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Topical Review

The Enantiomer of Cholesterol

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Abstract. Cholesterol plays a variety of significant roles in biological systems. However, the mechanisms by which cholesterol functions remain largely unclear. The enantiomer of cholesterol (*ent*-cholesterol)—which has identical physical properties, but opposite three-dimensional configuration compared to cholesterol—is a unique tool that can be used to better understand the mechanisms of cholesterol function. We review the literature pertaining to *ent*-cholesterol, focusing in particular on its use in biological studies.

Key words: Cholesterol — Enantiomer — *ent*-Cholesterol — Enantioselectivity

Introduction

Cholesterol

Cholesterol plays a variety of critical roles in cellular function. Having the appropriate amount of cholesterol in the appropriate place is essential for membrane structure, signal transduction, and human health.

At the cellular level, cholesterol is an essential structural component of cell membranes. Cholesterol increases membrane thickness and decreases transbilayer permeability (Demel & DeKruyff, 1976; Yeagle, 1993). Cholesterol facilitates phase separations of certain lipids, such as those responsible for the formation of lipid rafts in cell membranes (Simons & Ikonen, 1997; Brown & London, 2000). Lipid rafts are rich in cholesterol and sphingolipids and have been shown to play a role in regulating some signal transduction pathways (Simons & Toomre, 2000). Cholesterol also modulates the function of many different proteins.

Inappropriate distributions of cholesterol have been implicated in a number of human diseases. High blood levels of cholesterol correlate with cardiovascular disease, including atherosclerosis, coronary artery disease and hypertension (Brown & Goldstein, 1992; Goldstein, Hobbs & Brown, 2001; Harrison, 2001). Defects in the biosynthesis or metabolism of cholesterol are responsible for a number of less common disorders including Smith-Lemli-Optiz syndrome (defect in 7-dehydrocholesterol reductase (Harrison, 2001)), Wolman disease (inability to hydrolyze LDL particles (Assmann & Seedorf, 2001)), and Niemann-Pick type C (inappropriate intracellular trafficking of LDL-derived cholesterol (Harrison, 2001)). These disorders range in manifestation from limb defects and neurological disorders to death. Cholesterol has also been implicated in the progression of Alzheimer's disease (Wolozin, 2002).

Although cholesterol is known to be involved in many important biological processes, in most cases, the molecular mechanisms underlying cholesterol function are still largely unclear. In general, cholesterol may function by directly binding to a protein and/or by influencing membrane physical properties. Several approaches can be used to assess these modes of function.

One way in which investigators have attempted to probe the function of cholesterol is to either increase or decrease cellular cholesterol levels. Cholesterol can easily be added to or removed from cell membranes. Such experiments can indicate whether cholesterol is important for a given process. However, these experiments must be interpreted cautiously because changing cholesterol levels simultaneously affects both membrane physical properties and the availability of cholesterol to serve as a binding partner for proteins.

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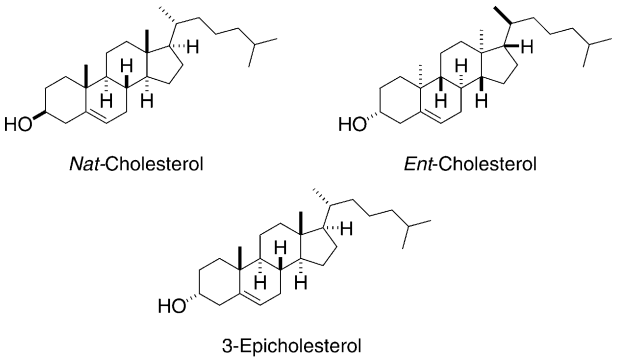


Fig. 1. Structures of cholesterol (*nat*-Cholesterol), its enantiomer (*ent*-Cholesterol), and its diastereomer (3-epicholesterol).

Another method investigators use in an attempt to differentiate between the membrane and protein effects of hydrophobic steroids, like cholesterol, is to replace the sterol with a related steroid analog—in particular, with a diastereomer. A diastereomer of cholesterol, for example, differs in configuration at one or more, but not all, stereocenters compared to cholesterol. Diastereomers have the same chemical constitution and bonding pattern, but a different relative configuration of the constituent atoms. Often, diastereomers can be distinguished by their chemical, physical and optical properties, and interact differently with other molecules.

One diastereomer of cholesterol is 3-epicholesterol, in which only the configuration at C-3 is inverted compared to cholesterol (Fig. 1). 3-Epicholesterol has been used in several biological studies to determine whether the shape of cholesterol is important for a given function, i.e., if cholesterol interacts with a protein. However, biophysical studies have shown that by some measures cholesterol and 3-epicholesterol behave quite differently in lipid mixtures. For example, in monolayers, cholesterol condenses lipids, but 3-epicholesterol does not (Demel, Bruckdorfer & Van Deenen, 1972a, 1972b). Cholesterol and 3-epicholesterol have different tilt angles with respect to the bilayer (Dufourc et al., 1984; Murari & Murari, 1986) and different effects on the thermodynamics of some lipid phase transitions (Cheetham et al., 1989). Thus, because diastereomers differ in both physicochemical properties *and* three-dimensional shape, discerning the basis for differences between the activities of diastereomers in biological studies can be problematic.

Cholesterol function can also be probed using a unique analog of cholesterol—its enantiomer (Fig. 1). Whereas diastereomers differ in configuration at one or more, but not all stereocenters, enantiomers differ in configuration at all stereocenters in the molecule. Enantiomers are mirror images of one another. Enantiomers have the same chemical composition, bonding pattern, and relative configuration. Both compounds share the same chemical and physical properties, and are only distinguished by their opposite three-dimensional shape, or absolute configuration. Their opposite absolute configurations allow enantiomers to be distinguished by plane-polarized light (optical rotation) or by interaction with another chiral molecule (e.g., a protein or a chiral chromatography resin).

By virtue of the enzymatic processes required to make steroids, cholesterol is found naturally in only one enantiomeric form, what we refer to as *nat*-cholesterol. Thus, any study of the unnatural enantiomer of cholesterol (*ent*-cholesterol) requires its chemical synthesis. Once synthesized, *ent*-cholesterol can be used to study cholesterol function, based on the following premise: processes (e.g., solubility or partitioning into a membrane) that depend solely on the physical properties of cholesterol will be identical for *nat*- and *ent*-cholesterol; processes that involve enantioselective recognition of cholesterol (e.g., binding to a protein) will be different for the two compounds.

While it is often convenient to think of cholesterol as having two separate effects—either on membranes or on proteins—in reality, the total effect of cholesterol in a system is the combination of its effects on membrane physical properties and on proteins. In many cases, there is probably some contribution from both. *Ent*-cholesterol is a particularly useful tool because when *ent*-cholesterol replaces *nat*-cholesterol, the membrane properties remain constant, thus allowing a disentangling of these two related effects of cholesterol.

Expectations of Enantioselectivity

Whether a process is enantioselective must be determined empirically. Nonetheless, principles that govern whether biological molecules have the potential to distinguish enantiomers have been formalized using geometrical arguments (Crossley, 1995; Mesecar & Koshland, 2000). Briefly, a chiral molecule, like cholesterol, can minimally be represented by a tetrahedron, with a central point *X*, and four surrounding points, *a, b, c, d*. *X* represents a single stereocenter with four attached atoms, *a* through *d*. Alternatively, *a* through *d* could represent functional groups at some distance from *X*, but having fixed orientations with respect to *X* (Crossley, 1995).

When the chiral molecule containing stereocenter *X* interacts with another chiral molecule, e.g., a protein, it may do so with various areas of the molecule, designated by *A, B, C*, and *D*. These areas represent fixed locations on the molecule that place constraints on its interaction with *X*. In order to satisfy each constraint, the appropriate letter in *X* must align with the lettered constraint in the receptor, i.e., *a* with *A, b* with *B*, etc. Based purely on geometrical consider-

Fig. 2. Discrimination of enantiomers by a generalized receptor. The molecules containing stereocenter *X* are enantiomers. The letters *A* through *D* represent areas of the receptor that place a constraint on the interaction of the receptor with the molecule. Alignment of the corresponding letter in the molecule with the receptor constraint satisfies the constraint. Four constraints on the interaction are required for discrimination of the two enantiomers. That is, if the receptor has four constraints (*A* through *D*) on the interaction with *X*, only one of the enantiomers can align the groups *a–d* to satisfy all of the constraints. The enantiomer on the right cannot satisfy all four constraints; a diagonal line through the lettered contraint marks unsatisfied constraints.

ations, there must be at least 4 constraints on the interaction in order to distinguish between the two enantiomers (Crossley, 1995; Mesecar & Koshland, 2000). This is illustrated in Fig. 2.

Constraints on the interaction may include hydrogen bonding, ion-pair formation, dipole-dipole interactions, π-π interactions, or steric constraints. In practice, it is difficult to predict the existence of constraints that may lead to enantioselectivity. Furthermore, if the "receptor" is not rigid, these geometrical arguments do not apply and it is difficult to theoretically predict whether an interaction will be enantioselective.

Applying these theoretical principles is difficult, in particular for cholesterol. For one, detailed structural information describing the interaction of cholesterol and its partners is not available in most cases. In addition, the molecule itself provides few clues: cholesterol has one hydroxyl group available for hydrogen bonding, a double bond for π-based interactions, and a rigid ring system that may contribute to steric constraints. The three-dimensional representations of *nat*- and *ent*-cholesterol shown in Fig. 3 reveal the similarity of these molecules. It is not immediately obvious what 4 constraints will contribute to enantioselectivity.

Nonetheless, a combination of theory and experimental data provide guidelines for whether or not to expect enantioselectivity. Table 1 summarizes our expectations for whether a given function of cholesterol will be enantioselective. Because *nat*- and *ent*-cholesterol have identical physical properties, these compounds should have the same effects on membrane properties. So, if cholesterol affects a given biological activity solely because of its effects on membranes, then we expect no difference in the activity of *nat*- and *ent*-cholesterol. By contrast, when direct, specific binding is required for cholesterol function, as in binding to a specific enzyme, there should be high enantioselectivity. Alternatively, cholesterol may bind to a protein, but in a non-enantioselective manner (i.e., in a way that does not require 4 constraints on the interaction, as when binding in a protein cleft). Thus, a finding of no enantioselectivity must be carefully interpreted and possibly further investigated.

In this article, we comprehensively review the literature related to *ent*-cholesterol. We review reports of the synthesis of *ent*-cholesterol, as well as biophysical and biological studies using *ent*-cholesterol. Readers interested in a general overview of all enantiomeric steroids are directed to a recent review by Biellmann (2003).

Synthesis of *Ent*-Cholesterol

To make the unnatural enantiomer of cholesterol, the configuration at each of the eight stereocenters in the sterol must be inverted relative to *nat*-cholesterol. There are no naturally-occurring steroids with the stereochemistry required for *ent*-cholesterol at carbons 8, 9, 10, 13, and 14, nor is there a simple way to invert the stereochemistry of these centers. It is possible by use of chemical methods to convert a steroid into an *ent*-steroid. The conversion of 19-nortestosterone to *ent*-19-nortestosterone has been described (Teerhuis, Huisman & Groen, 2001). However, application of this method to the synthesis of *ent*-cholesterol has not been described, and the method offers no advantages over the total synthesis approaches described below.

The comment by Arnett (1982) that "the enantiomer of cholesterol is not accessible without enormous synthetic effort" remains an accurate statement. To date, two fundamentally different approaches to the synthesis of *ent*-cholesterol have been reported. These approaches are summarized in Fig. 4. In the first method (Fig. 4*A*), the steroid ring system is constructed based on methods developed by Hajos (1974) and Ohloff (1983), resulting in the synthesis of *ent*-testosterone (*1*). Then, the side chain is added to *ent*-testosterone to make *ent*-cholesterol. Three different methods for the addition of the cholesterol side chain have been reported by Rychnovsky & Mickus (1992), Kumar & Covey (1999), and Westover and Covey (2003). In the second method (Fig. 4*B*), the cholesterol side chain is first constructed on a 5-membered ring, the precursor to the steroid D-ring. Then, the steroid ring system is elaborated. This method was reported by Jiang & Covey (2002).

In all, these methods allow for the synthesis of *ent*-cholesterol in 22 to 28 steps, with overall yields of ~2%. Since the synthesis of *ent*-cholesterol, the effects of this unique analog of cholesterol have been investigated in numerous biophysical and biological studies.

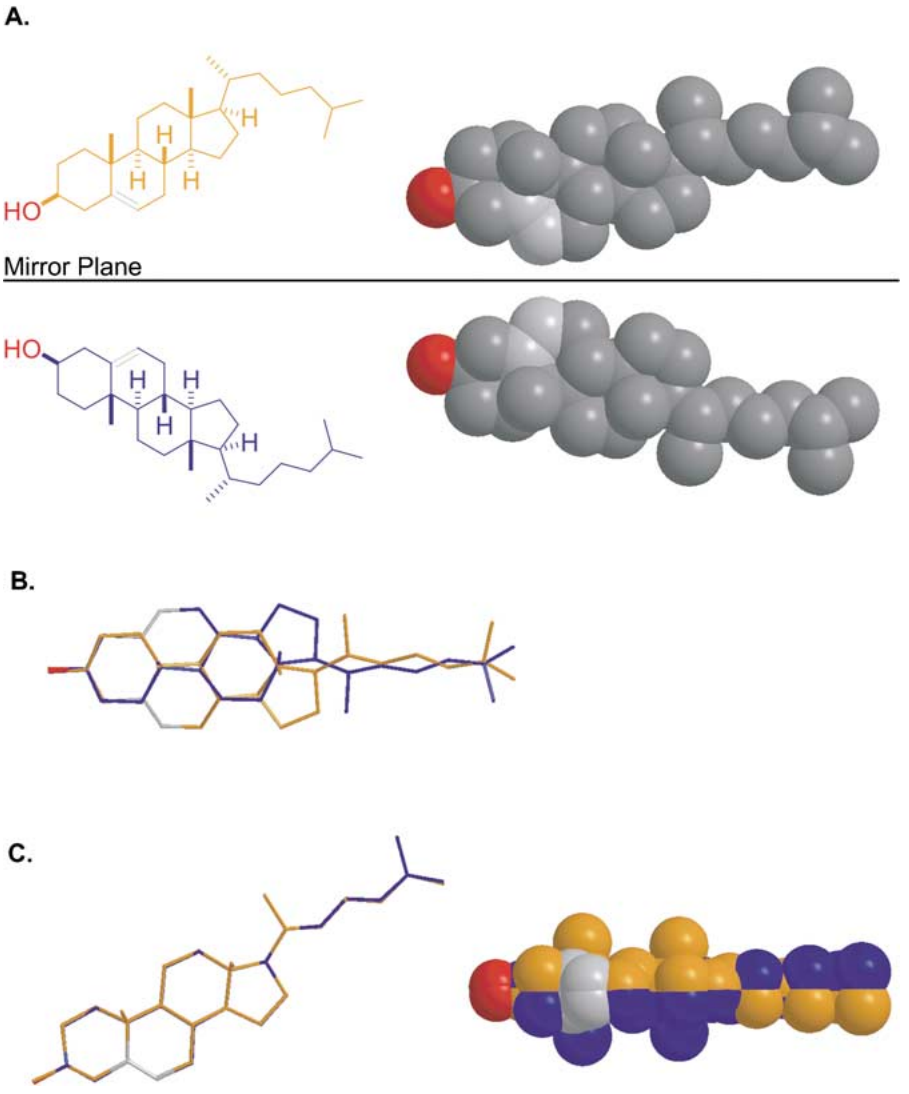


Fig. 3. Representations of cholesterol and its enantiomer. *Nat*-cholesterol (*orange*) and *ent*-cholesterol (*blue*) are shown in two- and three-dimensional representations. For clarity, all hydrogen atoms have been omitted from the three-dimensional models. In each representation, the oxygen of the 3-hydroxyl group is colored red and carbons 5 and 6 of the B-ring double bond are colored light grey. In (*A*) *nat*-cholesterol (*top*) and *ent*-cholesterol (*bottom*) are shown as if reflected through a mirror plane. In (*B*) and (C), *nat*- and *ent*-cholesterol have been superimposed. In (*B*) the distances between the 3-hydroxyl, 18-methyl and 19-methyl groups of the two enantiomers were minimized. The superimposed stick representations of *nat*- and *ent*-cholesterol are viewed from the β-face. This view shows the possibility for the close overlap of steroid rings A and C as well as the side chain. Compare upper left (*A*) with left representation in (*C*). In (*C*), the four rings of two enantiomers were aligned. Stick representations viewed from the *β*-face and space-filling models viewed from the side are shown. The side of the sterol containing the 5–6 double bond is closest to the viewer. This view highlights the possibility for the rings and side chain to occupy the same space with the 18- and 19-methyl groups projecting from opposite faces of the ring systems.

Table 1. Expectation for enantioselectivity

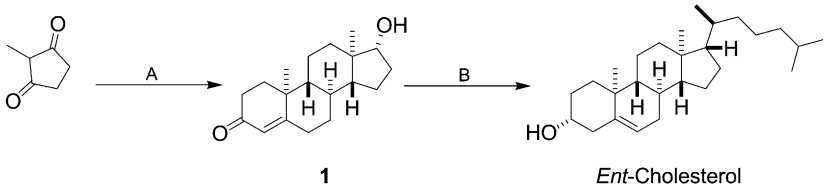
Low	High	Unknown
Lipid packing in membranes	Binding to enzymes	Membrane proteins
Non-receptor mediated interactions	Binding to specific transporters	
	Binding to nuclear receptors	

Biophysical Studies of *Nat*- and *Ent*-*Cholesterol*

Cholesterol is highly hydrophobic and usually found in lipid environments. Lipids are chiral molecules; glycerophospholipids have one chiral center and sphingolipids have two. Based on studies with cholesterol diastereomers, the interaction of cholesterol with lipids is sensitive to the stereochemistry of cholesterol. For example, cholesterol condenses lipids, but its diastereomer 3-epicholesterol does not (Demel et al., 1972a, 1972b). Therefore, it is possible that cholesterol-lipid interactions may also depend on the absolute configuration of the molecules.

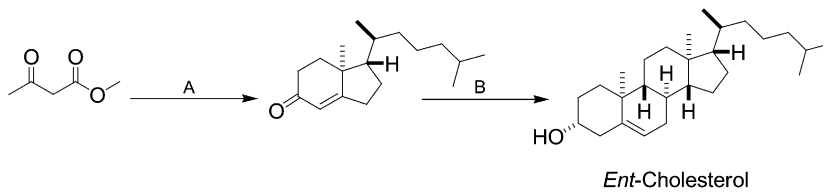
In the 1970's and 1980's, the enantioselectivity of sterol-phospholipid interactions was examined using phospholipid enantiomers (Ghosh, Lyman & Tinoco, 1971; Hermerter & Paltauf, 1982; Guyer & Bloch, 1983; Agarwal, Bali & Gupta, 1986). Notable results include the following. Monolayers of cholesterol with either *nat*- or *ent*-dipalmitoylphosphatidylcholine (DPPC) were identically compressible (Ghosh et al., 1971). The microviscosity of vesicles composed of *nat*-DPPC/cholesterol and *ent*-DPPC/cholesterol were identical based on diphenylhexatriene fluorescence depolarization (Guyer & Bloch, 1983). In mixtures of cholesterol with either *nat*-DPPC or *ent*-DPPC, phoshatidylcholine methylenes were characterized by identical NMR line widths (Hermetter & Paltauf, 1982). In all, studies with phospholipid en-

A. Synthesis of *Ent*-Cholesterol via *Ent*-Testosterone



Method	# Steps (A)	# Steps (B)	% Yield Overall	% Yield From 1
Rychnovsky & Mickus (1992)	12	10	2.7	7.7
Kumar & Covey (1999)	12	10	NR	9.7
Westover et al. (2003)	12	16	NR	15.2

B. Synthesis of *Ent*-Cholesterol via D-ring Intermediate



Method	# Steps (A)	# Steps (B)	% Yield
Jiang & Covey (2002)	13	13	1.6

Fig. 4. Overview of methods for the chemical synthesis of *ent*-cholesterol. *NR* = not reported.

antiomers have provided no evidence for significant enantioselective interactions between phospholipids and cholesterol. However, in these pre-1992 studies the chiral cross-check experiments—using *ent*-cholesterol—were not performed because this unnatural steroid was not available. Since the first synthesis of *ent*-cholesterol, the enantioselectivity of cholesterol-lipid interactions have been examined using several biophysical approaches.

Monolayers

Sterol-lipid interactions can be explored by measuring surface pressure versus mean molecular area during compression of mixed sterol-lipid monolayers on an aqueous surface. Cholesterol has a well-known condensing effect on the packing of both glycerophospholipids and sphingolipids.

Monolayers containing the same mol% of either *nat*- or *ent*-cholesterol co-spread with another lipid on an aqueous surface have now been studied. Monolayers of *nat*-DPPC or *nat*-palmitoyloleoylphosphatidylcholine (POPC) with either *nat*- or *ent*-cholesterol exhibited identical compression behavior (Lalitha et al., 2001a, 2001b; Westover et al., 2003). This finding provides the chiral cross-check, validating the earlier studies of cholesterol and phospholipid enantiomers by Ghosh and Lyman (1971).

Lalitha et al. (2001a; 2001b) reported that monolayers of egg sphingomyelin and *nat*-cholesterol compressed differently than monolayers composed of egg sphingomyelin and *ent*-cholesterol. However, subsequent studies have been unable to repeat these findings. Rather, Westover et al. (2003) report that monolayers of egg sphingomyelin with 30 mol% of either *nat*- or *ent*-cholesterol compressed with identical behavior. In addition, monolayers of 30 mol% of either *nat*- or *ent*-cholesterol with an enantiomerically pure *N*-stearoylsphingomyelin, namely (2*S*, 3*R*, 4*E*)-2-stearoylaminooctadec-4-ene-3-hydroxyl-1-phosphocholine), also compressed identically (Westover et al., 2003). This finding indicates that acyl chain heterogeneity in egg sphingomyelin does not obscure enantioselective interactions between cholesterol and lipids.

Bilayers

Sterol-lipid interactions can also be examined in the context of a lipid bilayer. To this end, multilamellar vesicles composed of sphingomyelin and either *nat*-or *ent*-cholesterol were analyzed for phase behavior, X-ray diffraction patterns and buoyant density (Mannock et al., 2003).

Cholesterol has multiple effects on the gel/liquid crystalline phase transition of bilayers composed of egg sphingomyelin and cholesterol: cholesterol increases the temperature of the gel/liquid-crystalline phase transition of egg sphingomyelin bilayers and decreases the cooperativity and enthalpy markedly. Mannock et al. (2003) reported that within experimental error, *nat*- and *ent*-cholesterol had identical effects on the temperature, cooperativity, and enthalpy of the gel/liquid-crystalline phase transition in bilayers with sphingomyelin.

Multilamellar vesicles composed of sphingomyelin and *nat*- or *ent*-cholesterol gave X-ray diffraction

patterns typical of stacked liquid-crystalline bilayers; each pattern consisted of a broad wide-angle band centered at 4.4 Å and 5 to 7 sharp, low-angle reflections that index as orders of a lamellar repeat period. Fourier analysis of these data indicated that bilayers containing *nat-* or *ent*-cholesterol were identical within experimental error (Mannock et al., 2003).

Fully-hydrated multilamellar vesicles of egg sphingomyelin and 30 mol% of either *nat-* or *ent*-cholesterol were found to have the same buoyant density. In summary, bilayers containing *nat-* or *ent*-cholesterol have similar thicknesses and molar volumes, and thus the area per lipid molecule must be very similar as well.

In all, no enantioselectivity in sterol-lipid interactions has been detected using monolayers or bilayers.

Biological Studies of *Ent*-Cholesterol

Cholesterol affects many important biological processes at both the cellular and organismal levels. In many cases, the exact role of cholesterol is not well understood. In order to better understand the molecular mechanisms underlying cholesterol activity, the effects of *ent*-cholesterol on several biological activities have been studied. The results are summarized in this section.

Monoclonal Antibody for Crystalline Cholesterol

Antibodies are well known for their specificity; indeed many antibodies demonstrate chiral discrimination between enantiomers (Geva et al., 2001). However, the ability of antibodies to discriminate enantiomers arranged on a planar surface was only addressed recently. Geva et al. raised a monoclonal antibody against cholesterol monohydrate crystals, which presents a regular crystalline array of cholesterol molecules for recognition by the antibody. Then, these authors tested the ability of this antibody to interact with monolayers of *nat*-cholesterol, *ent*-cholesterol, and 3-epicholesterol (Geva et al., 2001).

Pure monolayers of *nat-* versus *ent*-cholesterol are chemically and physically identical (e.g., same packing density, tilt angle), but mirror images of each other. By contrast, the packing of 3-epicholesterol in monolayers differs substantially from both *nat*-cholesterol (Izhaky & Addadi, 2000) *and ent*-cholesterol. In this study, monolayers of these sterols were formed at the air-water interface, and the fluorescently-labeled antibody was then introduced into the aqueous subphase. After equilibration, fluorescence at the surface was measured. The antibody interacted with monolayers of either *nat-* or *ent*-cholesterol with high affinity, and with each to the same extent. By contrast, the antibody did not interact with monolayers of 3-epicholesterol.

This "cholesterol antibody" clearly differentiates between cholesterol and its diastereomer, 3-epicholesterol, but not its enantiomer, *ent*-cholesterol. Thus, the relative configuration of the steroid hydroxyl group appears to be important for recognition by the antibody because this relative configuration is the same for *nat-* and *ent*-cholesterol, but different for 3-epicholesterol. By contrast, the absolute configuration of the sterol is not important for recognition by the antibody.

These data suggest that this antibody recognizes the packing of the sterol—as presented in the crystalline hapten. Geva et al. (2001) calculated that this antibody binds 10 to 12 cholesterol molecules at a time; this may obscure the effect of the absolute configuration of individual sterol molecules. It is likely that an antibody that recognizes a different aspect of the cholesterol molecule, e.g., a different crystal face or cholesterol free in solution, may demonstrate enantioselectivity.

Cholesterol Oxidase

The bacterial protein cholesterol oxidase catalyzes the conversion of cholesterol to cholest-4-en-3-one in a two-step process. First, the 3-hydroxyl group is oxidized with concomitant reduction of the cofactor flavin adenine dinucleotide (FAD). Then, the double bond is isomerized to the Δ^{4-5} position. Regeneration of the FAD cofactor is effected by reduction of O_2 to form hydrogen peroxide; the colorimetric detection of H_2O_2 underlies clinical assays for cholesterol (e.g., Wako Cholesterol CII).

Both Westover & Covey (2003) and Luker et al. (2000) have reported that *ent*-cholesterol is a substrate for cholesterol oxidase. However, *ent*-cholesterol is oxidized by the enzyme more slowly than *nat*-cholesterol. The fact that this enzyme appears to be enantioselective, but not enantiospecific may be better understood in light of the following. These studies were done using a kit containing cholesterol oxidase in which production of H_2O_2 is monitored colorimetrically. Thus, only the oxidation of the hydroxyl group (the first enzymatic step) is detected. Examination of the x-ray structure of cholesterol oxidase reveals that it is possible for both *nat-* and *ent*-cholesterol to present the 3-hydroxyl group identically in the active site (*unpublished observation*). However, such an active site alignment of the respective hydroxyl groups positions the double bond of each enantiomer in a different region in the active site. Thus, the double bond isomerization may not occur for *ent*-cholesterol. This could contribute to a slower off-rate of the enantiomeric substrate from the enzyme, leading to the result obtained in

these studies. Thus far, this issue has not been specifically addressed experimentally.

Amphotericin B

Amphotericin B is a polyene macrolide antibiotic that has been used for over 40 years to treat serious systemic fungal infections (Hartsel & Bolard, 1996). Amphotericin B is generally believed to form ion channels in sterol-containing cell membranes.[1] Amphotericin B has a high degree of selectivity for ergosterol-containing fungal membranes over cholesterol-rich mammalian cells. Whether amphotericin B is sensitive to the absolute configuration of cholesterol has now been addressed in artificial membrane systems as well as in fungal cells.

Mickus, Levitt & Rychnovsky (1992) measured channel conductances of amphotericin B in black lipid membranes consisting of soy azolecithin plus 5% of either *nat*-cholesterol or *ent*-cholesterol. In the absence of cholesterol, no channels formed even at amphotericin B concentrations as high as 2 μM. In membranes containing *nat*-cholesterol, single channels with conductance of 1–3 pS were found in the presence of 20 nM amphotericin B. By contrast, at these concentrations of amphotericin B, no channels were detected in *ent*-cholesterol-containing membranes; however, at 10-fold higher concentrations of amphotericin B, channels with high conductance (30–35 pS) were observed. Similar results were also found in membranes prepared with racemic glycerol monooleate in place of soy azolecithin. These data indicate that amphotericin B binds directly to cholesterol and suggest that amphotericin B activity is not simply dependent on sterol modification of membrane properties.

Recently, Richter et al. (2004) measured amphotericin B activity in the fungus, *Candida albicans*, on medium containing either *nat*- or *ent*-cholesterol. Addition of exogenous cholesterol to the fungal medium prior to treatment with amphotericin B suppresses the antifungal activity of amphotericin B, presumably because of competition for amphotericin B by exogenous versus membrane sterol. In this assay, it is unclear whether the exogenously added sterol incorporates into the membrane; nonetheless, the sterol does appear to interact with amphotericin B.

For this study, *C. albicans* was grown on medium containing *nat*-, *ent*-, or no cholesterol (control). Amphotericin B was introduced to the cultures on a disk; zones of inhibition surrounding the disk were then measured and expressed as a percentage of the control. At low sterol concentrations, no differences between *nat*- and *ent*-cholesterol were noted. However, at the highest dose given (60 ppm), *nat*-cholesterol was more effective than *ent*-cholesterol (86% versus 73% of control) in suppressing the antifungal activity of amphotericin B.

Although the context of the interaction with amphotericin B is unknown, this assay demonstrates at least some difference between the interaction of amphotericin B with *nat*- and *ent*-cholesterol. Together, these data suggest that an enantioselective molecular interaction between amphotericin B and cholesterol is important for the function of amphotericin B.

Gramicidin

Gramicidin is a 15-residue peptide with potent antimicrobial activity. Gramicidin elicits its cell-killing activity through disruption of lipid bilayer integrity. The ability of gramicidin to bind to and destabilize membranes is affected by the target membrane lipid composition. In particular, toxin binding is affected by membrane charge and fluidity, and membrane destabilization by the toxin increases membrane curvature (Prenner et al., 2001). However, lipid chirality does not appear to affect gramicidin channel activity. Providence et al. (1995) found that gramicidin channels with identical characteristics were formed in membranes composed of either *nat*- or *ent*-dioleoylphosphatidylcholine. In addition, both gramicidin and its peptide enantiomer also form identical channels (Providence et al., 1995)

In model membranes, cholesterol attenuates the effects of gramicidin by an unknown mechanism (Prenner et al., 2001). Mickus and coworkers (1992) found that membranes of soy azolecithin containing either *nat*- or *ent*-cholesterol supported the formation of identical gramicidin ion channels to a similar extent. Taken together, these data suggest that cholesterol affects gramicidin function through its influence on membrane physical properties.

Bacterial Pore-forming Toxins

Some bacteria secrete toxins as monomeric proteins that oligomerize to form large pores, thus permeabilizing and killing host cells. The pore-forming toxins selectively permeabilize host, but not bacterial, cells. Some toxins achieve this selectivity for host cells by only acting on membranes that contain cholesterol. This provides selectivity because, with rare exception

[1]There is some evidence that the in vivo antifungal activity of amphotericin B may not involve the formation of ion channels. Rather, amphotericin B may have multiple cell disrupting structures—including inducing non-bilayer or interdigitated lipid phases and defects (Hartsel, S., Bolard, J. 1996).

(Rohmer, 1999; Volkman, 2003) bacterial membranes do not contain sterols, whereas animal cells do.

Two distinct families of bacterial pore-forming toxins that require cholesterol for activity are represented by *Vibrio cholerae* cytolysin (VCC) (Ikigai et al., 1996) and streptococcal streptolysin O (SLO) (Bhakdi, Tranum-Jensen & Sziegoleit, 1985). Although both these toxins require cholesterol for their activity, the toxins are unrelated at the amino acid level and form pores of vastly different sizes. VCC requires cholesterol for membrane permeabilization, while SLO requires cholesterol for membrane binding. To further probe the role for cholesterol in pore formation by VCC and SLO, Zitzer et al. (2003) applied these toxins to calcein-laden liposomes containing no, *nat*- or *ent*-cholesterol.

Both VCC and SLO efficiently permeabilized *nat*-cholesterol-containing liposomes, but not liposomes containing no cholesterol. VCC did not permeabilize liposomes containing *ent*-cholesterol. These results demonstrate that the function of VCC requires stereospecific recognition of cholesterol. By contrast, SLO did permeabilize liposomes containing *ent*-cholesterol, although with less potency than in membranes containing *nat*-cholesterol. The finding that SLO is partially active in *ent*-cholesterol membranes was somewhat surprising since SLO is inactive in the presence of most cholesterol analogs. This result suggests that SLO may only recognize the most surface-exposed part of the cholesterol molecule.

For some bacterial toxins, cholesterol is not required for toxin function, but cholesterol does affect toxin activity. Two examples include *Staphylococcus aureus* α-hemolysin and *Streptococcus agalactiae* CAMP factor. Both of these toxins lyse erythrocytes; susceptibility of erythrocytes to the toxins is affected by their membrane lipid composition (Bortoleto et al., 1998; Lang & Palmer, 2003). To further investigate the effects of cholesterol on the activity of these toxins, α-hemolysin and CAMP factor were applied to calcein-laden liposomes containing either *nat*- or *ent*-cholesterol. Neither toxin was sensitive to the absolute configuration of cholesterol (personal communication, M. Palmer, University of Waterloo). This finding suggests that cholesterol-dependent membrane properties—not direct interactions of cholesterol with these proteins—affect the function of α-hemolysin and CAMP factor.

Multidrug Resistance P-Glycoprotein

Multidrug resistance P-glycoprotein (Pgp) confers resistance to structurally and functionally diverse chemotherapeutic drugs by decreasing intracellular concentrations of these compounds. There are two common models for the drug transport function of Pgp (Johnstone, Ruefli & Smyth, 2000): One, Pgp may act as a "pump," facilitating drug transport through the protein pore in an ATP-dependent manner. Two, Pgp may interact with a membrane-solubilized drug and act as a "flippase," translocating the drug from the inner to the outer membrane leaflet. The membrane transport function of Pgp is affected by cholesterol. In addition, there is some evidence that cholesterol itself binds to and is translocated by Pgp.

To further study the role of cholesterol in Pgp transport function, Luker et al. (2000) depleted cells of cholesterol with the cholesterol-complexing agent, methyl β-cyclodextrin. Upon cholesterol depletion, Pgp appeared to lose some of its functions: Cholesterol-depleted cells showed an increase in the accumulation of two different drugs, Tc-sestamibi and daunomycin. Luker also repleted cholesterol-depleted cells with either *nat*-cholesterol or *ent*-cholesterol. Repletion with *nat*-cholesterol completely restored Pgp function with respect to Tc-sestamibi and daunomycin. Repletion with *ent*-cholesterol reversed the cholesterol-depletion increase in Tc-sestamibi accumulation to the same extent as repletion with *nat*-cholesterol. By contrast, cells repleted with *ent*-cholesterol had less accumulation of daunomycin than cells repleted with *nat*-cholesterol.

Previous studies have shown that membrane fluidity affects Pgp function (Sinicrope et al., 1992). Thus, it is likely that cholesterol-dependent membrane properties have some effect on Pgp function. However, studies of *ent*-cholesterol and daunomycin clearly show that at least for some substrates, cholesterol appears to play another role—indeed, an enantioselective role in drug transport by Pgp. It is interesting to note that daunomycin is a chiral molecule, whereas Tc-sestamibi is symmetric and thus not chiral.

Nicotinic Acetylcholine Receptor

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated postsynaptic ion channel, whose function is modulated by lipids and cholesterol. In particular, cholesterol is required for agonist-stimulated channel opening. However, the basis for this role of cholesterol is unknown. Furthermore, cholesterol can be replaced by a host of other molecules, including cholestanol, 3-epicholesterol, cholesterol analogs with polar groups at C-3, and coprostanol (Addona et al., 1998, 2003). In addition, neutral "lipids," such as α-tocopherol, coenzyme Q_{10} and vitamins D_3 and K_1 also support activation of the nAChR (Sunshine & McNamee, 1992). These molecules vary in size, shape, and effect on membrane properties, making it difficult to discern the role of cholesterol.

To further explore the role of cholesterol in this system, Addona et al. (2003) assessed nAChR activity in the presence of cholesterol or its enantiomer using a stopped-flow fluorescence assay. The nAChR was affinity-purified from *Torpedo* and reconstituted into

bilayers composed of DOPC, dioleoylphosphatidic acid, and either *nat*- or *ent*-cholesterol. The authors reported that *ent*-cholesterol supported agonist-induced gating of the nAChR. The data provided only allow for a qualitative assessment.

Addona et al. conclude that there is a binding site for cholesterol with lax structural requirements; indeed, if there is a binding site it is neither stereo- nor enantioselective. These authors further proposed a periannular location for the binding site. Such a periannular binding site (a lipid-accessible protein crevice) would probably not be expected to be enantioselective, as described in the Introduction of this review.

Epidermal Growth Factor Receptor

The epidermal growth factor (EGF) receptor is a tyrosine kinase growth factor receptor that affects numerous cellular pathways. The EGF receptor has been found in cholesterol-rich lipid rafts. In addition, numerous studies have shown that cholesterol modulates EGF receptor-mediated signaling.

Westover et al. (2003) explored whether the effect of cholesterol on the EGF receptor is enantioselective by depleting A431 cells of cholesterol, then repleting with either *nat*- or *ent*-cholesterol. Depletion of cholesterol from cells disrupted lipid rafts and altered EGF receptor phosphorylation and signaling. However, repletion with either *nat*- or *ent*-cholesterol reestablished lipid rafts, as determined by probing protein distribution in sucrose density gradients. In addition, repletion of cells with either *nat*- or *ent*-cholesterol also equally reversed the effects of cholesterol depletion on basal and EGF-stimulated EGF receptor phosphorylation, intrinsic receptor phosphorylation, and receptor dephosphorylation.

These findings suggest that cholesterol most likely affects EGF receptor function because of its physical effects on membranes, not through direct interactions with a protein. Other studies have shown that membrane fluidity affects EGF receptor function (Ge, Wu & Lin, 2001), lending further support to this conclusion. However, we cannot rule out the possibility that cholesterol binds to the receptor in a non-enantioselective manner.

Serca ATPase

In advanced atherosclerotic lesions, macrophages accumulate large amounts of unesterified free cholesterol. Free cholesterol accumulation leads to macrophage apoptosis, which has been proposed to promote plaque destabilization (Tabas, 2002). A current model suggests that free cholesterol loading of the ER membrane leads to depletion of calcium stores and induces the Unfolded Protein Response, which triggers caspase activation and apoptosis (Feng et al., 2003).

To further probe the role of cholesterol in this process, Li et al. (2004) assessed the activity of SERCA2b, an ER calcium ATPase that pumps calcium from the cytosol into the ER lumen. Loading of ER membranes with unesterified *nat*-cholesterol decreased both the ATPase and calcium-sequestration activities of SERCA2b. Enrichment of ER membranes with *ent*-cholesterol inhibited SERCA2b to the same extent as enrichment with *nat*-cholesterol. By contrast, 3-epicholesterol did not inhibit SERCA2b activity. These data suggest that cholesterol-induced changes in ER membrane structure are the cause of SERCA2b inhibition.

Caenorhabditis Elegans

Caenorhabditis elegans requires exogenous cholesterol for normal growth, reproduction and behavior (Hieb & Rothstein, 1968; Lu, Newton & Stokstad, 1977; Chitwood et al., 1984). Cholesterol starvation has deleterious effects on all stages of *C. elegans* development (Shim et al., 2002).

Crowder et al. (2001) grew synchronized cultures of *C. elegans* on agar plates containing *nat*-cholesterol, *ent*-cholesterol, or no cholesterol. In the first generation, the growth and viability of animals grown on *ent*-cholesterol were not significantly different from those of animals grown on either *nat*-cholesterol or no cholesterol. By contrast, the locomotion of first generation *ent*-cholesterol animals was significantly slower than that of the *nat*-cholesterol animals. In the second generation, all of the *ent*-cholesterol animals died or arrested at early embryonic stages, while the *nat*-cholesterol animals continued to grow and develop normally. By growing the animals on deuterium-labeled *ent*-cholesterol, Crowder et al. demonstrated that *ent*-cholesterol was actually incorporated into the worms; so the effects of *ent*-cholesterol were not simply due to cholesterol deprivation. Additionally, the life-sustaining effects of *nat*-cholesterol were antagonized by *ent*-cholesterol.

This study demonstrated that the function of *nat*-cholesterol in a whole animal, *C. elegans*, is enantiospecific. It is yet unclear for which specific pathways/processes the absolute configuration of cholesterol is important. Given the diversity of proposed functions of cholesterol, the mechanisms underlying the effects of *ent*-cholesterol in *C. elegans* are likely to be complex.

Conclusions

Konrad Bloch (1983) suggested that cholesterol was selected through evolution as the end product of the sterol synthesis pathway because it "fits and interacts

Table 2. Summary of findings with *ent*-cholesterol

	Difference between enantiomers
Biophysical Studies	
Monolayers	None
Bilayers	None
Biological Studies	
Crystalline Cholesterol Antibody	None
Cholesterol Oxidase	Yes
Amphotericin B	Yes
Gramicidin	No
Vibrio Cholera cytolysin	Complete
Streptolysin O	Partial
α-Hemolysin	None
CAMP Factor	None
Multidrug Resistance P-glycoprotein	For some drugs
Nicotinic Acetylcholine Receptor	None
Epidermal Growth Factor Receptor	None
SERCA2b	None
Caenorhabditis elegans	Yes

effectively'' with membrane lipids, imparting cell membranes with specific properties. Bloch also suggested that sterols earlier in the biosynthetic pathway do not contribute the necessary properties to membranes. This theory is supported by the fact that diseases result when cholesterol is replaced by even a closely related sterol—like desmosterol (Clayton et al., 1996; FitzPatrick et al., 1998) or 7-dehydrocholesterol (Harrison, 2001). As reviewed above, studies with *ent*-cholesterol indicate that the unique physical properties of cholesterol, rather than its precise three-dimensional configuration, are important for its membrane functions. In addition, studies of *ent*-cholesterol with transmembrane proteins, like the EGF receptor and SERCA2b, indicate that the membrane properties imparted by cholesterol affect the function of some important transmembrane proteins.

However, as reviewed above, other activities of cholesterol depend on its absolute configuration. In fact, some proteins, including cholesterol oxidase, *Vibrio cholerae* cytolysin, and streptolysin O, clearly demonstrate enantioselectivity for cholesterol. In addition, the absolute configuration of cholesterol is essential for *C. elegans* viability.

The findings of each of the studies reported to date with *ent*-cholesterol are summarized in Table 2. Much remains to be learned about the functions of cholesterol, and the role of its absolute configuration in these functions. Some important questions to address include the following.

Does the absolute configuration of cholesterol affect the trafficking of cholesterol within a cell? This could be addressed by determining to which subcellular locations *ent*-cholesterol is distributed upon delivery to the cell. Such studies may provide new insights into the mechanisms of cellular cholesterol transport, an important issue in some disease states, including Niemann-Pick C.

Does the absolute configuration of cholesterol affect proteolysis events that occur within the membrane? Intramembrane proteolytic cleavage affects the function of several important proteins, including the amyloid precursor protein (APP), the sterol response element binding protein (SREBP), and a signal peptide peptidase (Rawson et al., 1997; Xia et al., 1997; Weihofen et al., 2002). Whether these proteolysis reactions are affected by cholesterol-dependent membrane properties or not may be clarified by studying the reactions in membranes containing *ent*-cholesterol.

Are transmembrane proteins containing ''sterol-sensing'' domains that ''detect'' cellular cholesterol levels sensitive to the absolute configuration of cholesterol? Examples of sterol-sensing proteins include HMG CoA reductase, NPC1, SCAP, and Patched (Osborne & Rosenfeld, 1998; Kuwabara & Labouesse, 2002). Cholesterol-dependent alterations in the activities of these proteins may involve direct interactions with cholesterol (or a related oxysterol) or cholesterol-dependent changes in membrane properties. These possibilities may be distinguished by the use of *ent*-cholesterol or a related *ent*-oxysterol.

Are membrane budding or fusion events affected by cholesterol-dependent membrane properties or the absolute configuration of cholesterol? This could be explored in model membrane systems, as well as in specific cellular events, such as synaptic vesicle formation or fusion involving the cholesterol-binding protein synaptophysin (Thiele et al., 2000).

Studies with *ent*-cholesterol have further characterized sterol-lipid interactions and provided new insights into a variety of important cellular processes. Still, there are many more potential uses for *ent*-cholesterol.

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