast apparently adapts readily to a foreign sterol by adjusting relative transacylation rates for saturated and unsaturated fatty acids. Whether this phenomenon is in any way related to the effects of cholesterol on phospholipid synthesis observed in mycoplasma[41] remains to be investigated.

Why yeast selects sterol side-chain methylation to achieve membrane disorder, presumably near the interior of the bilayer, can only be surmised. If indeed this structural disorder benefits yeast viability, the same degree of fluidity can be achieved by cholesterol, or other sterols lacking the $C_{24}$ methyl group in conjunction with formation of more unsaturated phospholipids. This alternative solution as demonstrated in the experiments cited here is certainly feasible but probably resorted to only under artificially imposed conditions. For *S.cerevisiae* sterol akylation as it occurs normally may be of special advantage because this organism synthesizes only mono-unsaturated fatty acids. The option of raising membrane fluidity by producing more highly unsaturated fatty acids is not available to *Saccharomyces.* Perhaps the device of side-chain alkylation chosen by plants and yeast is an example of evolutionary tinkering which succeeded and was therefore retained throughout the evolution of fungi and plants. Alkylated sterols are typically found in simple microbial eukaryotes as well as in plants, subject to widely varying temperatures in their natural habitats. The extra degree of fluidity created by the side-chain alkyl group may permit an organism to grow well and in fact optimally at relatively low temperatures.

For cells that produce ethanol, ergosterol may have another advantage. Yeast grown anaerobically on ergosterol is more resistant to ethanol than cells provided with cholesterol.[133] Similarly, $C_{24}$ methylase mutants are more ethanol sensitive than wild-type cells[59] as is strain GL7 when grown on 7-dehydrocholesterol instead of ergosterol.[22]

It is beyond the scope of this article to review the much more complex subject of plant sterol chemistry. Mention should be made however of the finding that for GL7 the most widely occurring plant sterols ($C_{24}$-ethyl) $\beta$-sitosterol and stigmasterol are as effective sterol sources as ergosterol.[26] Moreover, the plant sterols produce phospholipids with the same high content of saturated fatty acids as does ergosterol. That a side-chain ethyl group should have the same or perhaps even greater membrane disordering effect is to be expected. On the other hand, the special advantages apparently accruing to plants by addition of an ethyl instead of a methyl branch remain obscure, especially since plants have a vast capacity for synthesizing highly unsaturated fatty acids. If it is the principal conclusion from the studies described that ergosterol is specially designed to create membrane disorder or a relatively high membrane fluidity whereas, by contrast, cholesterol has the opposite effect in animal* or other cholesterol-containing membranes, then some qualifications are in order. Only about half of the total yeast

* Exceptions to this generalization have been reported for LM cell sterol mutants; cholesterol depletion causes an increase in the oleate content of the cellular phospholipids.[5]

sterols are associated with plasma membranes.[98,111] The remainder resides in other, not yet clearly identified organelles. A role for ergosterol in the biosynthesis of yeast mitochondria during aerobic adaptation has been suggested.[98] Whether the same structure-function relationships obtained for these and other, not necessarily fluidity related processes remains to be investigated.

It should be stressed again that the generalizations made here were derived largely from the behavior of a yeast mutant or yeast cultured in the complete absence of oxygen. Their responses are not necessarily representative of normal, wild-type cells. Observations which emphasize this point have been made with lanosterol as a sterol source. As discussed in Section X, wild-type yeast deprived of oxygen will not grow when supplied with lanosterol. On the other hand lanosterol elicits good growth of GL7 and of other mutant stains isolated by Gollub et al.[57] but only under anaerobic conditions. Aerobic cultures of GL7 do not grow on lanosterol unless adapted. Clearly the sterol requirements for the anaerobically raised mutant, though still absolute, is less specific, reflecting perhaps a more primitive metabolic or structural mode.

## XII. YEAST GROWTH ON CYCLOLAUDENOL AND CYCLOARTENOL

As described in an earlier section, lanosterol and the isomeric cycloartenol, both 4,4',14 trimethyl sterols, differ in their effects on artificial membranes and as sterol supplements for *M.capricolum*.[39] Cycloartenol raised the microviscosity of phosphatidylcholine vesicles substantially, whereas lanosterol did not. Likewise, mycoplasma cells grew more rapidly on the cyclosterol than they did on lanosterol. Conformational differences were invoked to explain the more cholesterol-like behavior of cycloartenol. Analogous studies have been carried out with the yeast mutant GL7. Cyclolaudenol, a 24-methyl derivative of cycloartenol (XV) was included for comparison.[26]

In model membranes cyclolaudenol, unlike cycloartenol does not elicit any significant condensing effects; microviscosity values remain low at all sterol concentrations.[39] It appears that perturbations by the extra methyl group in the side chain compensate for, or override, the acyl-chain ordering effect of the ring system as already noted above for ergosterol.

Since yeast prefers side-chain alkylated sterols (ergosterol over cholesterol) a comparison of a similar pair in the 9,19-cyclo sterol series, methylated and nonmethylated was of interest. Under both anaerobic and aerobic growth conditions, cyclolaudenol, the $C_{24}$ methyl derivative of cycloartenol, was the superior sterol source for GL7 (Table 6). The results further support the notion as discussed in the preceding section that yeast physiology benefits from the membrane disorder created by the bulky methyl group at $C_{24}$ in the bilayer interior.

For mycoplasma growth, the opposite is the case,[39] cycloartenol is superior to cyclolaudenol (Table 6). The respective microviscosities of the isolated mycoplasma membranes show similar differences, in keeping with the preference of these cells for sterols that condense or solidify the membrane bilayer.

The systematic comparison of sterol specificities for two microorganisms, one a mycoplasma strain and the other a yeast mutant, along with sterol responses of artificial membranes has led us to a less rigid, but perhaps more refined definition of sterol structure-function relationships. If one accepts growth rates as valid measures for the competence of an essential nutrient, then in many instances the biological response closely parallels the relative fluidity changes caused by sterols in artificial membranes. This parallelism holds for lanosterol, cycloartenol, and the various natural intermediates in the pathway to cholesterol. However, for a growing list of sterols — both natural and synthetic — the correlation is poor or nonexistent. The most striking examples so far are 3$\alpha$-methylcholesterol, ergosterol, and cyclolaudenol. Moreover, from the examples

**Table 6**
**RELATIVE EFFICIENCIES OF STEROLS (10 μg/mℓ) FOR GROWTH OF *M. CAPRICOLUM* AND YEAST MUTANT GL7**

| Sterol | M. Capricolum | | Yeast Mutant GL7 (maximum O.D. values) | |
|---|---|---|---|---|
| | Maximum O.D. values | $\bar{\eta}$[a] | Aerobic | Anaerobic |
| Cholesterol | 100 | 4.77 | 100 | 100 |
| Lanosterol | 69 | 3.16 | 3 | 69 |
| Cycloartenol | 90 | 4.31 | 45 | 90 |
| Cyclolaudenol | 76 | 3.34 | 90 | 103 |

[a] Microviscosities of isolated cell membranes.

Adapted from References 23 and 38.

given it is clear that a given sterol type may adequately satisfy the nutritional requirement of one organism or cell type but not of another. A universal membrane role for a given sterol structure can therefore not be defined. Only certain features, e.g., a rigid polycyclic ring system, appear to be essential and invariant. Secondary structural features, (e.g., presence or absence of side chain methyl groups) will determine whether the beneficial sterol-induced change is in the direction of membrane order (cholesterol) or disorder (fungal and plant sterols).

Perhaps a further caveat is called for here. The vast majority of studies on sterol function on biological membranes deal with sterol levels in the range employed or necessary for detecting fluidity changes in artificial phospholipid bilayers. Yet some cells or their membranes contain or require much smaller amounts of sterols. It may therefore be asked whether in such instances, e.g., when molar sterol-phospholipid ratios are 1:10 or less, the presence of sterol has any bearing on fluidity control. It was mentioned earlier that sterol-rich regions may exist locally in membranes that are otherwise sterol-poor and therefore exhibit low bulk fluidity. In these instances sterol could still exert structural effects as traditionally assumed but only in patches or restricted domains. Alternatively the phenomenon of sterol synergism (Section IX.) raises the possibility that in membranes of low sterol content, the sterol-dependent process is solely metabolic not structural. This may be the reason why the sterol requirement of yeast or yeast mutants is so low. Marginal sterol concentrations, i.e., less than 10 mol % are also found invariably in eukaryotic organelles other than the plasma membrane. These low sterol levels may in some instances be due to cross-contamination but the possibility that they are involved in some metabolic membrane-associated process cannot be excluded.

## XIII. MARINE STEROLS

Previous sections of this review offered an explanation for the benefits accruing to yeast and possibly also plants from adding extra alkyl groups to the isooctyl sterol side chain. Sterol structures bearing many more additional side chain alkyl groups have recently come to light from a systematic study of marine invertebrates.[47] It appears that six of the eight side-chain carbons are potential methylation sites, the extra alkyl groups conferring additional bulk to the flexible moiety of the sterol molecule. While a discussion of this intriguing subject is beyond the scope of this review, some brief comments bearing on related issues raised here earlier seem in order. For example it was proposed (Section XI.) that the $C_{24}$ methyl group of ergosterol is bound to disorder

interior regions of the phospholipid bilayer, compensating for the unavailability of more highly unsaturated fatty acid in yeast.[26] A similar critical relationship between the structure of sterol side-chains and that of the apposite fatty acyl chains was recently proposed by Walkup et al.[139] for certain marine invertebrates. Their hypotheis predicts that "the phospholipids of a marine organism will contain unique structural features which reflect their complimentarity to an alkylated sterol". The sponge *Aplysina fistulans,* known to contain the side chain alkylated ($C_{24}$, $C_{26}$) aplysterol (XI)[44] was analyzed for phospholipids as well. Several novel fatty acids were isolated, including two possessing monomethyl-branched chains, e.g., 11-methyloctadecenoic acid. Nearly all the "unusual fatty acids" contained either a methyl branch or a double bond at the $\omega 7$ carbon. Walkup et al.[139] suggest "that *A. fistularis* has evolved the synthesis of these substituted acids as a means for increasing the fluidity of its membranes in a region of the bilayer located near the ends of the fatty acyl chains". It is known from studies with artificial membranes that methyl branches on a fatty acyl chain and olefinic bonds have comparable effects on bilayers.[54,126] Attractive as these correlations are, they will be difficult to verify because both the sterol and fatty acid composition commonly found in marine invertebrates is highly heterogeneous. While aplysterol is the major (60% sterol of *A. fistularis,* the sponge contains eight additional sterols, including 11% cholesterol. The phospholipid composition (six types) and even more so the fatty acid pattern (26 components) is exceedingly complex. Whether a given sterol, such as aplysterol pairs specifically or preferentially with a "complimentary" phospholipid, e.g., one containing $\omega 7$-branched fatty acids, can obviously not be answered with currently available techniques. In any event it remains a mystery why certain cells, e.g., algae as well as marine invertebrates, deal with the problem of membrane fluidity by elaborating exceedingly complex arrays of sterols and phospholipids; while others, such as yeast, function competently with one dominant sterol and a much simpler fatty acid pattern. The natural habitat of a given organism probably determines the degree of diversity. Marine invertebrates are subject to widely varying environments, whether physical, chemical, or biological. While some of their lipids are undoubtedly of endogenous origin, others may be exogenous, i.e., adventitiously acquired from the food chain with little absorptive discrimination. Cells reared in culture in a defined environment might well afford lipid patterns of lesser complexity.

Two other structural departures found in the sterols of marine invertebrates are probably of minor functional consequence. 4$\alpha$-Methylsterols (e.g., lophenol) occur universally but usually as transient intermediates in lanosterol or cycloartenol demethylations. They are minor sterols in terrestial eukaryotes but, as has been noted, appear to be end-products of sterol biosynthesis in *Methylococcus capsulatus.* In marine organisms, 4$\alpha$-methylsterols are not uncommon, e.g., dinosterol (X) in *Gonyaulox tamarensis.*[124] Dinosterol accounts for as much as 60% of the total cellular sterol and is therefore a candidate for regulating bulk membrane properties in the dinoflagellate. It will be recalled that 4$\alpha$-methylsterols are the most immediate alkyl precursors of the cholestane system, only slightly inferior functionally to cholesterol proper, both in artificial and natural membrane systems. On the basis of this functional proximity both dinosterol and the 4$\alpha$-methylcholestenols of *Methylococcus capsulatus* can be looked upon as way stations in the evolution of the sterol pathway.

In another, more noteworthy departure from sterol metabolism in terrestial cells, nuclear demethylation at the sterol $\beta$-face occurs in the sponge *Axinella polypoides.*[81] The sterol in question, 19-norcholesterol, arises from cholesterol itself[45] and judging from the quantities present, qualifies as a functional membrane sterol. The fact that $\beta$-face demethylation does occur at all, albeit in a so far isolated instance, certainly weakens the hypothesis that nuclear demethylations in biological systems are confined

to the sterol α-face. However, since 19-norsterols are functionally less competent than cholesterol both in artificial membranes[72] and as growth factors (for *Mycoplasma carpricolum* and the yeast mutant GL7)[22] it remains defensible to argue that β-face demethylation — in contrast to those involving α-face methyl groups — is unproductive for sterol function in membranes. To escape the apparent dilemma we have suggested[72] that β-face demethylation, confined as it is to some primitive cells, may exemplify unsuccessful evolutionary tinkering which, for that reason, was abandoned during the evolution of organisms. Sponges appear to be dead-end branches in the phylogenetic tree.

## XIV. STEROLS AND PENTACYCLIC TRITERPENES IN PROKARYOTES

As recently as a decade ago, sterols were thought to be inventions of eukaryotic cells. The search for sterols in a variety of bacteria and blue-green algae proved either negative or, when positive results were claimed, documentation was inadequate. This author, among others, was attracted by the notion (no longer tenable) that intracellular organelles and sterol biosynthesis evolved concurrently. It therefore came as a surprise when Bird et al.[14] described the isolation of 4,4′-dimethyl- and 4α-monomethyl sterols in substantial quantity (0.22% of dry weight) from the obligate methanotroph, *Methylococcus capsulatus*. More recently, the conversion of squalene 2,3-epoxide to lanosterol has been demonstrated in methylococcus extracts.[113] The expectations that, if looked for, sterols might be found more widely in prokaryotes has not been born out. Over the years there have appeared several reports describing the isolation of cholesterol or plant sterols from bacteria usually after processing of large quantities of cells; however, with two exceptions, the sterol contents were at least one order of magnitude smaller than in eukaryotic microbes. The exceptions are the isolation of cholesterol from stable L forms of *Staphlococcus aureus*[60] and of cholesterol and β-sitosterol (0.03 to 0.05%) from *Cellulomonas dehydrogenans*.[141] In both instances the reported structural identifications were unambiguous.

The question remains why sterols are so rare in bacteria and why, when present, their quantities are so small. A content of, e.g., 0.01% would appear to be insufficient to effect bulk membrane fluidity significantly. In the few instances when they occur, a role for sterols in bacteria is not apparent. The usual classifications, e.g., Gram-positive or negative, presence or absence of the peptidoglycan cell wall, or morphology provide no guide for this limited distribution except that the few sterol-producing bacteria are obligate aerobes. This is consonant with the postulate that the emergence of the sterol biosynthetic pathway coincided with or followed the advent of aerobic cells.

Squalene, the universal acyclic sterol precursor occurs much more commonly though not ubiquitously in prokaryotes,[2,56,135] (Tornabene et al., 1969; Golberg and Shechter, 1978; Amdur et al., 1978) including the anaerobic Archebacteria, bacteria thought to be the most primitive.[136] The pathway to acyclic polyisoprenoids, including squalene, employs only anaerobic reactions. It could therefore develop early in evolution without the biosynthetic restraints imposed by the presumed absence of oxygen from the primitive biosphere.

Historically, it is of interest that mevalonic acid, the obligate precursor of isoprenoids, was discovered as a growth factor of *Lactobacillus* mutants[144] at a time when isoprene derived cell constituents were known in bacteria. The identification of bactoprenol[62,134,143] and isopentenyl adenine[127] in bacterial RNA, and of bacterial squalene came much later.

From what is known it appears that the acetate-mevalonate-polyprenol pathway is universal and developed in the most primitive forms of life. On the other hand,

prokaryotic squalene synthesis, while common, is not found in all taxonomic groups. It probably represents a point of evolutionary departure. Only cells that had acquired squalene synthetase could be antecedents of organisms equipped with squalene cyclizing enzymes as well. The various modes of further disposition of squalene, a molecule that has no known function per se, represents a striking example of biological diversity or, in the words of Jacob,[67] "evolutionary tinkering". By exploring conformational potentials of rigid polynuclear structures, cells have devised at least three types of cyclizations. Chemically, the hydrocarbon itself cyclizes with great facility.[68] In the most primitive biological mode, cyclizing enzymes act on the thermodynamically most favored all-"prechair" conformation of the hydrocarbon, affording, as in chemical systems, fully cyclized pentacyclic triterpenes, (e.g., the hopanoid diplopterol (XVI) or tetrahymanol (XVII). Apparently these processes are fully concerted, employing only $H^+$ as initiating and $OH^-$ as terminating reagents. Even though oxygen does not enter the reaction, anaerobic squalene producers apparently have not yet acquired enzymes capable of performing cyclizations. Whether one should regard some of the cyanobacteria (e.g., Anabena) which produce hopanoids[97] as exceptions depends on the classification one chooses. They are oxygen producers, but their metabolic patterns are anaerobic.

Hopanoids, originally isolated from some ferns, lichens, and mosses[13,79] have now been found in a sizable number of aerobic bacteria belonging to diverse taxonomic groups.[112] Whether their presence confers any selective advantage on the host organism is unknown since mutants lacking the cyclase have not been described. In model systems such as monolayers, tetrahydroxyl bacteriohopenes, mixed with dipalmitoylphosphatidylcholine exhibit condensing effects and quench phase transitions as does cholesterol.[104] It is therefore not unreasonable to view the hopanoids as primitive sterol equivalents and to assign to them a cholesterol-like, membrane-stabilizing function. Such a role can be imagined for prokaryotes adapted to extreme milieus (heat, pH), e.g., *Bacillus acidocaldarus,*[69] but is more difficult to rationalize for the hopanoid-containing bacteria growing in more normal environments.

Prior to the discovery of bacterial hopanoids, the universal presence of the peptidoglycan cell wall in prokaryotes was thought to obviate the need for additional membrane-stabilizing structures. That sterols are generally absent in bacteria was sometimes explained on this basis. Therefore, if hopanoids should prove in fact to play a structural, integrity-preserving role wherever they occur, then prokaryotes lacking polycyclic molecules might have devised alternate solutions, perhaps modified phospholipid structures for the same purpose.

Whether or not hopanoids and related pentacyclic triterpenes are functional antecedents of sterols, they do represent one early and successful device for transforming a flexible hydrocarbon into a rigid conformationally locked structure. It is important to note that cyclization of squalene to hopanes and their tetrahydroxypentane derivatives are terminal events. These polycyclic systems do not undergo any further metabolic modifications.[17] Stereomodels of these structures do in fact suggest, as do the model membrane effects cited above, that removal of branched alkyl groups might reduce rather than enhance condensing effects. In the context of the ultimate evolution of the sterol molecule, the hopanes and tetrahymanol are therefore dead-end structures.

A separate cyclization mode had to evolve for generating the tetracyclic steroids. This divergence of sterol evolution by oxygen-dependent squalene cyclization can actually be seen at work in *Methylococcus capsulatus.* In the early papers on methylococcus sterols,[14,19] only the isolation of partially demethylated lanosterol derivatives was noted. More recently, Rohmer et al.[113] discovered that *Methylococcus* extracts in fact catalyse two independent cyclizations. Squalene as the substrate yields the hopanoids diploptene and diplopterol while added (R/S) squalene epoxide is converted to lanosterol, 3-

epilanosterol and various hydroxyhopanoids. A squalene cyclase and a squalene epoxide cyclase, presumably distinct entities, therefore coexist in the methanotrophic organism. Interestingly the bacterial squalene epoxide cyclase(s) utilize both the R and S epimers; they lack the stereospecificity characteristic of the corresponding eukaryotic squalene epoxide cyclases.[8] Evidence for the existence of the requisite squalene epoxidase (NADPH-dependent) in *Methylococcus* was also obtained.[113] Whether this enzyme also lacks stereospecificity, i.e., produces the two epimeric epoxides was not stated. At any rate, Methylococcus emerges as a key organism for tracing sterol evolution to its beginning. Perhaps other more primitive bacteria elaborated squalene epoxidase but not the squalene epoxide cyclase. In principle, screening of bacteria for this enzyme is feasible but apparently has not been attempted.

As already stated the sterols isolated from *Methylococcus* cells are 4,4′-dimethyl and 4α-methylcholestenols, partially demethylated lanosterol metabolites. They do not ordinarily accumulate in eukaryotic cells.

That in *Methylococcus* the sterol pathway appears to stop short of completion, may be due to lack of a 4α-monomethyl oxidase. However, if this were the case one would be forced to postulate separate enzymes, specific for the metabolism of 4,4′-dimethyl and 4-monomethyl sterols, respectively. The evidence available for the corresponding rat liver systems suggests — but does not prove — that in the mammalian system a single, α-specific demethylase successively removes both C-4 alkyl groups, i.e., it has broad substrate specificity.[52] It would be surprising if the *Methylococcus* system, presumably more primitive, proved to be more highly specific as the arrest of demethylation at the stage of 4α-monomethyl sterol suggests.

A membrane function for the sterols *Methylococcus* produces has yet to be demonstrated. It is perhaps of interest that in some methylotrophic species an extensive system of intracytoplasmic membranes has been recognized. Squalene and sterols seem to be associated with these structures.[100]

## XV. TETRAHYMANOL

To date the occurrence of nonoxidative squalene cyclizations to pentacyclic triterpenes in eucaryotes has been recorded only in species of the genus *Tetrahymena*. *Tetrahymena pyriformis* and other ciliated protozoans normally contain tetrahymanol (Figure 2) in lieu of sterols[78] and produce it by proton-initiated squalene cyclization.[148] Sterols appear to be totally absent. This unique example of eukaryotic membrane chemistry — the retention of the prokaryotic nonoxidative mode of squalene metabolism — suggests that the more primitive squalene metabolites already possess some of the properties characteristic of sterols common to eukaryotic membranes.[84] When introduced into artificial phospholipid vesicles, tetrahymanol, like cholesterol, exerts a condensing effect above the transition temperature, a property it shares with diplopterol, the pentacyclic triterpene found in some bacteria.[104] Even more to the point, supplementation of *T. pyriformis* growth media with cholesterol (10 μg/mℓ) inhibits tetrahymanol synthesis without any effect on growth. Cholesta-5,7,22-trienol, derived intracellularly from exogenous cholesterol, replaces tetrahymanol in the protozoan cell membranes.[77] Thus, for the ciliate, a close cholesterol derivative if not cholesterol itself, appears to be functionally equivalent to tetrahymanol or vice versa. Ergosterol, a normally occurring 5,7,22-trienol, likewise enters *Tetrahymena* cells and when so doing inhibits tetrahymanol synthesis with equal efficiency.[77] In this case the sterol-tetrahymanol replacement causes the formation of more saturated fatty acids especially for phosphatidylethanolamine synthesis.[50,90] These shifts to more saturated fatty acid patterns are reminiscent of the changes that occur when ergosterol and cholesterol are the respective sterol sources for the sterol-requiring yeast mutant GL7.[26]

It may be assumed that the flexible, conformationally unrestrained isooctyl side-chain appended to the sterol ring system fluidizes rather than condenses the more interior segment of the membrane bilayer. Tetrahymanol, being fully cyclized lacks any structural feature of comparable mobility. Nevertheless, the two molecules are physiologically interchangeable in the instance of ciliated protozoa. Notably, *Tetrahymena pyriformis* also contains some diplopterol,[148] the anaerobic squalene cyclization product otherwise found only in some bacteria.

According to a brief report,[140] the primary site at which sterols inhibit tetrahymanol synthesis is located at the level of squalene synthetase, not HMG-CoA reductase. It seems remarkable that an organism apparently lacking the squalene-sterol pathway should nevertheless respond to a feedback inhibitor that it does not normally produce. The question may therefore be raised whether *T. pyriformis,* in spite of its apparent sterol independence, synthesizes some molecule of this class, but in amounts too small to be readily detected. The resistance of *T. pyriformis* to polyene antibiotics when grown without sterol supplement[35] seems to rule out this possibility. Nevertheless it is tempting to speculate that in *Tetrahymena* as in some other cells, (see Section IX). "sterol synergism" is at play. Assuming this to be the case, tetrahymanol would be expected to serve as the bulk membrane modifier while the trace sterol might have some metabolic function. Support for this notion, albeit indirect, may be seen in the observations that some ciliates greatly benefit from, if not absolutely require, a sterol for optimal growth. This is true for *Tetrahymena corlissi Th-X*[64] and *Tetrahymena setosa.*[65]On media of known composition, growth of these ciliate strains is stimulated markedly and equally by cholesterol and some of its derivatives, by ergosterol and by plant sterols. Only minute quantities of sterol (1 $\mu$g/m$\ell$) are needed for growth promotion. In *T. setosa* cells cultured on a fully synthetic medium with sterol supplements of this small magnitude, tetrahymanol synthesis is not impaired.[9] The inhibitory effect of sterol on tetrahymanol synthesis in *T. pyriformis* mentioned above is seen only at 20-fold higher sterol concentrations.

In recent experiments[58] we have found that as little as 0.05 $\mu$g/m$\ell$ of ergosterol supports the growth of *T. setosa.* Under these conditions the cellular amounts of tetrahymanol are normal (tetrahymanol:ergosterol = 100:1) and therefore likely to serve as bulk membrane components. The function and the localization of ergosterol or other sterols which *T. setosa* requires in trace amounts remains to be defined. The quantities of sterol incorporated are, in any event, too small to effect bulk membrane properties significantly.

Lanosterol and cycloartenol, the products of oxidative cyclization become functional only after metabolic removal of three nuclear methyl groups. Similar processing does not occur with tetrahymanol or, as previously mentioned, with the bacterial hopanes; the $C_{30}$ molecule remains intact and appears to be functionally competent as such. As the space-filling model shows, the unmodified pentacyclic structure displays two planar regions disposed favorably for interaction with phospholipid acyl chains. We suggest that the tetrahymanol (and diplopterol) ring systems, unlike lanosterol and cycloartenol, retained all of its alkyl branches, because their removal would not produce molecules that are functionally superior.

## SYNOPSIS

A biological system, whether an enzyme or any other cellular receptor responds to certain ligands but not to others. By systematically modifying the structure of a natural ligand or substrate and determining the kinetic consequences of the responding system one arrives at certain correlations between structure and function. Chemical groups that are essential, beneficial, or deleterious for biological activity thus can be defined. We

have taken a different approach to the problem of specificity. Evolution at the molecular level means that for a given purpose nature has chosen certain molecules but not others by a process of extensive experimentation. Guided by this maxim we have scrutinized the late stages of an existing biosynthetic process by examining the functional consequences of substituting sequential intermediates for the final product of a pathway. From this analysis it is concluded that the pathway nature has chosen leads to progressive improvement and therefore is directed by evolutionary pressures.

One cannot rationalize functional competence or perfection, either intuitively or deductively, simply by inspecting chemical structures per se, i.e., without reference to the environment in which it functions. If, however, the molecule is viewed as a component of a system, interaction with a chemically defined partner will be determined by complimentarity. Affinity or fitness becomes a determinable parameter even if the interaction occurs without chemical change.

Cholesterol intercalated between acyl chains of a phospholipid bilayer represents such a complimentary system. A sterol that fits and interacts effectively with the phospholipid partner will alter the physical state of the membrane more than a molecule that is poorly accommodated. Lanosterol, the earliest intermediate in the sterol pathway fits least and cholesterol, the end product, fits best. The fitness of partially demethylated intermediates lies in between. This has been shown by appropriate measurements both with artificial and natural membranes. We therefore conclude that the temporal sequence of steps nature has chosen for the sterol pathway evolved in time in response to selection pressures. Improvement of function was the driving force.

Prevailing views assign to membrane sterols a role that is predominantly or exclusively structural, modulation of membrane fluidity and, perhaps secondarily, control of various physiological events. The majority of the experiments described here are consistent with this view; however exceptions have been encountered. Certain unnatural sterols are highly effective growth factors for sterol auxotrophs even though they are without influence on membrane fluidity. Sterols may therefore play a second role in membranes, unrelated to fluidity control. For example, since the sterol ring system is bulky and rigid, its insertion into the bilayer will separate not only contiguous fatty acryl chains but also phospholipid head groups. As a result, electrostatic interactions or charge densities at the membrane-water interface may be affected without necessarily changing bulk fluidity.

Certain sterol auxotrophs can be raised on mixtures consisting of a relatively ineffective sterol and trace amounts of a sterol that in larger amounts is optimal for promoting growth of the organism. The two sterols act synergistically. So far the evidence suggests that the synergistic effect is likewise unrelated to fluidity control but involves some metabolic, membrane-associated events. In mycoplasma, cholesterol specifically stimulates some step in the biosynthesis of phospholipids, tentatively identified as the uptake or activation of unsaturated fatty acids. The apparent linkage between cholesterol supply and phospholipid synthesis may have a bearing on coordinated events in cellular growth and membrane assembly.

For obvious reasons, membranes rich in sterols (e.g., the red cell or plasma membranes) have been the most popular materials for studying sterol function. Little attention has been paid to the role of sterols in the more numerous membranes that are sterol-poor. Clearly, the smaller the sterol content the smaller the likelihood that in such membranes the bulk physical state of the bilayer is under sterol control. Perhaps there are membranes, organelles, or cells which require sterol in small amounts solely for control of some metabolic process.

This review also addresses the vexing question why side-chain alkylated sterols normally predominate in the membranes of yeast. This specialized structural feature

appears to be the solution chosen by yeast for enhancing membrane disorder in the bilayer interior as an alternative to producing more highly unsaturated fatty acids. The broader problem of single or multiple side-chain alkylations, not only in yeast but also in plants and marine invertebrates, is becoming an area of growing interest.

Finally we comment on recent research in several laboratories which raises intriguing new questions about the evolution of sterol-like molecules. Early in biological time, probably prior to the advent of the oxidative route from squalene to tetracyclic sterols, certain bacteria acquired a one-step anaerobic process for cyclizing squalene to pentacyclic triterpenes. These molecules, the hopanes and tetrahymanol (found only in ciliated protozoa) appear to be functional sterol equivalents. However this primitive alternative to oxidative squalene cyclization is of limited potential. Higher cells (except for tetrahymena) did not adopt it. Once formed, these pentacyclic triterpenes remain structurally intact, i.e., metabolically inert. Probably they are antecedents but not precursors in the biochemical sense of the tetracyclic sterols which arose and evolved independently. Thus, once squalene became available, primitive cells, experimenting with the acyclic hydrocarbon, found a chemically very simple one-step process for forming a rigid and bulky molecule. This solution however was of limited scope. Later cells chose the more complex but ultimately more versatile oxidative route to the sterol structure, the advent of which marks a turning point in the evolution of organisms.

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