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Lyotropic Liquid Crystals (Myelin Forms) From Some Sterols and Water-Soluble Amphiphiles

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The formation of myelin tubes, a variety of lyotropic liquid crystals easily observable with a microscope, has been studied in different systems involving sterols (or some metabolically or phylogenetically related substances) and various water-soluble amphiphiles. Different factors influencing myelin tube formation have been investigated: temperature, concentration of the amphiphile solution, and the chemical structure of both partners of the association. The prominent position of cholesterol among numerous related substances is emphasized.

Keywords: myelin tube formation, water-soluble amphiphiles, sterols and phylogenetic precursors

INTRODUCTION

Myelin forms (or myelin figures) appear as lyotropic liquid crystalline structures which can be observed by simple optical microscopy.[1] Myelin forms are frequently produced, when amphiphiles, such as lecithin, are brought into contact with water. The tubes which rapidly develop appear as the ordered morphological structures arising from the orientation of the phospholipid molecules, following the attraction of water dipoles by the ionized polar heads and the hydrophobic effect[2] directed towards the aliphatic hydrocarbon chains. Rod-like, fluid structures are also formed when an insoluble amphiphile (such as cholesterol) is immersed in an aqueous solution of a water-soluble amphiphile (for instance a sodium soap).

In previous papers,[3,4] we have shown that a number of sterols closely related to cholesterol form myelin tubes when their crystals are immersed in an aqueous solution of sodium oleate and we have outlined the main structural features which appear to be necessary for the production of well-formed myelin tubes. The direct observation of myelin tube formation from sterols and sodium oleate closely parallels the results obtained by more sophisticated techniques for studying the association of sterols with the constituents of biological membranes. We have developed our initial work following three distinct and often interrelated lines: a) the influence of the temperature and of the concentration of the sodium oleate solution has been investigated; b) several hydrosoluble amphipathic compounds have been compared to sodium oleate with respect to their ability to give myelin tubes with cholesterol; c) the interaction between sterols related to cholesterol (either in a metabolic or a phylogenetic sense) and aqueous solutions of different amphiphiles has been studied.

MATERIALS AND METHODS

Experimental procedure

The technique for examining myelin tube formation has been described in our previous paper.[3] It consists in observing, under the microscope (G = ×50 or 150), a few crystals of a sterol deposited on a slide after allowing a few drops of an aqueous solution of a surface-active agent to run under the cover-glass. The instrumentation is limited to an optical microscope equiped with a thermostatic chamber and a polarizing device.

Materials

Sterols. The sterols were obtained from the following sources: cholest-5-en-3β-ol (cholesterol) from Calbiochem AG (San Diego, Cal., USA); cholest-5-en-3β,7α-diol, cholest-5-en-3β,7β-diol, cholest-5-en-3β-ol-7-one, cholest-5-en-3β,20α-diol, 3β-hydroxy-chol-5-enic acid from Steraloids Inc. (Wilton, NH); 19-*nor*cholesterol, 17-ethinyl-androst-5-en-3β-ol, cholecalciferol, 25-hydroxycholecalciferol, from Roussel Uclaf (Romainville, France); cholest-5-en-3β,22(*S*)-diol, cholest-5-en-3β,22(*R*)-diol from Sigma (St. Louis, MO, USA); thiocholesterol from Aldrich (Beerse, Belgium); samples of squalene, 9:19-*cyclo*-lanost-24-en-3β-ol (cycloartenol), 4,4-dimethyl-14-methylene-9:19-*cyclo*-cholestan-3β-ol (cycloeucalenol), urs-12-en-3β-ol (α-

amyrin), olean-12-en-3β-ol (β-amyrin), lup-20(29)-en-3β-ol (lupeol), hopan-22-ol (diplopterol), hopan-29-ol-22($R+S$) (nerifoliol), bis-homohopan-32-ol-22(R), bacteriohopanetetrol were prepared in the laboratory of Professor G. Ourisson (University of Strasbourg); tetrahymanol samples were gifts of Doctor E. Caspi (The Worcester Foundation for Experimental Biology, Shrewsbury, Mass., USA) and of Professor Y. Nozawa (Gifu University School of Medicine, Japan); cholest-5-en-3β,26-diol, 17-methylamino-androst-5-en-3β-ol and 22(R)-aminocholesterol were provided by Professor A. Crastes de Paulet (University of Montpellier, France).

Amphiphiles. Most sodium salts of the carboxylic acids were prepared in our laboratory; the fatty acid was dissolved in warm absolute ethanol and excess ethanolic NaOH was added. After cooling, the sodium neutral soap precipitated and was collected by filtration.

The fatty acids and the different amphipathic compounds have the following origin: C_{10}, C_{11}, C_{12}, C_{14}, C_{19} and C_{20} saturated linear acids, elaidic acid, erucic acid, dodecylamine, N,N-dimethyldodecylamine-N-oxide, ethyl-dimethyl-dodecylammonium bromide, N-lauroylsarcosine sodium salt, octyl-β-D-gluco-pyranoside, from Fluka (Buchs, Switzerland); 12-hydroxy-stearic acid, ricinoleic acid, sodium ricinelaidate, linolenic acid, γ-linolenic acid, sodium linoleate, sodium arachidonate, Triton X100 from Sigma; sodium hexyl-, octyl-, decyl-, lauryl- and myristyl-sulphates from Mann Research (New York, USA); sodium alkanesulphonates (C_8 to C_{16}) from Aldrich; α-hydroxymyristic and α-hydroxypalmitic acids from Supelco, Inc. (Bellefonte, PA, USA); sodium oleate from Merck (Darmstadt, Fed. Rep. Germany); trimethylcetylammonium bromide, hexadecanoic and octadecanoic acids from Prolabo (Paris, France); Tweens from Atlas Powder Co. (Wilmington, Del., USA).

RESULTS

1. Influence of experimental conditions on myelinization of cholesterol by sodium oleate

Cholesterol crystals placed in a 25 mM sodium oleate aqueous solution give rise to myelin tubes which grow rapidly at room temperature (25°C); the first tubes are observed a few seconds after the crystal has been brought into contact with the soap solution; at first the tubes increase in both width and length but their diameter rapidly reaches a limit in value, ca. 10–15μ, and then remains constant,

whereas a steady lengthening is observed for 5–10 minutes, giving long cylindrical forms still attached to the birefringent fringe around the crystal (Figure 1).

When the growth stops, some swelling usually appears at the end of the myelin form, which may separate from the cylindrical part of the tube, to give an isolated freely floating vesical. Occasionally, a long cylindrical myelin form can turn back and twist upon itself, giving a double-helix structure; however such twisted tubes, also described with phospholipid liposomes,[5] occur much more rarely than those occurring with fatty acid–hydrazine systems.[6,7]

Very similar results were obtained by diluting the oleate concentration to 3 mM. With still lower concentrations (1.5 or 0.75 mM), the myelin forms appear very slowly and are limited to short thin cylindrical tubes. The limit of myelinization is observed with ca. 0.35 mM, a concentration for which a very narrow fringe is still visible around the crystal.

The thermostatic chamber allowed us to investigate the influence of the temperature on cholesterol myelinization by 25 mM sodium

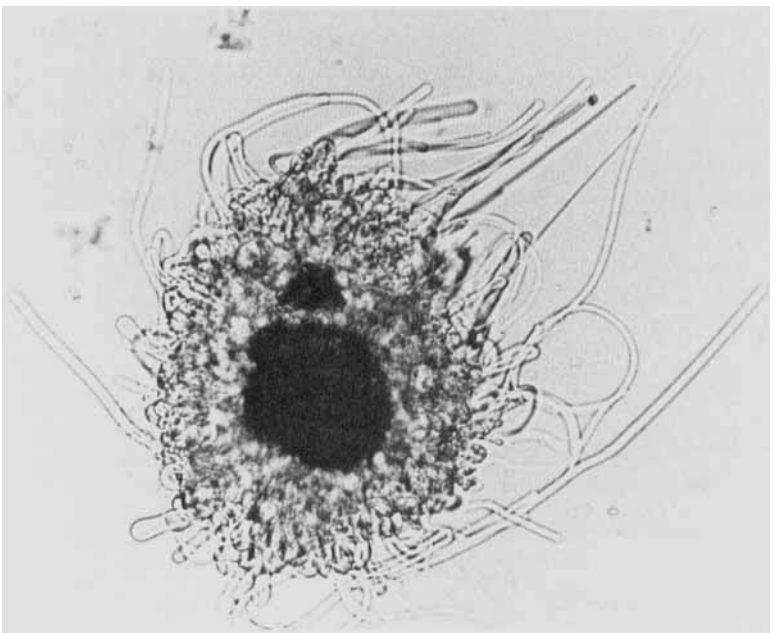


FIGURE 1 Myelin tube formation from cholesterol and 25 mM sodium oleate. Microscope aspect (G × 50) after 10 minutes.

oleate, within the range +4° to 80°C. Myelin tubes were observed over the full range of temperature examined. Apart from the more rapid growth of the myelin forms at higher temperatures, the only noticeable effect of temperature was a greater proportion of twisted structures at higher temperatures.

The techniques of varying the concentration of the aqueous solution of an amphipathic molecule and carrying out observations at different temperatures were largely used in the subsequent experiments.

2. Myelinization of sterols by other hydrosoluble amphiphiles

a. Anionic agents Our previous experiments on the myelinization of cholesterol (and of some related sterols) by sodium oleate[3] have been extended to other sodium soaps, prepared from saturated (and unsaturated) fatty acids with differing chain lengths (and with different numbers, positions and configurations of the double bond).

Saturated linear chain carboxylic amphiphiles (Table I) gave myelin forms with cholesterol from C_{11} and up; the limit caprate/undecanoate, observed at 25°C with concentrated (25 mM) soap solutions of high quality-grade products, is close to that observed (caprylate/pelargonate) by Dervichian and Magnant.[8] Clear, aqueous 25 mM solutions of longer chain soaps could only be investigated at higher temperatures. With Na nonadecanoate and Na arachidate, the highest concentration compatible with obtaining transparent solutions was 12.5 mM, even at 65°C. The lowest sodium soap concentration giving myelin tubes with cholesterol ranged from 6.2 mM (laurate; 35°C) or 3.1 mM (myristate; 45°C) to 0.75 mM (palmitate; 55°C). Six sterols, structurally related to cholesterol and also leading to myelin forms when immersed in sodium oleate solutions,[3] were compared with respect to the influence of the chain length of the sodium soap on myelinization; only fucosterol and stigmasterol differed significantly from cholesterol, which in every case appeared to be the more active sterol.

Branched-chain saturated fatty acids are only represented in our investigations by phytanic acid, a C_{20} compound identified as 3,7,11,15-tetramethylpalmitic acid which is present in patients with Refsum's disease.[9,10] At room temperature, cholesterol treated with 5 mM sodium phytanate led to the immediate formation of typical myelin tubes.

Unsaturated sodium soaps (Table II). These were examined under similar conditions of concentration (25 mM) and temperature (25°C) with the exception of sodium elaidate (50°C) and sodium erucate

TABLE I

Myelin tube formation from cholesterol (or related sterols) by aqueous solutions of sodium soaps of some saturated fatty acids

Sterols	C_{10} 25 mM 25°C	C_{11} 25 mM 25°C	C_{12} 25 mM 35°C	C_{14} 25 mM 45°C	C_{16} 25 mM 55°C	C_{18} 25 mM 55°C	C_{19} 12.5 mM 65°C	C_{20} 12.5 mM 65°C
Cholesterol	0	T	T	T	T	T	T	T
Cholestanol	0	0	T	T	T	T	T	T
Desmosterol	0	T	T	T	T	T	T	0
7-Dehydrocholesterol	S	S	T	T	T	T		
β-Sitosterol	0	0	T	T	T	T	T	T
Fucosterol	0	0	0	T	T	T	T	S
Stigmasterol	0	0	0	0	T	T	T	S

Abbreviations used: *T*, well-formed cylindrical myelin tubes; *S*, swelling fringe; 0, no morphological modification of the crystal.

TABLE II

Myelin tube formation from cholesterol (or related sterols) by aqueous solutions of sodium soaps of some unsaturated fatty acids.

Sterol	C_{18},Δ_9 (*cis*)	C_{18},Δ_9 (*trans*)	$C_{18},\Delta_{9,12}$ (all,*cis*)	$C_{18},\Delta_{9,12,15}$ (all,*cis*)	$C_{18},\Delta_{6,9,12}$ (all,*cis*)	$C_{20},\Delta_{5,8,11,14}$ (all,*cis*)	C_{22},Δ_{13} (*cis*)
Cholesterol	T	T	T	T	t	S + t	T
Cholestanol	T	T	t	S	S + t	S	t
Desmosterol	T	T	T	T	T	S + t	0
7-Dehydrocholesterol	T	T	S + t	S + t	t	t	
β-Sitosterol	T	T	S + t		0	0	t
Fucosterol	T	T	S	S + t	0	0	S
Stigmasterol	T	T	0	0	0	0	t

Abbreviations used: T, well-formed cylindrical myelin tubes; t, short thin tubes, with delayed and limited growth; S, swelling fringe; 0, no morphological modification of the crystal.

(18.75 mM; 45°C). There are only minor differences between the two conformers of Δ_9-octadecenoic acid as the lower limit of cholesterol myelinization by oleate or elaidate rises from 0.37 to 0.75 mM; the same limit was observed with sodium erucate, a C_{22} monoethenic compound.

Desaturation of sodium oleate (Δ_9), leading to linoleate ($\Delta_{9,12}$) and to linolenate ($\Delta_{9,12,15}$), does not appreciably modify the myelinizing aptitude or the morphology of the tubes; the di- and triethenic compounds were, however, less efficient in dilute solutions, and the lower concentration still active was ca. 3 mM. In contrast, this limiting concentration rose to 12 mM for sodium γ-linolenate, which differs from the above-mentioned compounds by the presence of a double bond close to the hydrophilic head; furthermore, only thin short tubes were observed.

Sodium arachidonate, the soap of a tetraethenic fatty acid (of profound biological interest as the precursor of prostaglandins) appeared as a poorly reactive compound giving only a swelling fringe and some occasional poorly developed tubes.

Hydroxylic sodium soaps. Introducing a hydroxyl group on the α-carbon of a saturated sodium soap does not significantly modify the myelinizing properties towards cholesterol; the lowest active concentrations and the morphology of the tubes formed with 2-hydroxy-myristate and 2-hydroxy-palmitate were comparable to those of the parent compounds. When the hydrophilic group is more distant from the acidic head, as in sodium 12-hydroxy-stearate (6 mM; 50°C), only a limited number of myelin forms appeared. Cholesterol crystals were not modified by a 25 mM solution of sodium ricinoleate (the 12-

hydroxy derivative of oleate) unless the temperature was raised to 60°C. The *trans*-isomer, sodium ricinelaidate gave only negative results.

N-Lauroylsarcosine (NLS), an amphipathic amide with a carboxylic group is used as its sodium salt to solubilize various membrane proteins from *E. coli*,[11] from spiroplasms[12] or from thymic cells.[13] When studied under the same conditions (25°C; 25 mM) as those used for sodium oleate in our first paper,[3] sodium N-lauroylsarcosinate showed some specificity for cholesterol as only this sterol led to myelin forms, whereas several structurally related sterols gave negative results (cholestanol, β-sitosterol, fucosterol, desmosterol, stigmasterol, zymosterol). The number of myelin tubes obtained with cholesterol is, however, smaller with NLS than with sodium oleate; accordingly, the limit of myelinization rises from 0.375 mM (sodium oleate) to 12.5 mM (NLS). When the observations are made at higher temperatures, the specificity for cholesterol progressively disappears (Figure 2).

Sodium alkane sulphonates. Nine representatives of this series, with the general formula:

$$H—(CH_2)_n—SO_3Na$$

were tested at 25°C *vs.* cholesterol, in aqueous solutions ranging from 0.75 to 25 mM (Table III); experiments with sodium octadecane sulphonate were conducted at 60°C due to the low solubility of the longer chain compound.

We have also studied the sodium salts of some sulphate esters of the fatty alcohols possessing a C_6, C_8, C_{10}, C_{12} or C_{14} chain; the compounds:

$$H—(CH_2)_n—O—SO_3Na$$

differ from the alkane sulphonates in having an additional oxygen atom. Only sodium decylsulphate and its higher analogues led to

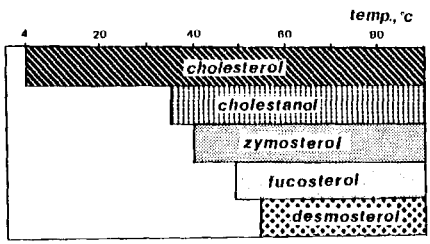


FIGURE 2 Myelinization of various sterols with a 25 mM sodium N-lauroylsarcosinate. Ranges of temperature leading to positive results.

TABLE III

Myelin tube formation from cholesterol and some sodium alkane sulphonates

Concentration	$H-(CH_2)_n-SO_2Na$ n = 8	9	10	11	12	13	14	15	16
25 mM	0	0	T	T	T	T	T	T	
12.5 mM			0	T	T	T	T	T	
6.25 mM				0	T	T	T	T	T
3.1 mM					T	T	T	T	T
1.5 mM					0	T	T	0	0
0.75 mM						0	0		

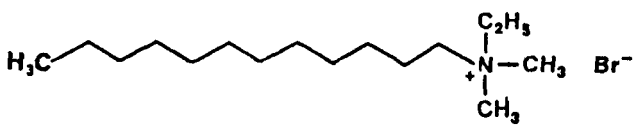
Abbreviations used: T, well-formed cylindrical myelin tubes; 0, no morphological modification of the crystal.

myelinization. Myelin tube formation was observed at lower concentrations with these ester salts: for instance, the lower limit of 0.375 mM, found for sodium lauryl sulphate (C_{12}) can be compared to the value of 3.1 and 1.5 mM obtained respectively with sodium dodecane sulphonate (a C_{12} compound) and with sodium tridecane sulphonate (if one assumes that the oxygen atom of the ester linkage has approximatively the same volume as a carbon atom).

b. Cationic agents. We have investigated one representative of the long carbon chain primary amines and some amphiphilic quaternary ammonium compounds.

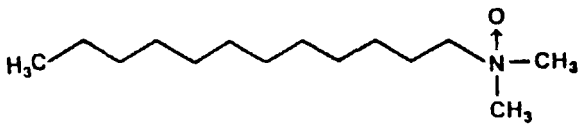
Dodecylamine hydrochloride. A clear 25 mM solution of dodecylamine hydrochloride can be easily prepared by dissolving the amine crystals in a stoichiometric amount of aqueous hydrochloric acid. This solution appeared to be a very efficient myelinizing reagent for cholesterol, leading to immediate luxuriant tubes, rapidly forming numerous twists. Upon dilution, myelin forms were still observed at a concentration of 3 mM. Desmosterol, β-sitosterol and fucosterol were less reactive than cholesterol.

Amphiphilic quaternary ammonium compounds. Ethyldimethyldodecylammonium bromide is a quaternized derivative of dodecylamine. This quaternization led to a small decrease of the myelinizing



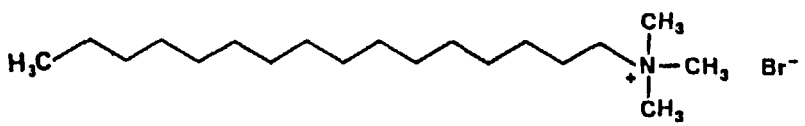
properties *vs.* cholesterol, as tubes (but no twists) could be observed only from 6 mM solutions.

N,N-dimethyldodecylamine N-oxide may be compared to the pre-



ceding compound on structural grounds, as it only differs by the replacement of the ethyl group by an oxygen atom. However, the N-oxide has lost the character of a quaternary ammonium compound. N,N-dimethyldodecylamine N-oxide, a molecule used for the isolation of photosynthetic complexes[14,15] and rhodopsin,[16] showed the same myelinizing properties *vs.* cholesterol as the corresponding quaternary ammonium.

A longer chain compound, cetyltrimethylammonium bromide be-



haved towards cholesterol as sodium oleate; moreover at 0.375 mM, this ammonium compound allowed myelinization of β-sitosterol, fucosterol and stigmasterol, whereas, as stated above, higher concentrations were needed for the unsaturated soap.

c. Non-ionic agents. Most non-ionic amphipathic agents are derived from the action of ethylene oxide on long carbon chain molecules attached to polycyclic compounds, the polymerization being conducted until hydrosoluble compounds are obtained. Tritons® and Tweens® are the classical representatives of the polyoxyethylenic non-ionic agents. We have studied 25 mM aqueous solutions of Triton X-100 and of Tweens 20, 40, 60 or 80. Neither gave myelin tubes with cholesterol.

Alkylglucosides are a newer class of non-ionic agents. Octyl-β-D-glucopyranoside has been recently used for solubilizing rhodopsin[17] or for the extraction of monoamine-oxidase from Rat liver[18]; with this compound (25–12 mM), cholesterol led to a slight myelin tube formation; desmosterol was also myelinized with the 25 mM solution.

3. Myelinization of sterols and related substances; new results

Since our first paper,[3] we have extended our field of investigation to thiocholesterol and to other sterols (or related substances) of animal, plant or bacterial origin.

a. Thiocholesterol. The sulfhydryl analogue of cholesterol has been used to examine the role of hydrogen bonding in cholesterol–phospholipid interactions[19] and in biological cross-linking experiments.[20] Thiocholesterol failed to give any myelin tubes, when immersed in 25 mM sodium oleate, in the 25°–65°C temperature range. As thiocholesterol may be transformed into the corresponding disulfide on storage, experiments were also performed in the presence of 1% dithiothreitol and the same negative results were recorded.

The discrepancy between cholesterol and thiocholesterol concerns not only the hydroxyl/sulfhydryl replacement, but also the fact that thiocholesterol is an anhydrous product, whereas the natural sterol is usually obtainable as its monohydrate. Fine needles of anhydrous cholesterol, in the presence of 25 mM sodium-oleate, slowly gave typical myelin tubes, the velocity of formation being the only difference from cholesterol hydrate.

b. Compounds related to cholesterol. The unsuccessful attempts to obtain myelin figures with the polyprenic acyclic precursor of cholesterol, squalene, with several amphiphiles is predictable as no hydrophilic group is present on this carbon chain.

In our previous paper[3], the negative results found for lanosterol (the first cyclic product in the biosynthetic pathway leading to cholesterol) were explained by the steric hindrance between the hydrophilic 3β-hydroxyl group and the two *gem*-methyl groups situated on the vicinal C-4; the lack of myelin tube formation contrasted with the myelinization of 4,4-dimethylcholesterol by sodium oleate and led us to re-examine this case. The initial sample of 4,4-dimethylcholesterol was found to be contaminated with cholesterol: a specially purified product, obtained by courtesy of Prof. Pete, from Reims University, gave the same negative results as lanosterol, with 25 mM sodium oleate and with all of the amphiphiles mentioned above. A demethylated derivative of cholesterol, 19-*nor*cholesterol, also gave myelin tubes with sodium oleate. Myelinization was slow and meager and limited to concentrations higher than 12.5 mM.

Hydroxysterols have recently attracted the attention of biochemists; we have investigated some compounds belonging to this group. The first step in the pathway from cholesterol to bile acids is cholest-5-en-3β,7α-diol, resulting from the action of a specific 7α-hydroxylase. This hydroxycholesterol consists of tabular crystals, resembling those of cholesterol hydrate. Typical myelin tubes were observed with sodium oleate, with a lower concentration limit of 3 mM; some short tubes could also be observed at room temperature with other 25 mM

sodium unsaturated soaps (linoleate, γ-linolenate); higher temperatures were required for myelinization by 25 mM sodium laurate (35°C), myristate (45°C), palmitate (35°C) and elaidate (50°C). In contrast, the thin needles of the stereoisomeric 7β-hydroxy-cholesterol only led to negative results, whereas cholest-5-en-3β-ol-7-one mimics 7α-OH-cholesterol.

The biochemical process leading to steroid hormones, through the oxidative rupturing of the side chain by a desmolase, starts with the formation of hydroxy derivatives in positions C_{20} and C_{22}. Cholest-5-en-3β,20α-diol, or 20(*S*)-hydroxycholesterol showed the same myelinization characteristics as 7α-OH-cholesterol; with sodium oleate the tubes appeared rapidly; the 20(*R*) compound was not tested. The two stereoisomers of 22-hydroxycholesterol behaved differently; cholest-5-en-3β,22(*S*)-diol is the only one myelinizable, with 6 mM as a lower concentration limit for sodium oleate. When studied under the same experimental conditions, the nitrogenous compound 22(*R*)-aminocholesterol was unreactive. The myelinization previously noted with 3-β-OH-pregn-5-ene[3] must be contrasted with the negative results given by treating 17-ethinyl-androst-5-en-3β-ol or 17(methylamino)-androst-5-en-3β-ol with 25 mM sodium oleate.

We have previously observed[3] that a hydroxy derivative of cholesterol possessing an additional polar group at the end of the side chain (25-OH-cholesterol) could not be myelinized. The 26-OH-cholesterol gave the same negative results also obtained with 3β-hydroxychol-5-en-24-oic acid, a compound in which the increased polarity on the shortened side chain is conferred by a carboxylic group.

Cholecalciferol (vitamin D_3), the uv-irradiation product of 7-dehydrocholesterol, has lost the B ring. 25 mM Sodium oleate gave no myelin tubes at room temperature; myelinization could however be observed when the temperature is raised above 35°C. The hydroxylation of vitamin D_3 first occurs in the liver, to give 25-OH-cholecalciferol; this latter compound, which has obviously lost its amphiphilic character, gave no myelin forms, whatever the temperature and the nature of the amphiphiles.

c. Plant sterols. Some plant 3β-OH sterols are closely related to cholesterol or to its metabolic precursors, e.g. two compounds previously investigated[3]: stigmasterol, from calabar and soya beans[21] and fucosterol, from *Fucus vesiculosus*.[22] The negative results observed with ergosterol/sodium oleate at 25°C[3] contrast with our present observation of tube formation at a higher temperature (80°C); myelin forms could also be found with 25 mM solutions of C_{14}, C_{16},

C_{18} saturated sodium soaps or sodium elaidate, at the temperatures required for obtaining clear solutions.

The structure of the other plant sterols studied are given in Figure 3. Cycloartenol (9:19-*cyclo*-lanost-24-en-3β-ol), a C_{30} sterol isolated from *Strychnos nux vomica*,[23] only differs from lanosterol by a cyclopropane ring, and by the desaturation of ring B. These minor structural differences markedly change the behavior towards 25 mM sodium oleate or different sodium soaps: cycloartenol, unlike lanosterol, was easily myelinized at the appropriate temperature. Similar positive results were obtained with α-amyrin (urs-12-en-3β-ol),[24] which also gave myelin tubes with 25 mM N-lauroylsarcosine (53°C).

In contrast, despite varying the nature of the soap molecule, its concentration or the temperature of observation, myelin tubes could be obtained neither with cycloeucalenol, a plant sterol from *Eucalyptus microcorys*[25] closely related to cycloartenol, nor with the β-amyrin (olean-12-en-3β-ol) and lupeol (lup-20(29)-en-3β-ol).[26]

d. Hopanoids. Hopanoids are triterpene pentacyclic molecules with the hydroxyl group(s) situated opposite ring A. The five compounds used for experimentation (Figure 4) differ by a progressive increase of the distance between ring E and the hydrophobic group.

Tetrahymanol, from *Tetrahymena pyriformis* membranes,[27,28] diplopterol from *T. pyriformis*[29] and from *Methylococcus capsulatus*[30]

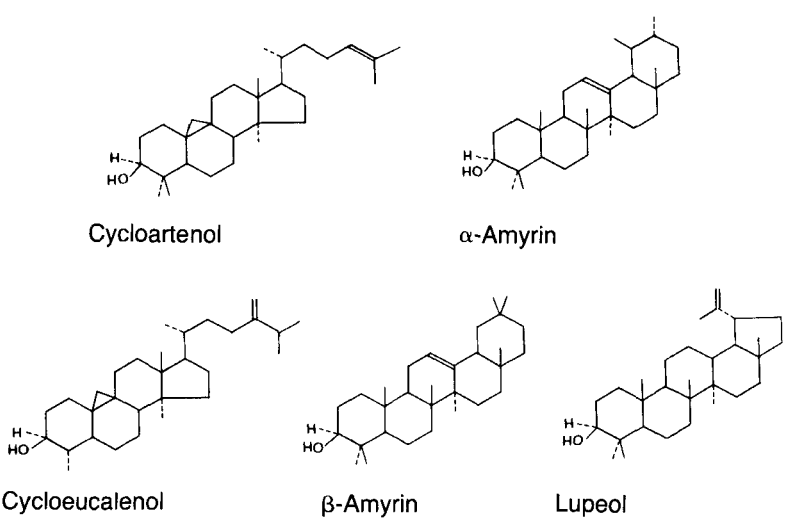


FIGURE 3 Chemical structure of some plant sterols and related compounds.

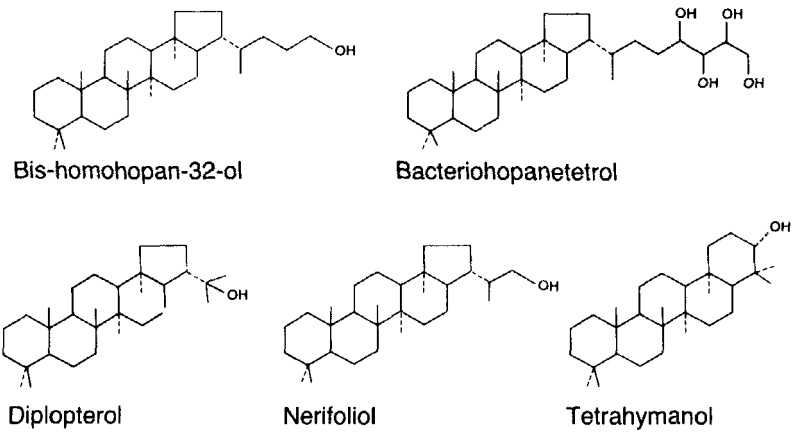


FIGURE 4 Chemical structure of hopanoids.

and nerifoliol from ferns[31] could not be myelinized by 25 mM sodium oleate over a large temperature range.

We have tested two isomers of bis-homohopan-32-ol: the 22(*R*) stereomer, in presence of sodium oleate, gave rise to typical myelin tubes which could be observed even with dilute solutions (lower limit, 6 mM); positive results were also noted with clear solution of sodium laurate, myristate, palmitate and elaidate. The configuration at C-22 appears to be crucial, as bis-homohopan-32-ol-22(*S*) could not be myelinized by sodium oleate (25–75°C). Bacteriohopanetetrol, a C_{35} pentacyclic hopanoid, was first isolated from *Acetobacter xylinum* by Förster *et al.*[32] It also exists in the glycolipid fraction of the thermoacidophilic species *Bacillus acidocalcarius*[33] and in many bacteria.[34,35] The minute quantity of bacteriohopanetetrol available limited our investigations to 25 mM sodium oleate (35°C) and sodium palmitate (55°C); the two soaps slowly myelinized the hopanoid, but gave very well-formed tubes.

DISCUSSION

The basic structure of the myelin form is a bilayer, analogous to the phospholipid bilayer found in all biological membranes. The bilayer may be homogenous (lecithin) or may result from the molecular

association between two amphiphilic compounds, differing with regard to their solubility in water (cholesterol and the soap anion). The bilayer, approximatively 50 Å thick, bends to give an elementary tube. The myelin form consists of a series of concentric tubes (ca. 1000), separated by a layer of water, forming a multilamellar structure.[36] Entry of water causes an expansion resulting in the telescopic extension of the myelin tube[37]; a consequence of the sliding of the bimolecular layers on the intercalated water molecules.

Observation of myelin tube formation thus appears to be a simple technique for studying the possible fluid molecular associations between two amphiphilic compounds: one hydrosoluble and the other non water-soluble; in such associations, the formation of myelin forms is compatible with structural variations of both partners of the association.

The hydrosoluble partner

The specificity of the hydrosoluble amphiphilic molecule appears to be very low: myelin tubes can be obtained with several compounds differing in the hydrocarbon chain as well as in the hydrophilic polar head.

The paraffinic chain of the hydrosoluble amphiphile comes into close contact with the hydrophobic part of the sterol, as a consequence of the hydrophobic effect more than as the occurrence of weak van der Waals forces.[38] The spontaneous formation of a multilamellar structure requires a degree of fluidity of the bilayer. In the external part of the bilayer, the rigid steroid ring is associated with a fully extended hydrocarbon chain; the fluid character of the myelin tube depends on the fluidity of the central part of the bilayer, where the aliphatic part of the sterol is associated with the terminal moiety of the amphiphilic agent. This is the reason why a carbon chain with a minimum of 10–11 carbon atoms is required to allow myelinization, either in the carboxylic or in the sulphonic series. Myelinization is favoured by increasing the number of carbon atoms and can also be observed with C_{20} branched-chain sodium phytanate. However, with long-chain sodium soaps such as stearate, the increase of the intermolecular forces between the hydrophobic tails requires an increase of temperature above the transition temperature to obtain a clear aqueous solution. This temperature still allows the association between the soap chains and the hydrophobic rings and the side chain of the sterol.

The occurrence of one double bond does not markedly affect the lower concentration limit, in *trans*-isomers: sodium elaidate and stearate gave similar results.

The bent carbon chain of oleate is apparently well adapted to form fluid associations with cholesterol; additional *cis* double bonds (linoleate, linolenate) reduce the aptitude of the soap for myelinizing cholesterol, especially when the double bond approaches the hydrophilic head (γ-linolenate) and modifies interactions between the polar heads and the hydrophilic group of the sterol; arachidonate, with its four *cis* double bonds, showed a low degree of myelinization.

The hydrophobicity of the paraffinic chain is not affected when a single hydrophilic group is introduced in the vicinity of the polar head (α-hydroxy soaps). In N-lauroylsarcosine, the chain is equivalent in length to a C_{15} linear compound. The occurrence of the oxygen and nitrogen atoms of the amide group, near but not actually adjacent to the ionized carboxylic group considerably diminish the myelinizing aptitude and sharpen the specificity towards cholesterol.

Replacing the carboxylic acid group of a fatty acid by a sulphonic group does not markedly change the myelinizing properties of the sodium salts, despite the increase in the solubility of these sulphur compounds which permits experiments to be performed at room temperature. The tridecane and tetradecane sulphonates appeared to be the most efficient, even when the temperature was raised to jallow a direct comparison with the corresponding carboxylic analogues.

The extra oxygen atom which distinguishes alkylsulphates from alkane sulphonates may lead to an additional hydrogen bond, hence giving the higher hydrosolubility and myelinizing power of the sulphate esters.

A complete change in the nature of the ionized group of the hydrophilic head does not suppress the myelinizing properties: dodecylamine hydrochloride, a cationic amphiphile appeared to be very efficient towards cholesterol, not only with respect to the lowest active concentration but also as the myelin tubes rapidly form twists. The ability of this aliphatic amine to give myelin forms is also apparent under different experimental conditions, as a droplet of pure dodecylamine, when immersed in 0.1 N HCl gives, by itself, well formed tubes and twists.[4] Quaternization of the amine lessens the myelinizing properties; however, a number of invert soaps studied by Polonovski[39] gave positive results.

The role of a ionized polar head, either anionic or cationic, in the amphiphilic molecule, is emphasized by the negative results obtained with several non-ionic amphiphiles, with the exception of octylglucopyranoside.

The non-hydrosoluble partner

The new sterols investigated in this paper confirm the role of the structural features necessary to obtain myelinization.[3]

The equatorial hydroxylic group on ring A appears to be especially important as a hydrogen bond-forming center; neither cholest-5-en-3-one (no hydroxy group) nor epicholesterol (with an axial hydroxy group) gave myelin tubes over the large range of temperatures (up to 80°C) investigated. Thiocholesterol possesses the same morphological characters as cholesterol and differs only by a O/S replacement, leading to a thiol group with a lesser ability to generate hydrogen bonding. No myelin forms could be observed with the sulphur analogue.

The slow formation of myelin forms with anhydrous cholesterol, compared to the rapid formation of abundant myelin tubes with cholesterol monohydrate (the ordinary cholesterol crystals), suggests that the sterol monohydrate is linked to the polar head of the amphiphilic hydrosoluble molecule by more than one hydrogen bond, the water molecule acting as a second hydrogen bond-forming center.

The introduction of an axial supplementary hydroxy group in ring A (cholest-5-en-3β,4β-diol) or in ring B (cholest-5-en-3β,7α-diol) does not suppress the myelinization with sodium oleate; it limits tube formation to solutions with a concentration higher than 3 mM. In contrast, introducing a further equatorial hydroxy group in ring B of cholesterol molecule (cholest-5-en-3β,7β-diol) led to negative observations. The corresponding ketone, 7-ketocholesterol, behaves as 7α-hydroxycholesterol, thus emphasizing the lack of participation of the keto group in establishing a fluid association with the hydrosoluble amphiphile; this was already noted at position 3, as cholest-5-en-3-one is unreactive.

The inability of lauroylsarcosine to myelinize 7α-hydroxycholesterol may be related to the formation of a supplementary hydrogen bond between the amide group of the hydrosoluble amphiphile and the 7α-OH group of the steroid; such an association, which can be confirmed using molecular models, obviously suppresses the rectilinear character necessary to obtain the close molecular packing of the bilayer.

A supplementary hydroxy group in the side chain completely suppresses myelinization when this group may be freely hydrated, thus impairing the amphiphilic character of the molecule: this is what was observed with 25- and 26-hydroxycholesterol, as well as with 22(*R*)-hydroxycholesterol. The formation of myelin tubes from 22(*S*)-hydroxycholesterol can be explained in terms of the hydrophobic environment of the —OH group created by the vicinal methyl groups

on C-18, C-21 and C-26 (Figure 5, A). A similar situation occurs for the hydrophilic group on C-20 of 20(*S*)-hydroxycholesterol which is also partially masked by the same methyl groups (Figure 5, C). In 22(*R*)-hydroxycholesterol, the hydroxyl group of the side chain points towards the α-face of the sterol molecule; the steric hindrance caused by C-21 and C-26 is lessened (Figure 5, B) and the hydroxy group in C_{22} can be freely hydrated: 22(*R*)-hydroxycholesterol should not therefore be regarded as an amphiphile.

The same consideration applies to the unreactive 22(*R*)-aminocholesterol; the primary amino group and the hydroxylic group appear to possess very similar hydrophilic properties.

The simultaneous loss of both amphiphilic character and of the aptitude for myelinization is also evident for shorter side chain steroids, as 3β-OH-chol-5-enic acid and pregnenolone.[3] The nitrogenous compound 17-(methylamino)-androst-5-en-3β-ol behaves as pregnen-

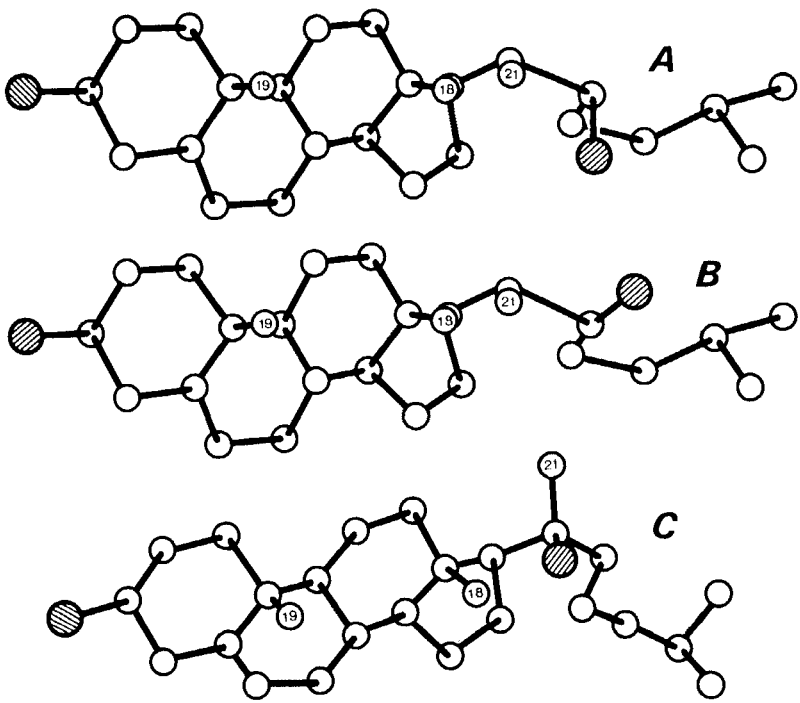


FIGURE 5 Molecular models of 22 (*S*)-hydroxycholesterol (A), 22(*R*)-hydroxycholesterol (B) and 20(*S*)-hydroxycholesterol (C). Only carbon and oxygen atoms are represented; the models are redrawn from photographs of Cochranes (Oxford) molecular models.

olone, a further proof of the similar hydrophilic properties of —NH_2 and —OH groups.

Two C_{21} steroids, 3-β-OH-pregn-5-ene (a compound with an ethyl side chain) and 3-β-OH-17-ethinylandrost-5-ene, differ by the degree of saturation of the side chain and by their aptitude to give myelin tubes with sodium oleate. Following the same line of thought, one may suggest that the hydrophilic character of the side chain may be due not only to a —OH or a —NH_2 group, but may also result from the polarization of the acetylenic bond, allowing the attraction of water dipoles. A similar hydration at a polarized ethenic bond of the side chain may also offer an explanation of the limited aptitude to give myelin tubes with sodium oleate of desmosterol,[7] fucosterol[7] and of ergosterol which is reactive at 80°C, a temperature which lessens water bonding.

The large range of temperature (4°–80°C) over which myelin tubes can be observed with cholesterol/25 mM sodium oleate encompasses the endothermic lattice change of cholesterol at 40°C.[40]

Metabolic aspects

The fair degree of correspondence between the structural features of the sterol molecule necessary to obtain fluid associations with natural phospholipids,[41–44] with sodium oleate[3] or with the simple amphiphilic molecules discussed in this paper allow us to discuss our results from a physiological viewpoint.

In a mammalian adult, the total cholesterol content (1–2 g/kg) is represented mostly by free cholesterol (ca. 75%). Cholesterol esters appear chiefly in plasma, adrenals, hair, skin and liver.[45] A large percentage of the unesterified cholesterol is localized in membranes.

It clearly appears from Figure 6 that zymosterol and desmosterol, the immediate precursors of cholesterol in the biosynthetic pathway, are the only molecules which can be easily myelinized by water soluble amphiphiles, although to a lesser degree than cholesterol itself. Similar remarks can be made concerning the catabolic lines leading to bile acids, steroid hormones or to antirachitic factors. One may thus assume, for instance, that 20α-hydroxy- or 22(*S*)-hydroxycholesterol, two myelinizable compounds, may be retained in membranes whereas non-myelinizable pregnenolone, the next metabolic step, leaves the membrane towards its final hormonal fate.

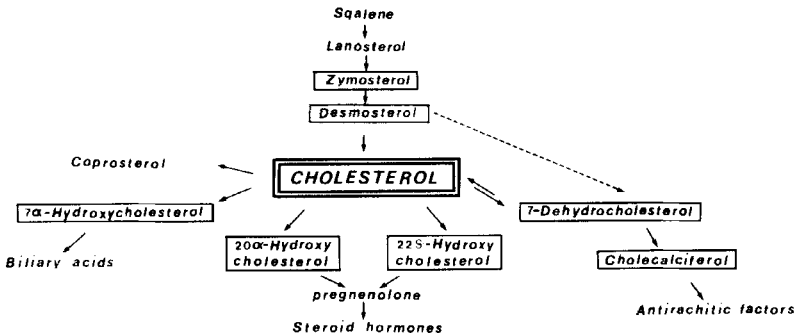


FIGURE 6 Major pathways of cholesterol metabolism. The molecules leading to typical myelin tubes are boxed.

Phylogenetic aspects

A number of prokaryotic or plant polyterpenes are now considered as phylogenetic precursors or structural equivalents of sterols.[46,47] Lanosterol and cycloartenol are both derived from the cyclization of squalene (3*S*)-2,3-epoxide; Ourisson and Rohmer[48] have recently described the mechanisms which can lead to the 8.9 double bond of lanosterol in vertebrates and fungi or to the formation of the cyclopropane ring of cycloartenol in higher plants.

The demethylation step between lanosterol and cholesterol may be interpreted as a result of an evolutionary drift to obtain a compound better able to give fluid molecular associations with phospholipids (shown in the experimental part of in this paper with other amphiphiles). A further demethylation reduces this ability: 19-*nor*-cholesterol is a rare normal sterol only detected in lower marine organisms as sponges[49] or gorgones.[50]

The myelin tubes easily observed with cycloartenol contrast with the negative results obtained with lanosterol. These negative results could be explained in terms of the steric hindrance in the vicinity of the hydrophilic group at C_3 brought about by the two *gem* methyl groups on C_4, as dimethyl-4,4-cholesterol was shown to be unreactive and as zymosterol (the 4,4-dimethylated derivative of lanosterol) gives myelin tubes.[3] The presence of the cyclopropane ring may then alter the conformation of cycles A and B, thus modifying the steric hindrance at C_4. The non-myelinization of cycloeucalenol (a compound with a cyclopropane ring possessing only one methyl group at C_4) may then be understood as a consequence of the hydration of the side chain following the polarization of a particularly accessible dou-

ble bond, as is also observed for the non-myelinizable lupeol. The opposite behaviour of the two isomers of amyrin is linked to minor structural differences as β-amyrin possesses a second *gem*-dimethyl group on the hexagonal ring opposite to the hydroxyl, instead of the two separated methyl substituents of α-amyrin; this proved to be sufficient to suppress the sodium oleate test. Comparing the two amyrins is a further example of correspondence between the crystalline form and the ability to undergo myelinization (which is usually associated with a tabular form of the crystals). The α- and β-amyrins respectively crystallize as tablets or needles; this relationship cannot however be generalized, as the crystalline tablets of lupeol could not be myelinized.

Regarding the five hopanoids studied, it is clearly apparent that the formation of myelin tubes cannot be observed with tetrahymanol, diplopterol and nerifoliol, three compounds in which the hydrophilic hydroxy group is situated in the vicinity of the cyclic part of the molecule.

The positive results observed with bis-homohopan-32-ol-22(*R*) can be paralleled with the associations obtained between this compound and natural lecithins, using Corey-Pauling-Kolten molecular models; the non-myelinizable isomer only showed loose associations.[57] Finally it may be noted that in bacteriohopanetetrol (the polar head of which is distant from the rings) and which gave typical tubes, a 22(*R*) conformation also occurs.

Among numerous steroids which were shown to form myelin tubes in the presence of water-soluble amphiphiles, the particular place of cholesterol is obvious. This membrane sterol, compared to its metabolic or phylogenetic precursors, as well as to some of its degradation products, appears to possess the molecular structure most adapted to forming fluid molecular associations. This may be paralleled to the wide distribution of cholesterol within a large range of organisms. Our results also emphasize the value of the simple technique of observing the formation of myelin tubes for studying the molecular associations of sterols and related molecules, possible even with a minute quantity limited to a few crystals of microscopic size.

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