Biochimica et Biophysica Acta, 641 (1981) 11—19

BBA 79114

STRUCTURAL REQUIREMENTS OF STEROLS FOR MYELIN TUBE FORMATION WITH SODIUM OLEATE

E. NEUZIL, J. FOURCHE, R. JENSEN, H. JENSEN and G. MORIN

Laboratoire de Biochimie Médicale, UER 3 Sc. Méd., Université de Bordeaux II, 146, rue Léo Saignat, 33076 Bordeaux Cedex (France)

(Received July 21st, 1980)

Key words: Sterol structure; Myelin tube formation; Sodium oleate

Summary

Cholesterol crystals treated with an aqueous solution of sodium oleate give rise to cylindrical lamellar associations which appear under the microscope as rapidly growing tubes. Myelin forms are also obtained with other membrane sterols (desmosterol, cholestanol, 7-dehydrocholesterol) but not with lanosterol, a metabolic precursor of cholesterol, nor with the catabolic products of cholesterol (coprosterol, cholecalciferol, pregnenolone). The structural requirements for obtaining myelin tubes from sterols and sodium oleate closely agree with the results obtained by studying sterol-lecithin associations using other experimental techniques (unimolecular films at the air/water interface and permeability of liposomes), association of sterols with an erythrocyte protein and cholesterol liquid crystals.

Introduction

The morphological aspects described by Virchow [1] in several pathological and normal tissues which are generally known as myelin (or myelinic) forms (or figures) can be obtained using very simple chemical systems. Typical myelin tubes are easily observed when a fragment of lecithin, deposited on a microscope slide, is surrounded by a drop of water which is allowed to run under the cover-glass. Myelin tubes are also formed by immersing a crystal of cholesterol in a dilute soap solution [2]: the crystal becomes at once covered with minute, clear, colourless out-growths which rapidly increase in size and appear like intestinal villi in shape [3]. The molecular structure of myelinic figures was fairly well interpretated by Nageotte [4] who emphasized the role of the amphiphilic character of the molecules (lecithin) or of the

molecular associations (cholesterol-soap) and their arrangement in tubularly orientated bilayers. The basic structure of the myelin form then consists of a series of concentric tubes, each elementary tube being separated from the adjacent inner and outer tubes by a layer of water. Entering of water into the fragment of lecithin or entering of the soap solution into the crystal of cholesterol generates a swelling process and orientates the amphiphilic molecules (or the amphiphilic molecular associations). The telescopic extension of the myelin tubes occurs through the sliding of the bimolecular layers on the intercalated water molecules; the growth of the myelin forms also requires a convenient degree of fluidity of the bulk of amphiphilic molecules (or molecular associations), a necessity for a continuous growth of the developing bilayers.

The associations between water-soluble and non-water-soluble molecules, specially studied by Dervichian and Magnant [5], give rise to myelin forms: (a) when both types of molecule are amphiphilic; (b) when the intermolecular forces are sufficient to maintain the cohesion of the bilayer structure and, at the same time, weak enough to assure a fluid character of the films; this implies some structural similarities, in a very broad sense, between the two types of molecule; (c) when the molecular association corresponds to a state intermediary between complete dispersion in water and total lack of solubility in this medium.

Myelin forms, regarding their bilayer structure as well as their fluidity, thus appear as simple models of biological membranes. As sterols are normal constituents of these membranes, studying the formation of myelin forms from sterols appears to be a promising field of investigation. Dervichian and Magnant [5] have focused their attention on the myelin forms obtained from cholesterol (and a few cholesterol esters) by the action of a number of water-soluble amphiphilic substances, such as alcaline soaps or alkyl sulphates of various chain lengths; myelin forms are also observed when cholesterol is associated with cationic amphiphilic molecules, for example, heptadecylamine [5], myristylcholine hydrochloride [5] or different synthetic inverted soaps [6]. In this paper, we have examined the myelinisation of a variety of sterols in an aqueous solution of sodium oleate in order to elucidate the structural factors which are responsible for myelin tube formation.

Experimental Procedure

Materials

The sterols were obtained from the following sources: cholest-5-en-3β-ol (cholesterol), from Calbiochem AG, Lucerne, Switzerland; cholest-5,24-dien-3β-ol (desmosterol), 24-ethylcholest-5,24(28)-dien-3β-ol (fucosterol), cholest-4-en-3β-ol (allocholesterol), cholest-5-en-3α-ol (epicholesterol), cholest-4-en-3α-ol (epiallocholesterol), cholest-5-en-3-one, cholest-5-en-3β,4β-diol, cholest-5-en-3β,25-diol, 5α-cholestan-3β-ol (cholestanol), 5β-cholestan-3β-ol (coprostanol), 5β-cholestan-3α-ol (epicoprostanol), 5α-cholest-7-en-3β-ol (lathosterol), 5β-cholanic acid-3α-ol (lithocholic acid), cholest-5-en from Steraloids, Inc., Wilton, NH; cholest-5,7-dien-3β-ol (7-dehydrocholesterol) from Sigma, St. Louis, MO, U.S.A.; 24β-methylcholest-5,7,22-trien-3β-ol (ergosterol), 24-ethylcholest-5,22-dien-3β-ol (stigmasterol), 5α-cholest-8,24-dien-3β-ol (zymosterol),

cholecalciferol, pregn-5-en-3β-ol-20-one, cholest-4-en-3-one, androst-5-en-3β-ol-17-one (dehydroepiandrosterone) from Roussel Uclaf; 24-ethylcholest-5-en-3β-ol (β-sitosterol) from Hoffmann-La Roche; 5α-cholestan-3-one from Mann Research Laboratories; samples of androst-5-ene, androst-5-en-3β-ol, androst-4-en-3β,17β-diol and 17-methylene-5α-androstan-3β-ol were kindly provided by Professor A. Crastes de Paulet (University of Montpellier); Pregn-5-en-3β-ol and two C_{24} and C_{29} analogues of cholesterol were gifts of Professor K.E. Suckling (Department of Biochemistry, University of Edinburgh Medical School, Edinburgh U.K.); 4,4-dimethylcholest-5-en-3β-ol (4,4-dimethylcholesterol) was kindly given by Professor G. Ourisson (University of Strasbourg).

Sodium oleate was purchased from Prolabo, Paris.

Methods

Method A. A few crystals of the sterol to be tested were deposited on a microscope slide and covered by a cover-glass resting on two small fragments of another cover-glass which acted as small shims. The preparation was examined at room temperature ($t = 25°C$) under a microscope ($G = 50$ or 150) and, by means of a capillary Pasteur pipette, a few drops of a 25 mM sodium oleate solution were allowed to run under the cover-glass. When no myelin tube was found after 15 min of observation, the test was considered as negative.

Method B. Sterols giving negative tests were also examined using a different method: an aliquot of the sterol and its equivalent molar weight of sodium oleate were dissolved in absolute ethanol, and the solution thus obtained evaporated to dryness on a water bath. A fragment of the pasty residue was examined using a procedure similar to that used in method A, with the only difference being that the sodium oleate solution was replaced by distilled water.

Results

As soon as the aqueous solution of 25 mM sodium oleate came into contact with the crystals of cholesterol, a swelling fringe appeared immediately from which cylindrical tubes rapidly evolved and grew longer (Fig. 1); myelin forms could also be obtained with more dilute solutions of sodium oleate; the lowest efficient concentration was 0.4 mM. Identical myelin tubes were observed with method B by hydrating a cholesterol/sodium oleate mixture.

A number of sterols closely related to cholesterol and listed in Table I also gave rise to typical myelin tubes, using either method A or B. These sterols differ from cholesterol in the modifications involving the cyclic moiety of the molecule (saturation, further desaturation, methylation, further hydroxylation), the aliphatic chain (shorter aliphatic chains, alkylation, associated or not with desaturation) or both parts of the molecule.

On the other hand, modifying some structural factors of the molecule of cholesterol suppressed the ability to produce myelin tubes using method A (25 mM sodium oleate); the corresponding sterols (Table II) are classified using the same criteria as in Table I.

Fig. 1. Microscopic depiction of myelin tube formation from cholesterol and sodium oleate (method A).

Most of the sterols leading to negative results with method A have also been examined using method B. Lathosterol led to swelling forms among which some tubular structures could be found: however, the short and thick tubes observed were quite different from the thin and rapidly growing tubes illustrated in Fig. 1. A number of sterols (epicholesterol, allocholesterol, epiallocholesterol, coprosterol, androstenol, 17-methylene-androstanol, 25-hydroxycholesterol, cholestenone, lithocholic acid) only gave round-shaped or ovoid swelling forms, devoid of any tubular structure. With ergosterol, no swelling form at all was observed.

TABLE I

STEROLS FORMING MYELIN TUBES WITH SODIUM OLEATE AT 25°C

Cholesterol
Sterols differing from cholesterol in the cyclic moiety
Cholestanol (5α-cholestan-3β-ol)
7-Dehydrocholesterol (cholest-5,7-dien-3β-ol)
4,4-Dimethylcholesterol (4,4-dimethylcholest-5-en-3β-ol)
4-Hydroxycholesterol (cholest-5-en-3β,4β-diol)
Sterols differing from cholesterol in the side chain
C_{21} analogue of cholesterol (pregn-5-en-3β-ol)
C_{24} analogue of cholesterol (20-*n*-propylpregn-5-en-3β-ol)
β-Sitosterol (24-ethylcholest-5-en-3β-ol)
Desmosterol (cholest-5,24-dien-3β-ol)
Stigmasterol (24-ethylcholest-5,22-dien-3β-ol)
Fucosterol (24-ethylcholest-5,24-(28)-dien-3β-ol)
Sterol differing from cholesterol in both parts of the molecule
Zymosterol (5α-cholest-8,24-dien-3β-ol)

TABLE II

STEROLS UNABLE TO GIVE MYELIN TUBES WITH SODIUM OLEATE (METHOD A) AT 25°C

Sterols differing from cholesterol in the cyclic moiety
Cholest-5-ene
Cholesterol esters
Epicholesterol (cholest-5-en-3α-ol)
Allocholesterol (cholest-4-en-3β-ol)
Epiallocholesterol (cholest-4-en-3α-ol)
Coprostanol (5β-cholestan-3β-ol)
Epicoprosterol (5β-cholestan-3α-ol)
Cholest-4-en-3-one
Cholest-5-en-3-one
5α-Cholestan-3-one
Lathosterol (5α-cholest-7-en-3β-ol)
Sterols differing from cholesterol in the side chain
Androst-5-en-3β-ol
Dehydroepiandrosterone (androst-5-en-3β-ol-17-one)
Pregnenolone (pregn-5-en-3β-ol-20-one)
25-Hydroxycholesterol (cholest-5-en-3β,25-diol)
C_{29} analogue of cholesterol (26-ethylcholest-5-en-3β-ol)
Sterols differing from cholesterol in both parts of the molecule
Androst-4-en-3β,17β-diol
17-Methylene-5α-androstan-3β-ol
Lithocholic acid (5β-cholanic acid-3α-ol)
Ergosterol (24β-methylcholest-5,7,22-trien-3β-ol)
Lanosterol (4,4,14α-trimethyl-5α-cholest-8,24-dien-3β-ol)

Discussion

We have already pointed out that the formation of myelin tubes from amphiphilic water-insoluble substances such as sterols requires the association with amphiphilic water-soluble molecules. Sodium oleate has been selected because

this soap is readily soluble in water at room temperature and because oleic acid is a major component of membrane lipids; moreover, the occurrence of a double bond in the carbon chain of oleic acid is responsible for its low melting point and thus favours a fluid character for its associations.

The hydroxyl group at C_3 represents the hydrophilic part of the cholesterol molecule, the elimination of which obviously suppresses the possibility of myelin tube formation (cholestene). Negative results are also obtained following less drastic modifications maintaining the oxygen atom of the hydroxyl group, such as esterification (sterides) or oxidation (cholestenone). The association of the sterol with sodium oleate is thus not only linked to the presence of a hydrophilic group, as the remaining oxygen atom is still able to attract water molecules, but also requires the possibility of hydrogen bonding given by the hydroxyl group. The spatial position of this hydroxyl group plays an important role: for example, epicholesterol, the 3α epimer of cholesterol, does not give myelin tubes, despite the close structural relationship between the two molecules. A certain degree of association between sodium oleate and epicholesterol apparently occurs and is responsible for the ovoid swelling forms observed when using method B, a consideration also applying to cholestenone.

A further hydroxylation of the cholesterol molecule does not affect the ability of myelinisation when the supplementary group appears at C_4: the amphiphilic character of the molecule is preserved whereas this character is lost in 25-hydroxycholesterol, a compound possessing two hydrophilic groups at opposite parts of the molecule.

The hydrophilic/lipophilic balance within the molecule of cholesterol may be modified by changes affecting the aliphatic side chain. The more lipophilic, but still amphiphilic C_{29} analogue of cholesterol does not form myelin tubes with sodium oleate; this negative result may tentatively be explained by the fact that the association with sodium oleate still occurs, but that the increased cohesion forces between the long straight side chains do not allow the fluidity required for tube formation.

Another C_{29} analogue of cholesterol, β-sitosterol, on the contrary, is a myelin tube-forming sterol; the two supplementary carbon atoms do not really lengthen the aliphatic side chain but introduce a ramification which does not increase the intermolecular cohesion forces. Stigmasterol and fucosterol, two unsaturated derivatives of β-sitosterol, show the same behaviour, indicating the minor influence of a double bond in the side chain, as confirmed by the fact that desmosterol reacts in the same way as cholesterol.

The eight-carbon atom side chain of cholesterol may be reduced to a C_5 (C_{24} analogue) or C_2 chain (pregnenol) without affecting the ability to produce myelin forms, whereas the complete elimination of the aliphatic chain (androstenol) appears as a negative factor. The influence of the amphiphilic character previously discussed is also obvious when comparing pregnenol with pregnenolone, a steroid possessing a hydrophilic ketone group on its short side chain and giving no myelin form. The hydrophilic/lipophilic balance is also modified by introducing two methyl groups at the C_4 position: however, this modification does not affect myelin tube formation, thus demonstrating the limited steric hindrance on the vicinal hydroxyl group.

Typical myelin forms are obtained with cholestanol, the reduction product

of cholesterol possessing an asymmetric centre C_5 at the juncture of rings A and B. On the contrary, no tube was noted with coprostanol, the 5β epimer of cholestanol; the main structural difference between the two epimers is the *cis* or *trans* configuration of rings A and B, the *trans*-structured cholestanol possessing a planar nucleus whereas the *cis*-structured coprostanol corresponds to a much thicker bended molecule. The role of the planarity of the sterol skeleton is also emphasized by the comparison of the planar cholesterol with the non-planar allocholesterol, a substance unable to give myelin tubes.

The above-mentioned remarks concerning the requirement of a free 3β-OH group also applies to cholestanone, which led to negative results; other negative results obtained with epicoprostanol, epiallocholesterol and 17-methyleneandrostanol are easily interpreted as the first two sterols possess two unfavourable structure factors, non-planarity and a 3α-OH group, whereas 17-methyleneandrostanol, despite its free 3β-OH group and its 5α configuration, has a chain reduced to a single carbon atom.

Myelin tubes are readily given by 7-dehydrocholesterol, a planar 3β-OH sterol, and by zymosterol, an unsaturated derivative ($\Delta_{8,9}$) of desmosterol possessing the 5α configuration.

Lanosterol possesses a favourable 3β-OH group, a 4,4-dimethyl substitution (as in 4,4-dimethylcholesterol) and the same unsaturation degree ($\Delta_{8,9}$ and $\Delta_{24,25}$) as in zymosterol: the negative results obtained with lanosterol therefore appear related to the 5β configuration of the molecule, a configuration also present in lithocholic acid; moreover, the latter molecule has no amphiphilic character on account of the presence of the carboxylic group on the side chain. Cholecalciferol, which also does not form myelin tubes, is linked to sterols by its biological origin but the opening of the ring B results in a molecular structure quite different from that of usual sterols.

Among the different molecules investigated, lathosterol shows a particular behaviour towards sodium oleate: the crystals, when contacted by the soap solution, quickly disaggregate but do not form the myelin tubes observed with two closely related sterols, cholestanol and 7-dehydrocholesterol (lathosterol appears as a $\Delta_{7,8}$ derivative of cholestanol or as a 5α-structured reduction product of 7-dehydrocholesterol). The disaggregation may be related to the weakness of the intermolecular forces in the crystals of lathosterol, a hypothesis supported by its low melting point (F = 119°C; cholesterol, F = 148°C). Weak cohesion forces between lathosterol and oleate may also explain the results obtained with method B, if one supposes that the microscopic aspects observed (short and thick myelin tubes) are linked to the easy distension of the individual bilayers.

The absence of myelin tube formation with ergosterol is surprising, since ergosterol only differs from the reactive 7-dehydrocholesterol in minor features of the side chain. Moreover, the side chain of ergosterol is very similar to the side chain of the myelin tube-forming stigmasterol, and the intermolecular forces in the crystals of these two sterols should be very comparable as their melting points are closely related (ergosterol, F = 168°C; stigmasterol, F = 170°C).

Most of the sterols giving myelin tubes with sodium oleate are present in animal membranes. Cholesterol is the most widely distributed; the cholesterol :

lipid ratio in several membranes (liver, erythrocytes) appears close to unity. Cholesterol is often accompanied by cholestanol, 7-dehydrocholesterol, desmosterol and lathosterol. Plant sterols (β-sitosterol, stigmasterol, fucosterol) also lead to positive results, with the exception of ergosterol (the predominant sterol in yeast and fungi).

Considering now the chief steps of cholesterol metabolism, we have found that lanosterol, the first cyclic sterol formed in the isoprenic synthesis, does not form myelin tubes; lanosterol is not present in biological membranes. The other proved or postulated precursors of cholesterol, i.e., zymosterol, desmosterol and 7-dehydrocholesterol, give myelin forms with sodium oleate and are found in natural membranes. The de novo synthetized cholesterol or the cholesterol of dietary origin is catabolized following four major different pathways: (a) the action of a specific hydroxylase on the side chain of cholesterol leads to the 20α-OH derivative, which is rapidly converted to pregnenolone, the precursor of progesterone, androgens, oestrogens and corticosteroids; (b) cholesterol, via 7-dehydrocholesterol, is photochemically transformed into cholecalciferol; (c) cholesterol is stereospecifically reduced by intestinal bacteria to coprostanol, a sterol which is not reabsorbed and which is eliminated in the faeces; (d) cholesterol, after several hydroxylation reactions, gives different cholic acids, which are partly transformed by microbial enzymes to lithocholic acid. The catabolic products, pregnenolone, cholecalciferol, coprostanol as well as lithocholic acid, have lost the ability of forming myelin tubes with sodium oleate; none of these molecules is a constituent of normal membranes.

Our findings concerning the structural factors which govern the ability of sterols to give myelin tubes with sodium oleate mostly parallel the results obtained by other authors in other related experimental fields [7]. Demel et al. [8] have shown that the interaction of different sterols and steroids with lecithin in monomolecular films at the air/water interface is dependent on a planar sterol nucleus and on the presence of a 3β-OH group: cholesterol, cholestanol, 7-dehydrocholesterol give high collapse pressures, whereas *cis*-structured sterols and ketosteroids show high areas per molecule. The same group of workers proved that similar structural features are required in order to observe a marked reduction of the permeability properties of lecithin liposomes towards glucose [9], glycerol [9] or erythritol [10]. Bloch [11] has also recently emphasized the importance of a dealkylated planar α-face in sterols for membrane function, for example, reduction of glucose permeability or increase in the microviscosity of lecithin vesicles [12,13]. The influence of the length of the cholesterol molecule has been thoroughly studied by Suckling et al. [14—16] with spin-labelling techniques: side chains shorter or longer than that of cholesterol caused significantly less ordering; the influence of the length of the side chain was less marked in our myelin tube formation experiments, as only 17-methyleneandrostanol and the C_{29} analogue of cholesterol were ineffective.

Klappauf and Schubert [17] have studied the interaction of band 3 protein from human erythrocyte membranes with cholesterol and cholesterol analogues: modifications of the polar group (shift of the OH group from the 3β to the 3α configuration, or its conversion into a keto group) and the absence

of the side chain show the same negative influence as in our own experiments, while the omission of the double bond in ring B or the introduction of a second double bond in this ring have no effect. Finally, our results also generally agree with the conditions required for observing liquid crystal properties of sterol derivatives [18]: cholestanol exhibits the same cholesteric behaviour as cholesterol, whereas epicholesterol and coprostanol do not give liquid crystals [19].

In conclusion, the technically very simple method of microscopic observation of myelin forms, when applied to the sterol-sodium oleate systems, shows that the structural factors required for obtaining well formed myelin tubes are very comparable to those defined for sterol-lecithin associations, using more sophisticated techniques. The assimilation of myelin forms to a simple model of biological membranes is thus reinforced and an extension to other biomolecules of the method of myelin tube observation can be logically projected.

Acknowledgements

The financial support of the University of Bordeaux II is acknowledged. We are grateful to Mrs. Monique Malgat for her expert technical assistance and to Mrs. Chateaureynaud for her help in our photographic work.

References

1 Virchow, R. (1854) Virchow's Arch. Pathol. Anat. Physiol. 6, 562—570
2 Brücke, E. (1879) Sitzungber. Math.-Naturwiss. Kl. Kaiserslautern Akad. Wiss. 79, 267—276
3 Brockbank, E.M. (1896) Gall-Stones, pp. 14—23, J. and A. Churchill, London
4 Nageotte, J. (1936) Morphologie des Gels Lipoïdes, Hermann and Cie, Paris
5 Dervichian, D. and Magnant, C. (1946) Bull. Soc. Chim. Biol. 28, 419—426
6 Polonovski, J. (1946) C.R. Soc. Biol. 140, 19—21
7 Demel, R.A. and de Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109—132
8 Demel, R.A., Bruckdorfer, K.R. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 311—320
9 Demel, R.A., Bruckdorfer, K.R. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 321—330
10 De Kruijff, B., de Greef, W.J., van Eijk, R.V.W., Demel, R.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 298, 479—499
11 Bloch, K. (1976) in Reflections on Biochemistry (Kornberg, A., ed.), pp. 143—150, Pergamon Press, Oxford
12 Lala, A.K., Lin, H.K. and Bloch, K. (1978) Bioorg. Chem. 7, 437—445
13 Dahl, C.E., Dahl, J.S. and Bloch, K. (1980) Biochem. Biophys. Res. Commun. 92, 221—228
14 Suckling, K.E. and Boyd, G.S. (1976) Biochim. Biophys. Acta 436, 295—300
15 Craig, I.F., Boyd, G.S. and Suckling, K.E. (1978) Biochim. Biophys. Acta 508, 418—421
16 Suckling, K.E., Blair, H.A.F., Boyd, G.S., Craig, I.F. and Malcolm, B.R. (1979) Biochim. Biophys. Acta 551, 10—21
17 Klappauf, E. and Schubert, D. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1225—1235
18 Gray, G.W. (1976) in Advances in Liquid Crystals (Brown, G.H., ed.), Vol. 2, pp. 62—72, Academic Press, New York
19 Wiegand, C. (1949) Z. Naturforsch. 4, 249—251