An analogous K^+/H^+ symporter has been proposed for *Streptococcus faecalis* (Bakker & Harold, 1980).

In either model the K^+ transporter would contain at least one binding site for K^+ and one for Na^+, presumably the normal substrates. In the presence of Na^+, Tl^+ accumulation closely parallels that observed for K^+ in that both the TrkA and the Kdp systems are stimulated and the observed discrimination against Rb^+ is similar to that observed by Rhoads et al. (1977) for K^+ transport. Li^+ inhibits both Tl^+ and K^+ accumulation via the TrkA system. Perhaps Li^+ is a very poor substrate for this system. Li^+, however, stimulates Tl^+ uptake via the Kdp system and is a much more effective stimulator than Na^+ at concentrations over 20 mM. This is not true for K^+ uptake. Li^+ also decreases the discrimination of the Kdp system for Rb^+. Possibly when Li^+, with a smaller crystal ionic radius than Na^+, interacts with the Na^+ site of the Kdp system, the effective size of the K^+ site is increased somewhat. This might decrease the interaction of the site with K^+ and facilitate the binding of the larger Tl^+ and Rb^+ ions. This could account for the large enhancement of Tl^+ uptake seen in the Li^+ assay buffer compared to the Na^+ assay buffer (Figure 4) in contrast to the decreased uptake of K^+ in the Li^+ assay buffer compared to the Na^+ assay buffer (Figure 8). In any case, although Li^+ and Tl^+ are probably not physiological substrates of the Kdp and TrkA K^+ transport systems, they are useful as probes for the interactions of Na^+ and K^+ with these systems.

In preliminary studies of the growth of TK2240 and T-K1001 on agar plates, we have been unable to observe a requirement for Na^+ for growth, although a requirement for K^+ is easy to observe. Thus, the cells can probably maintain internal levels of K^+ sufficiently high for growth even when very little Na^+ is present. Perhaps some other cation (H^+, NH_4^+, or choline) can serve well enough as a substitute for Na^+ to allow for growth.

The observations in this paper indicate that Na^+ is probably involved in the mechanism of K^+ accumulation via the Kdp and TrkA systems in *E. coli*. Future efforts will concentrate on determining what model best explains the effect of Na^+ on each K^+ transport system. At least one of these systems, the Kdp system, may be very similar to the Na^+/K^+-ATPase of higher organisms.

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Effect of Alkyl-Substituted Precursors of Cholesterol on Artificial and Natural Membranes and on the Viability of *Mycoplasma capricolum*†

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ABSTRACT: Various alkyl-substituted sterols and stanols representative of the intermediates in cholesterol biosynthesis from lanosterol have been compared with respect to (a) their effect on the physical state of lecithin vesicles, (b) their efficacy as growth factors for the sterol auxotroph *Mycoplasma capricolum*, and (c) their effect on the physical state of the respective mycoplasma membranes. By all three criteria, sterol effectiveness progresses in the order lanosterol < 4,4-dimethylcholestanol ≤ 4β-methylcholestanol < 4α-methylcholestanol < cholestanol < cholesterol. Since the corresponding steps in cholesterol biosynthesis occur in the same order, we conclude that the nuclear modifications of the lanosterol structure by oxidative demethylation serve to improve the membrane function of the sterol molecule.

The presence of sterols in membranes of all but the most primitive cells suggests that they perform an essential function in higher forms of life. At the physiological or biochemical level, this membrane function is not well understood. It can, however, be readily demonstrated that experimentally induced changes in cholesterol concentration alter the physical state of membranes and in turn modulate a broad range of cellular processes including lateral diffusion of receptors (Frye & Edidin, 1970), ion transport and solute permeability (Wiley & Cooper, 1975; Kimelberg & Papahadjopoulos, 1972), cell–cell interactions (Edelman, 1976), and the shape of cell surfaces (Heiniger et al., 1976).

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The rigid, polycyclic cholestane system appears to be uniquely designed for modulating membrane properties as studies with artificial membranes have shown (Oldfield & Chapman, 1972; Demel & DeKruyff, 1976). By inference, the same is true for natural membranes, but information on sterol structure–function relationships in biological systems is much more limited.

In a recent speculative paper from this laboratory dealing with certain aspects of sterol evolution, structure, and function (Bloch, 1976), reasons were given for the compulsory removal during the late stages of cholesterol biosynthesis of methyl substituents linked to the α face of lanosterol. Demethylation at C-14 and C-4, it was argued, results in a planar α face rendering the molecule more effective for interacting hydrophobically with contiguous phospholipid fatty acyl chains in the lipid bilayer. Subsequently, and in support of the hypothesis, sterols bearing alkyl substituents at C-4 and C-14 were shown to be inferior to cholesterol in reducing glucose permeability or increasing the microviscosity of lecithin vesicles (Lala et al., 1978). Likewise, carbon-13 nuclear magnetic resonance studies showed lanosterol to be much more mobile in phospholipid bilayers than cholesterol (Yeagle et al., 1977). Normally, only traces of lanosterol are found in eucaryotic membranes (Schneider et al., 1957). Moreover, lanosterol fails to support the growth of the sterol-requiring hide beetle *Dermestes vulpinus* (Clark & Bloch, 1959) and accumulates in a mutant line of Chinese hamster ovary cells which die when deprived of cholesterol (Chang et al., 1977). On the other hand, lanosterol, 4,4-dimethylcholesterol, and 4-monomethylsterols were recently shown to support the growth of *Mycoplasma capricolum*, a sterol-requiring procaryote (Odriozola et al., 1978). They do so without being metabolized. This unique sterol auxotroph has made it possible to study the effects of cholesterol precursors on growth and cell viability and on the physical state of a biological membrane. Parallel studies show an excellent correlation between the competence of various methyl-substituted cholesterol derivatives as growth-promoting sterol sources and their ability to influence the physical state of artificial membranes.

Materials and Methods

Chemicals. Cholesterol (Sigma) was recrystallized from ethanol and dried in vacuo. Lanosterol (Sigma) was purified according to established procedures (Block & Urech, 1958). The following compounds were received as gifts: 4β- and 4α-methylcholestan-3β-ols from T. A. Spencer; 4,4-dimethylcholesterol (4,4-dimethylcholest-5-en-3β-ol) and 4,4-dimethylcholestanol (4,4-dimethylcholestan-3β-ol) from R. B. Woodward; dihydrolanosterol from R. B. Clayton. All sterols were checked before use by gas–liquid chromatography and found to be 99% pure. Cholestanol, 5α-cholestane, heptadecanoic acid, and elaidic acid were purchased from Sigma; palmitic acid was from Supelco; egg yolk phosphatidylcholine (PtdCho)[1] was from Avanti.

Growth of M. capricolum and Isolation of Membranes. M. capricolum (California Kid strain 14, ATCC 27342) was cultured on a modified Edward medium (Razin & Rottem, 1976) after the bactoheart infusion broth, bactopeptone, and yeast extract components had been exhaustively extracted with chloroform–methanol (2:1 v/v). Thallium acetate was omitted and the PPLO–serum fraction was replaced with 4 mg/mL essentially fatty acid free bovine serum albumin (Fraction V, Sigma), 10 μg/mL sterol, 5 μg/mL palmitate, and 6.5 μg/mL elaidate. Ethanolic solutions of sterols and fatty acids were mixed and added to the medium. The final concentration of ethanol in the medium was 0.5% by volume. One hundred milliliters of medium in a 125-mL Erlenmeyer flask was inoculated with 0.1 mL of a cholesterol-grown culture and incubated statically in a 37 °C water bath. Growth was followed spectrophotometrically by reading the absorbance at 640 nm against a water blank and by monitoring the pH of the medium. Mass doubling times were determined from semilog plots of the increase in absorbance of the culture with time after correcting for an initial absorbance reading of 0.04. In the late logarithmic phase of growth when the pH had declined to 6.7, cells were harvested by centrifugation at 12000*g* for 10 min. Cells were washed with 0.25 M NaCl and lysed in deionized water at 37 °C (Razin & Rottem, 1976). Membranes were pelleted at 30000*g* for 30 min followed by a 1-min treatment at 37 °C with 10 μg of deoxyribonuclease in 0.05 M NaCl and 0.01 M sodium phosphate buffer, pH 7.4. Membranes were then washed once in 0.05 M NaCl and 0.01 M sodium phosphate buffer, pH 7.4, and once in deionized water. The membranes were suspended in deionized water for lipid extraction and microviscosity studies. Membrane protein was determined by the procedure of Lowry et al. (1951).

Lipid Analysis of M. capricolum Membranes. Membrane lipids were extracted according to Folch et al. (1957). Heptadecanoic acid (50 μg) and 5α-cholestane (20 μg) were added as internal standards to extracted lipids prior to saponification. Lipids were saponified by heating in 0.5 N KOH in 90% methanol for 1 h at 70 °C, and the unsaponifiable lipids were extracted with petroleum ether. The aqueous layer was then acidified with 6 N HCl and extracted with petroleum ether. Fatty acid methyl esters were generated with 14% boron trifluoride in methanol. Lipids were quantitated by gas–liquid chromatography (GLC) on a Perkin-Elmer instrument (Model 900) equipped with a 1.8-m column of 3% SP2250 (Supelco) at 260 °C for sterols and 10% SP2330 (Supelco) for fatty acid methyl esters with a temperature program from 150 to 210 °C increasing at 6 °C/min.

Thin-layer chromatography analysis of the total lipid extract of cholesterol- or lanosterol-enriched membranes on silica gel G in chloroform–methanol–acetic acid–water (80:13:8:0.3 v/v) showed the lipid to be comprised predominantly of phospholipids and sterols. No glycolipids were detected. Major phospholipids were tentatively identified as phosphatidylglycerol and cardiolipin by comparison of their R_f values with known standards. On the basis of this identification, the amount of phospholipid in the total membrane lipids of *M. capricolum* was calculated from one-half the molar fatty acid content by using 748 as the average molecular weight for phospholipid.

Vesicle Preparation. Vesicles containing different sterols were prepared by a modification of the procedure of Szoka & Papahadjopoulos (1979). The desired amounts of sterol and PtdCho (1.29 μmol) in hexane were mixed in a 25-mL round-bottom flask and evaporated to dryness under argon. To the film of lipid was added 1.0 mL of NaCl (10 mM) and 3.0 mL of diethyl ether. This biphasic mixture was sonicated for 3–5 min in a Branson sonicator (bath) at 4 °C to give a homogeneous opalescent dispersion. The organic solvent was then removed under reduced pressure (water aspirator) at 25 °C. The aqueous solution containing the lipid vesicles was then placed on a high vacuum line to remove any remaining organic solvent. The vesicle preparation was passed through

[1] Abbreviations used: PtdCho, phosphatidylcholine; GLC, gas–liquid chromatography; $\bar{\eta}$, microviscosity; DPH, 1,6-diphenyl-1,3,5-hexatriene.

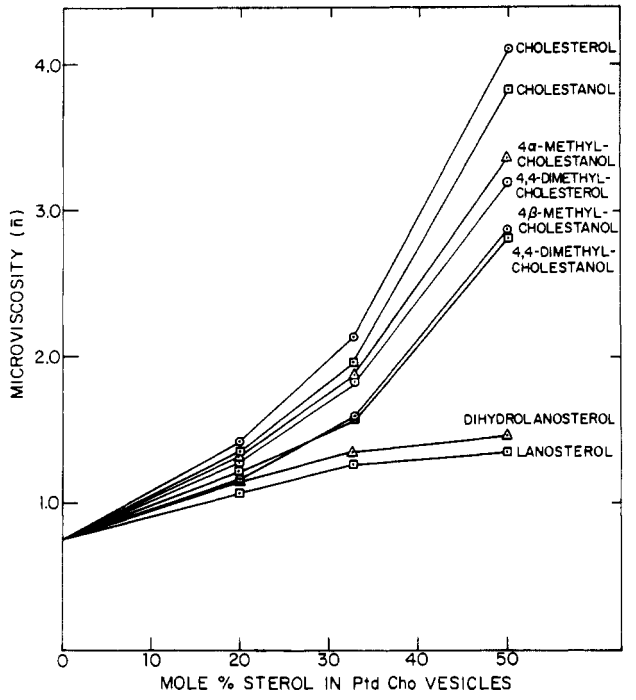

FIGURE 1: Effect of sterol alkyl groups on the microviscosity ($\bar{\eta}$) of PtdCho vesicles at 25 °C.

a Sepharose 4B column to remove any unincorporated sterol in order to analyze the sterol content of the phospholipid vesicles. The amounts of sterol and phospholipid in the eluted vesicles were quantitated by GLC as described above and found to be present in the ratios as added originally.

Microviscosity Measurements of Vesicles and Mycoplasma Membranes. Microviscosities were determined in an Elscint microviscosimeter, Model MV-1A, using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe (Shinitzky & Inbar, 1974; Shinitzky & Barenholz, 1974).[2] To 1 mL of the vesicle preparation (1.29 μmol in PtdCho) or 1 mL of mycoplasma membranes (1 mg of protein per mL) was added 1 mL of 1 μM solution of DPH in 10 mM NaCl. This mixture was incubated at 37 °C for 30 min. The fluorescence depolarization of DPH was then measured at 25 °C for PtdCho vesicles and at 37 °C for mycoplasma membranes, and the microviscosities were calculated according to Shinitzky & Inbar (1976).

Results

Microviscosities of Model Membranes Containing Various Methyl-Substituted Sterols. In extension of an earlier study (Lala et al., 1978) we now present additional data on the microviscosities of PtdCho vesicles containing 4,4,14-trimethyl-, 4,4-dimethyl-, or 4-monomethylcholesterol derivatives. The series includes fully saturated test sterols (except for the lanostane derivatives) and in some instances the corresponding Δ^5-sterols. Therefore, effects of a single structural change, methyl substitution, or ring unsaturation can be compared and assessed. The results of microviscosity determinations using the fluorescent probe DPH are given in Figure 1. At all concentrations, but especially above 30 mol %, cholesterol and cholestanol raised the microviscosity of PtdCho vesicles to a much greater extent than any of the methyl-substituted Δ^5-sterols or -stanols. Sterols lacking the 14α-methyl group caused substantial microviscosity increases, intermediate between those given by lanosterol and cholesterol with the smallest effect shown by 4,4-dimethylcholestanol and the greatest by 4α-methylcholestanol. Notably, the microviscosity values for vesicles containing 4β-methylcholestanol (axial 4β-methyl) and 4,4-dimethylcholestanol were essentially identical and distinctly lower than those containing 4α-methylcholestanol (equatorial 4α-methyl). Clearly the orientation of alkyl substituents at C-4 influences the membrane sterol behavior. In the two instances tested (Figure 1), the Δ^5-sterols were significantly more effective than their ring-saturated counterparts.

Table I: Effect of Various Methylated Cholestane Derivatives on the Growth Rate and Cell Yield of *M. capricolum*

sterol added to growth media (10 μg/mL)	mass doubling time (h)	absorbance of late log culture at 640 nm
cholesterol	1.8	0.45
cholestanol	2.5	0.40
4α-methylcholesterol	4.0	0.32
4,4-dimethylcholesterol	4.6	0.31
4,4-dimethylcholestanol	5.6	0.30
4β-methylcholestanol	5.4	0.23
lanosterol	6.0	0.18
dihydrolanosterol	9.0	0.12

Growth of M. capricolum on Various Methyl-Substituted Sterols. The same series of sterols characterized with respect to their ability to raise the microviscosity of artificial membranes (Figure 1) have been tested as growth supplements for the sterol-requiring *Mycoplasma* strain. *M. capricolum* growth responses to some of the test compounds have already been reported (Odriozola et al., 1978) and are confirmed by the present results, at least qualitatively. However, we consider the data obtained in the more recent investigation more reliable for the following reason. For our previous study the growth media used were supplemented with bovine serum albumin (Calbiochem) that contained cholesterol, as subsequently discovered. The amounts apparently varied markedly from batch to batch (K. Lala and A. K. Lala, unpublished experiments). Since the presence of even small amounts of cholesterol obscures the true efficiency of the sterols that were to be compared with cholesterol, we have chosen for the present study a more rigidly delipidated bovine serum albumin preparation (less than 3 μg of cholesterol per g of protein). The amount of cholesterol found to be attributable to the lipid-extracted basal medium without serum albumin added was less than 0.01 μg/mL. The levels of contaminating cholesterol in the unsaponifiable fractions from cells of the various cultures were checked by GLC and were found in all instances to be less than 2% of the total sterol. Cell growth can therefore be attributed with confidence to the test sterol proper without any significant contribution by contaminating cholesterol. The same GLC analyses have confirmed our earlier conclusion (Odriozola et al., 1978) that *M. capricolum* is unable to modify any of the sterol structures utilized.

In the absence of any sterol supplement measurable growth did not occur. The relative efficacies of the various sterols are shown and expressed both as mass doubling times (hours) and as absorbance of a given culture in the late logarithmic phase of growth (Table I). The sterols are arranged in the order of their growth-promoting capacity which is greatest for cholesterol and smallest for dihydrolanosterol. Mass doubling

[2] We are aware of recent reports (Chen et al., 1977; Dale et al., 1977; Kawata et al., 1977; Tilley et al., 1979) questioning the validity of absolute microviscosity values obtained from fluorescence depolarization measurements. In the present work we report relative $\bar{\eta}$ values of membranes differing only in the structures of the sterol component and we consider these to be valid.

Table II: 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)				satd/unsatd fatty acid ratio	$\bar{\eta}$
		16:0	18:0	18:1	18:2		
cholesterol	30.6	41.7	2.2	52.9	<0.1	0.88	5.05
cholestanol	32.0	43.2	1.9	53.8	0.9	0.81	4.72
4α-methylcholestanol	27.5	43.2	1.5	55.0	0.2	0.81	4.53
4,4-dimethylcholesterol	28.0	39.4	2.3	57.1	1.0	0.72	4.31
4,4-dimethylcholestanol	33.0	44.5	1.6	53.5	0.3	0.85	4.09
4β-methylcholestanol	21.8	42.6	1.3	55.7	0.3	0.78	3.69
lanosterol	24.9	47.6	1.5	50.8	<0.1	0.97	3.17
dihydrolanosterol	24.4	57.5	1.3	41.2	<0.1	1.40	3.54

times range from a low of 1.8 h for cholesterol to 9 h for dihydrolanosterol and the corresponding cell yields (absorbance at 640 nm) from 0.45 to 0.12. There is an inverse relationship between mass doubling time and absorbance. All compounds previously found to support mycoplasma growth (Odriozola et al., 1978) gave positive results also under the present more controlled conditions, i.e., elimination of cholesterol as a possible contaminating sterol source.

Examination of Figure 1 and Table I reveals a remarkably close correlation between the ability of the various sterols to increase the microviscosity of artificial membranes and their efficacy as growth factors of *M. capricolum*. A given structural modification changes both parameters in the same direction. The behavior of lanosterol, though not contradicting this conclusion, is somewhat surprising since lanosterol has only a minimal effect on microviscosity in artificial membranes (Figure 1), yet it supports mycoplasma growth at a substantial rate [see Dahl et al. (1980)]. The correlation between the physical properties of artificial membranes and mycoplasma growth holds also for Δ^5-unsaturated vs. saturated sterols, the latter being the less effective by both criteria.

Lipid Composition. Present evidence suggests that cells employ two devices for adjusting membrane fluidity in response to environmental changes: raising or lowering the ratio of saturated to unsaturated fatty acids (Marr & Ingraham, 1967; Sinensky, 1971) and changing the levels of membrane-associated sterol (Sinensky, 1978). Since the present study reveals a wide range of microviscosities in artificial membranes as a function of sterol structure—of course at constant fatty acid compositions—and since mycoplasma growth rates are similarly dependent on sterol structure, it seemed of interest to examine the fatty acid composition, sterol content, and microviscosities of the membrane fractions isolated from each of the sterol-grown cells listed in Table I. The data are given in Table II. As shown by GLC, in all instances the test sterol added to the medium was recovered unchanged and accompanied by a peak (<2% of total sterol) corresponding in retention time to cholesterol. This cholesterol probably arises in part from the cholesterol-grown inoculum and in part from the very low amounts of cholesterol in the growth media. In the accompanying paper (Dahl et al., 1980) we present evidence that these small amounts of cholesterol (<0.1 μg/mL) in the growth media are insufficient to support measurable growth without additional sterol.

The sterol content of isolated membranes ranged from 21.8 to 33 mol % and tended to be higher for the sterols that supported growth more effectively. However, there is no clear correlation between sterol content per se and rate of growth. For example, 4,4-dimethylcholestanol showed as high an enrichment in the membrane as cholesterol, yet it supported growth relatively poorly (Table I). Similarly, whereas cells grew at approximately the same rate on 4,4-dimethylcholestanol and 4β-methylcholestanol (Table I), cell sterol content was substantially higher with the former than with the latter. Growth rates are therefore not a direct function of total sterol incorporation. Structure rather than total uptake determines the efficiency of a given sterol.

More closely related with mycoplasma growth rates are the microviscosities of the membranes isolated from the various cells (Table II). The more efficient a sterol as a mycoplasma growth factor, the greater the microviscosity ($\bar{\eta}$) values of the isolated cell membranes. Therefore, under the conditions of the present experiments, sterol structure appears to determine growth rates in proportion to its ability to raise the microviscosity of the receptor membrane.

While the absolute $\bar{\eta}$ values of the artificial membranes (Figure 1) and mycoplasma membranes (Table II) are not comparable because they were determined at different temperatures and because the membranes differ otherwise in chemical composition (primarily protein and the nature of the polar lipids), the order in which the various sterols affect this physical parameter of the two membrane types is remarkably similar.

The fatty acid profiles of the various membrane phospholipids and hence the ratios of saturated to unsaturated acids fell into a relatively narrow range. For lanosterol and particularly dihydrolanosterol, these ratios were, however, significantly higher. For a source of fatty acid *M. capricolum* relies entirely on the external medium. As shown in Table II, these cells appear to have only a limited ability to control phospholipid composition by altering the proportional uptake of palmitate and elaidate. In the absence of an effective mechanism for changing fatty acid composition, mycoplasma cells apparently cannot compensate for other factors which lower or raise membrane fluidity, in the present instance the structural characteristics of individual external sterol.

Discussion

An amphipathic orientation of sterols in the bilayer such that the polar 3β-hydroxyl substituent is near some polar phospholipid moiety and the hydrophobic moiety is parallel to the fatty acyl chains seems well established (Demel & DeKruyff, 1976). According to currently favored models, sterols, when so positioned, condense the bilayer above the transition temperature by hydrophobic association between the rigid tetracyclic nucleus and the C-2 to C-10 segment of paraffinic acyl chains (Rothman & Engelman, 1972). The planarity of the cholesterol α face is held primarily responsible for the strength of these interactions (Bloch, 1976). Consequently and predictably, any bulky groups protruding from the α plane in the contact region will weaken the sterol–phospholipid interactions, i.e., increase bilayer fluidity. Some

evidence in support of this hypothesis has already been published. Thus, lanosterol and some of the partially demethylated intermediates in the lanosterol–cholesterol pathway were found to satisfy the sterol requirement of *M. capricolum* to varying degrees but less effectively than cholesterol (Odriozola et al., 1978). At the same time, we have shown that the observed effects of some methylcholestane derivatives on glucose permeability and microviscosity in artificial vesicles follow on the whole the pattern predicted by the hypothesis (Lala et al., 1978). In the present study we have defined sterol structure–function relationships more precisely by comparing sterol effects on (a) the microviscosity of lecithin vesicles, (b) the growth rates of the sterol auxotroph *M. capricolum*, and (c) the microviscosity of the respective mycoplasma membrane fractions. By all three criteria, the sterol competence follows the same order: lanosterol < 4,4-dimethylcholestanol ≤ 4β-methylcholestanol < 4α-methylcholestanol < cholestanol. We have deliberately chosen this series of ring-saturated stanols (with the exception of lanosterol) in order to evaluate a single parameter, the presence or absence of methyl groups linked to the sterol α face. The fact that none of these saturated sterols occur normally in membranes does not, in our opinion, invalidate the comparison since we are dealing with structural rather than metabolic effects. In two instances the corresponding Δ^5-sterols (cholesterol and 4,4-dimethylcholesterol) were slightly more effective by all three criteria. Other natural intermediates in the pathway [4,4-dimethyl-8(9)- or 4,4-dimethyl-7(8)-cholestenols] (Gautschi & Bloch, 1958) remain to be tested. It should be stressed that 4β-methylcholestanol (or stenol) has not been found in biological systems, a fact of interest in the context of sterol structure and function as discussed below.

The superior position of cholesterol in the series tested reflects the almost universal selection of cholesterol (or side-chain substituted, B-ring unsaturated stenols) for function in biological membranes. In order of importance, cholesterol appears to owe this superiority to the following features: planarity of the α face in the region of C-14, absence of methyl substituents at C-4, and insertion of a 5,6 double bond. Lanosterol, a 4,4,14-trimethyl derivative and the first cyclic intermediate in sterol biosynthesis, is least effective as a sterol source for *M. capricolum* and as a modulator of membrane fluidity. There is by now ample evidence that the 14α-methyl group of lanosterol projecting from the sterol α plane interferes most seriously with essential hydrophobic interactions, thereby increasing the opportunity for rapid trans–gauche rotational isomerizations of the fatty acyl chains in that potential contact region. In fact, 14α-methylcholestanol shows the same behavior in model membranes as lanosterol (Lala et al., 1978). On the other hand, neither of the two methyl groups at C-4 (4β-axial and 4α-equatorial) project into the sterol α plane as shown by Dreiding stereomodels or space-filling models. Steric interference with fatty acyl-chain interactions at the α face is therefore not a likely cause for the lower effectiveness of the 4-alkylsterols. Notably, however, the behavior of the 4α-monomethyl derivative is distinctly closer to that of cholestanol than the behavior of either 4,4-dimethylcholestanol or 4β-monomethylcholestanol, the latter two having essentially identical effects on either microviscosity or cell growth. The equatorial 4α-methyl group lies in the plane of the ring system and for that reason should, as it does, only minimally interfere with packing. On the other hand, the axial orientation of the 4β-methyl group adds bulk to the sterol and changes the contour of the β face, which may also be important for hydrophobic interactions (Huang, 1977). We believe it to be significant that the monomethyl intermediates in the lanosterol–cholesterol pathway are always the 4α-stenols and never the 4β compounds (Nes & McKean, 1977). This is true for animal tissues (lophenol, 4α-methylcholest-7-en-3β-ol) and wherever else 4-monomethylsterols accumulate [e.g., in *Methylococcus capsulatus* (Bird et al., 1971; Bouvier et al., 1976) and in dinoflagellates (Shimizu et al., 1976)].

The enzymes that catalyze the first step in the oxidative removal of alkyl substituents at C-4 are specific for methyl groups in the equatorial α orientation. However, contrary to expectation, monodemethylation of 4,4-dimethylcholesterol produces the 4α-monomethyl derivative and not the expected β epimer. Rahman et al. (1970) have clarified this apparent paradox. The methyl group remaining at C-4 epimerizes to 4α-methyl by way of the Δ^4-3-ketone (Sharpless et al., 1969). Cells thus avoid formation of the 4-monomethyl epimer that appears less competent for membrane function.

Finally, we wish to reemphasize that every biosynthetic step in the sequential removal of methyl substituents from the lanosterol structure renders the sterol molecule functionally more competent as judged by the parallel gradient in effectiveness for growth of a sterol auxotroph and the ability to raise the microviscosity of model membranes. In summary, the results presented here support the earlier hypothesis invoking evolutionary pressures as the driving force for nuclear modifications during the terminal phase of cholesterol biosynthesis.

Acknowledgments

A number of experiments similar to those described here and in the accompanying paper were first carried out by Krishna Lala and Dr. Anil Lala. The interpretation of the results was complicated by the inadvertent and then unrecognized presence of cholesterol in the mycoplasma growth medium. We, however, acknowledge their important contributions to the subject under investigation.

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Sterols in Membranes: Growth Characteristics and Membrane Properties of *Mycoplasma capricolum* Cultured on Cholesterol and Lanosterol†

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ABSTRACT: *Mycoplasma capricolum*, a procaryotic sterol auxotroph, shows optimum growth on cholesterol and substantial growth on lanosterol. The effect of the two sterols on membrane fluidity, their ability to support growth on a broad range of fatty acid combinations, and a possible synergism of low amounts of cholesterol and high amounts of lanosterol were studied. When the cholesterol content of *M. capricolum* membranes rose from 14 to 28 mol % of the total lipid, their microviscosities increased from $\bar{\eta}$ = 3.3 to 4.5, whereas membranes containing 14–23 mol % lanosterol showed a constant microviscosity value of $\bar{\eta}$ = 3.1. Arrhenius plots of the microviscosity values of membranes rich in cholesterol were linear over a 40 °C range, but those rich in lanosterol exhibited prominent discontinuities at 20 and 25 °C. Cholesterol allowed the cells to grow on media containing a wide variety of fatty acid supplements, whereas lanosterol supported growth only with certain fatty acid combinations. Finally, low levels of cholesterol unable to support the growth of *M. capricolum* produced a synergistic effect on growth when combined with lanosterol. The results demonstrate the superiority of cholesterol compared to lanosterol as a membrane sterol and suggest a possible role for cholesterol in addition to or other than to regulate bulk membrane fluidity.

Recent studies in this laboratory have disclosed marked differences in the membrane behavior of cholesterol and lanosterol, a trimethyl-substituted precursor of cholesterol. Incorporated into model membranes such as phosphatidylcholine vesicles, lanosterol, in contrast to cholesterol, neither raises the microviscosity nor reduces the exit of vesicle-entrapped glucose (Lala et al., 1978). Carbon-13 nuclear magnetic resonance studies have shed light on the physical basis for these differences; lanosterol is much less immobilized in lecithin lipid bilayers than cholesterol (Yeagle et al., 1977). In eucaryotic sterol auxotrophs lanosterol has also been shown to be incompetent as a substitute for cholesterol (Clark & Bloch, 1959; Chang et al., 1977). Unexpectedly, however, *Mycoplasma capricolum*, a sterol-requiring procaryote, was found to grow moderately well on lanosterol, without modifying it, as well as on cholesterol (Odriozola et al., 1978). This relatively broad sterol specificity offers the opportunity for evaluating the possibility of multiple sterol functions in membranes and the importance of membrane physical state as a determinant of mycoplasma growth. To this end, we have (a) compared the relative ability of cholesterol and lanosterol to modulate membrane fluidity and (b) examined the effect of supplementing lanosterol-containing growth media with low levels of cholesterol. We find that quantities of cholesterol, inadequate by themselves, will, when combined with lanosterol, support mycoplasma growth in a synergistic manner. We also

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