

Cholesterol-to-Coprostanol Conversion

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Cholesterol (examples of synonyms 3 β -hydroxy-5-cholestene or 5-cholesten-3 β -ol) in the intestine may be either absorbed or undergo microbial conversion to different metabolites, of which non-absorbable coprostanol (examples of synonyms 5 β -cholestanol or 5 β -cholestan-3 β -ol) is the end and predominant product found in feces. Note that multiple synonyms can be employed for a same steroid molecule, which does not help the reader to find his way. Cholesterol is a 27-carbon molecule with a structure formed by a polycyclic ring skeleton with a trans A/B ring junction, a β -hydroxyl group in the equatorial position at C3 (i.e., in plane of the molecule), a double bond at C5 (Δ^5 double bond), two methyl groups at C10 and C13, and a side chain at C17 (A).

Keywords: cholesterol ; coprostanol ; gut microbiome ; intestine ; bile acids ; feces

1. Introduction

Cholesterol sustains life of most higher animals and birds as a main element of cell membrane architecture, a unique natural precursor for the synthesis of all five classes of steroid hormones (glucocorticoids, mineralocorticoids, progestins, androgens and oestrogens), vitamin D, and bile acids. Yet, an excess of cholesterol in blood is also detrimental in humans since it is recognized as a major risk of cardiovascular disease, which is a leading cause of mortality in developed countries ^[1]. Excess cholesterol in blood and other organs comes from an imbalance between input and output. Input originates from endogenous synthesis mainly in the liver and the small intestine, plus exogenous food intake of animal origin. Output proceeds via bioconversion to bile acids in the liver, steroid hormones in diverse tissues, mainly the adrenal cortex, testis, and ovary, cell renewal, plus enteric metabolism into virtually non-absorbable microbial derivatives, which are eliminated in the feces. Among them, non-absorbable coprostanol is by far the most predominant and of highest clinical interest for removal of cholesterol from the body. **Figure 1** gives a rough estimation of daily cholesterol input and output in the healthy adult.

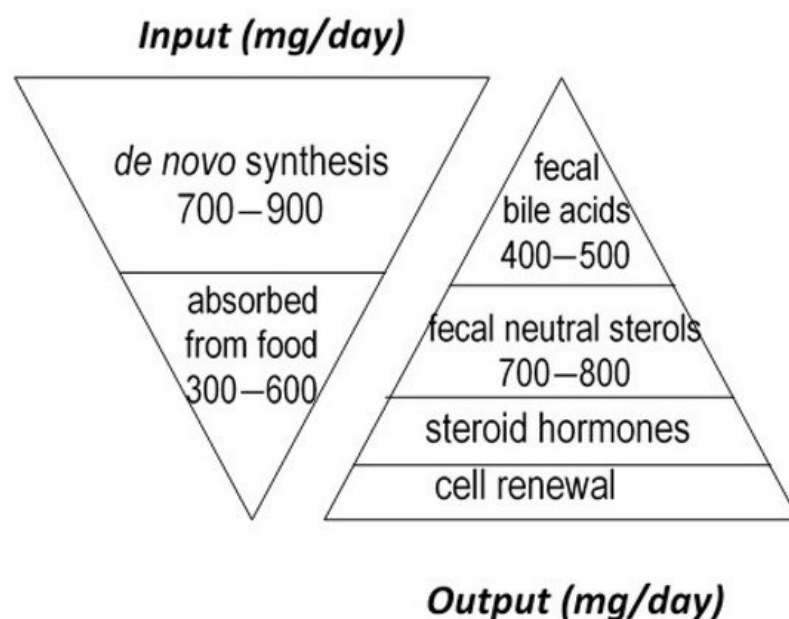


Figure 1. Estimation of daily cholesterol input and output in the healthy adult.

Considerable effort has thus been devoted to develop, question, and update reliable clinical interventional strategies aimed at lowering blood cholesterol through either depleting input, stimulating output, or both. A simple way of getting there would be to limit cholesterol input by lowering its dietary supply from animal products and/or its intestinal absorption by sequestering or competing ingredients ^[2], which unfortunately may not be sufficient in a number of cases where an

inherited propensity to synthesize too much endogenous cholesterol limits the effectiveness of a healthy diet. For that reason, a number of medicines have been proposed to inhibit synthesis of cholesterol by the liver and intestine, the most popular and widely prescribed being statins. A complementary strategy would be to increase the use of cholesterol for bile acid synthesis. This could be done through oral intake of bile acid sequestering agents, which partially remove bile acids from the enterohepatic circulation and boost their compensatory de novo synthesis from the unique precursor cholesterol. A fourth and last strategy, which was proposed several decades ago, but failed to be put into practice up to now, would be to increase the bioconversion of cholesterol in the gut lumen to its end, non-absorbable bacterial metabolite coprostanol, which could be achieved through supplementation with either cholesterol-metabolizing microbes or microbial enzymes.

2. Gut Microbial Metabolites of Cholesterol and Suspected Pathways

Cholesterol (examples of synonyms 3β -hydroxy-5-cholestene or 5-cholesten- 3β -ol) in the intestine may be either absorbed or undergo microbial conversion to different metabolites, of which non-absorbable coprostanol (examples of synonyms 5β -cholestanol or 5β -cholestan- 3β -ol) is the end and predominant product found in feces. Note that multiple synonyms can be employed for a same steroid molecule, which does not help the reader to find his way. Cholesterol is a 27-carbon molecule with a structure formed by a polycyclic ring skeleton with a trans A/B ring junction, a β -hydroxyl group in the equatorial position at C3 (i.e., in plane of the molecule), a double bond at C5 (Δ 5 double bond), two methyl groups at C10 and C13, and a side chain at C17 (**Figure 2 A**). Cholesterol metabolism by gut bacteria seems to be limited to the end product coprostanol and its intermediates, with no degradation of the side chain, and no fission of the steroid rings, which, in aerobic soil bacteria, lead to the complete degradation of the steroid molecule up to CO₂ [3][4]. This could be interpreted as a symbiotic relationship between the gut cholesterol-metabolizing microbiota and the host, which participates in the elimination of excess cholesterol from the body without altering the integrity of the intestinal cell membranes. On the other hand, the significance of cholesterol-to-coprostanol reduction to the physiology of the bacteria remains to be elucidated. Eyssen et al. [5] speculated that cholesterol acted as a terminal electron acceptor in cholesterol-reducing bacteria, thereby supplying energy by means of electron transport. However, several active strains do not require cholesterol for growth, indicating that some cholesterol reducing microorganisms can use alternate electron acceptors, or generate energy by other ways [6].

Coprostanol is the saturated analogue of cholesterol. In addition to the saturation of the Δ 5 double bond, the structure of coprostanol differs from that of cholesterol by a cis A/B ring junction, which causes the A ring to be bent into a second plane at approximately a right angle to the B:C:D rings, and the 3-hydroxy group to be in the axial position, i.e., out of plane of the molecule (**Figure 2 B**). Such a structure would be mainly responsible for the poor ability of coprostanol to be esterified within the intestinal mucosa, and therefore to be absorbed [7][8][9].

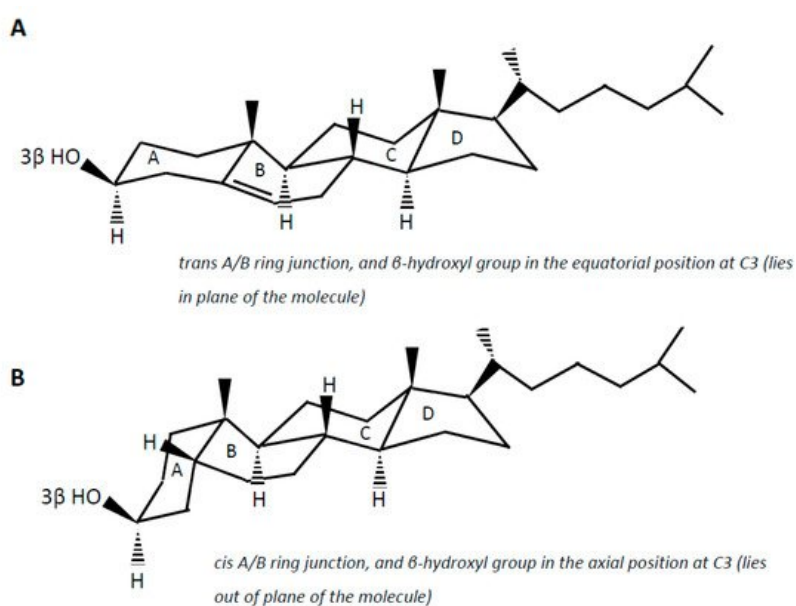


Figure 2. Planar representation of cholesterol (**A**) and coprostanol (**B**).

Two metabolic pathways have been proposed to explain the conversion of cholesterol to coprostanol by intestinal microorganisms [10][11]. One pathway is the direct stereospecific reduction of the Δ 5 double bond [10] (**Figure 3**). The other pathway involves the intermediate formation of Δ 4-cholesten-3-one (or 4-cholesten-3-one, or cholestenone) and coprostanone [11] (**Figure 3**). A series of complex in vivo and in vitro experiments using labeled and double-labeled steroid molecules was conducted over almost two decades, to follow the outcome of the hydrogen atom of the 3-hydroxy

group [10][11][12][13] and prove the formation of the intermediates 4-cholesten-3-one and coprostanone [11][14]. This led to the incontrovertible evidence that conversion of cholesterol to coprostanol proceeds at least in part, if not largely, via the intermediate-involving pathway, while participation of a direct pathway is not proved but cannot be excluded [11]. Since then, many patterns of neutral fecal sterols by gas liquid chromatography, coupled or not with mass spectrometry, have referred to the presence of coprostanol, 4-cholesten-3-one and coprostanone in feces from humans and conventional animals, while all three metabolites are totally absent in germ-free models [15][16][17][18].

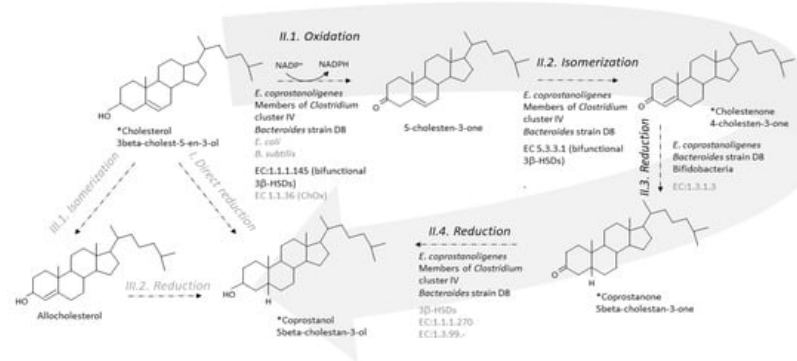


Figure 3. Direct and indirect pathways for the conversion of cholesterol to coprostanol by the gut microbiota. The indirect pathway (II, large grey arrow) is by far the most probable as evidenced by identification of the intermediates in crude feces and cultures of pure active strains. The direct pathway (I) cannot be excluded but never received any proof of evidence. Note that isomerization of cholesterol to allocholesterol and then reduction of allocholesterol to coprostanol (pathway III) are chemical reactions that, until proved otherwise, do not proceed in any organism. * Intermediate seen in GC-MS analyses of feces or pure active cultures. Micro-organisms and enzymes involved in the considered reaction are mentioned; black and grey characters correspond to confirmed and uncertain knowledge, respectively. A micro-organism is confirmed if it has been shown to process to the reaction in pure culture. An enzyme is confirmed if it has been purified and characterized in at least one gut micro-organism.

3. Step-by-Step Conversion of Cholesterol to Coprostanol

Evidence of cholesterol reduction via the indirect pathway being established, efforts have been made to characterize each step involved, and to identify and purify the corresponding enzymes. Here is where it gets tricky, as limited progress has been made since the isolation of the first active strains.

The first step of the indirect pathway is the oxidation of cholesterol to 4-cholesten-3-one. Two types of known enzymes are able to catalyse this reaction, cholesterol oxidase (ChOx, EC 1.1.36) and 3 β -hydroxy- Δ 5-steroid dehydrogenase (3 β -hydroxy- Δ 5-HSD, EC 1.1.1.145) [19]. Both are bifunctional enzymes, which carry out two separate, sequential reactions. The enzymes first oxidize the 3 β -hydroxy group of cholesterol into the 3-keto group of 5-cholesten-3-one, and then isomerize the double bond in the steroid ring backbone, from Δ 5 to Δ 4, giving 4-cholesten-3-one (EC 5.3.3.1, steroid Delta-isomerase) (**Figure 3**).

Importantly, ChOx are oxygen-dependent enzymes, and they are found in hundreds of various aerobic cholesterol-degrading soil microorganisms, including bacteria, molds, and yeasts [20][21]. ChOx is the first enzyme in the oxic pathway of cholesterol mineralization to carbon dioxide [22]. To our knowledge, only two inhabitants of the gastrointestinal tract, both facultative anaerobes, namely, one *Escherichia coli* strain isolated from the feces of a colon-cancer patient [23], and one *Bacillus subtilis* strain isolated from fecal tiger excreta [24], were reported to support ChOx activity in aerobic cultures. We also found genes that share 30–60% sequence identity with ChOx in the genome of *Bacteroides* strain D8 (unpublished data). Although it is unlikely that ChOx could participate in the conversion of cholesterol to 4-cholesten-3-one in the anaerobic environment of the lumen, it cannot be ruled out that the mucosa-associated microbiota could oxidize cholesterol in an oxygen-dependent enzymatic reaction. No homologs of any queried ChOx could be found in the genome of *E. coprostanoligenes* or among almost six million non-redundant complete genes assembled from the metagenomic sequencing of more than three thousand human fecal samples from various countries of the world [25]. In contrast, within the same study [25], a 3 β -HSD was found in *E. coprostanoligenes* ATCC 5122. The enzyme, named IsmA for “Intestinal steroid metabolism A,” was overexpressed in *E. coli* , purified using HisPur™ Ni-NTA Resin, and partially characterized [25]. It is an NADP⁺ dependent, oxygen independent, and cholesterol inducible HSD that oxidizes cholesterol to 4-cholesten-3-one and coprostanol to coprostanone, but does not accept the 3 α -hydroxy bile acids cholic and chenodeoxycholic acids as substrates. Based on massive amount of correlations combining huge metagenomics and metabolomics datasets from several cohorts in the world, six homologs of IsmA were also tracked from uncultivated

members of the human gut microbiota [25]. They were overexpressed in *E. coli*, and all six lysates were confirmed to oxidize cholesterol to 4-cholesten-3-one as well as coprostanol to coprostanone. When binning co-abundant genes into metagenomic species (MSPs) across more than 3000 metagenomics datasets, 20 different MSPs containing *ismA* genes were identified. They formed a coherent clade in the phylogenetic neighbourhood of *Clostridium* cluster IV, which contains species linked to host health, including short-chain fatty acid producers. *IsmA*-encoding MSPs had a high relative abundance (average 1.4%) in human metagenomes, and the percentage of metagenomes containing at least one *IsmA*-encoding MSP varied from 37% to 92%, which supports the idea that *IsmA*-encoding bacteria are prevalent constituents of the human gut microbiome [26]. However, they do not exclude implication of other enzymes, since functional predictions based on sequence similarity may be difficult in the particular case of enzymes where the actual enzymatic reactions or substrates may differ even at high sequence similarity, and vice versa [26][27].

In 1973, Björkhem and coworkers [14] obtained a crude enzyme preparation from supernatants of freeze-pressed cecal contents of rats, which, when incubated with [4-¹⁴C]cholesten-3-one, produced coprostanone as the only product. The 3-oxo- Δ 4-steroid 5 β -reductase activity required NADH as cofactor. The semi-purified enzyme preparation also catalyzed the reduction of the Δ 4-double bond of progesterone and testosterone but not the Δ 5-double bond of cholesterol, pregnenolone, or dehydroepiandrosterone. The mechanism of reduction of Δ 4-double bonds in 3-oxo- Δ 4-steroids by the microbial 3-oxo- Δ 4-steroid 5 β -reductase was found to involve transfer of hydrogen from the 4 B -position of NADH to the 5 β -position of the steroid. We could not find any recent proof of 3-oxo- Δ 4-steroid 5 β -reductase activity in the gut microbiota, and no bacterial 3-oxo- Δ 4-steroid 5 β -reductase; EC:1.3.1.3, has never been purified nor can be found in the databases (**Figure 3**). However, three genes predicted to encode cholestenone 5 β -reductase, EC 1.3.1.3, were found to be upregulated when *B. bifidum* PRL2010 was grown in presence of cholesterol [28] (**Figure 3**). One of them had a sequence that was found to be highly conserved (identity greater than 80 % and coverage 100 %) in the genomes of ten other bifidobacteria, all isolated from the mammalian gut [29]. When a large daily inoculum is administered (10⁹ cells per day of overnight cultures over 20 days) to the hypercholesterolemic apoE-KO mice, *B. bifidum* PRL2010 tended to lower plasma cholesterol in this small-sized experiment (5 mice treated and 5 untreated). Genes sharing 30–60% sequence identity with cholestenone 5 β -reductase, EC 1.3.1.3, were also found in the genome of *Bacteroides* strain D8 (unpublished data).

4. Dietary Regulation

Any dietary strategy aimed at decreasing cholesterol absorption in the proximal intestine and increasing cholesterol-to-coprostanol biotransformation in the hindgut would be beneficial for blood cholesterol lowering, provided that the coprostanol producing phenotype is present. Many studies report the results of dietary strategies to lower blood cholesterol in man or diverse animal models. However, few of them include a profile of fecal sterol excretion among which we can gather little significant information on cholesterol-to-coprostanol transformation and excretion rates. Importantly, the purpose of this paragraph is not to summarize the extensively documented dietary regulation of blood cholesterol, but to bring together what we know about the effect of the diet on biotransformation of cholesterol in the gut. Some effects of dietary sugars, fatty acids, proteins, and amino acids, as well as specific diets, are reported below.

Fecal concentration of cholesterol and its microbial metabolites, either expressed per g of dry [29] or wet [30] feces, is generally reported to be highest in omnivores, lowest in vegans, and intermediate in lacto-ovo vegetarians, but total excretion of neutral sterols per 24 h would be higher in vegetarians [29]. Interestingly, mean efficiency of microbial cholesterol metabolism, expressed as the ratio of cholesterol to its microbial products, would not essentially differ between dietary habits [29][30], and the same percentage of low converters (around 20%) would be found in omnivores and vegetarians [29]. In a way similar to cholesterol, plant sterols are metabolized by the gut microbiota. In this respect, main bacterial metabolites are ethylcoprostanone, ethylcoprostanol, and sitostanol for β -sitosterol, methylcoprostanone and campestanol for campesterol, and stigmastanol and ethylcoprostanol for stigmastanol [31]. Independent studies agree that cholesterol metabolism by the gut microbiota is less efficient when high supraphysiological doses of plant sterols are consumed daily by healthy human volunteers [31][32], suggesting that the gut microbiota could preferably use plant sterols as substrates when present in greater proportions than cholesterol [31]. The same was observed in hamsters fed a hypercholesterolemic diet [33], providing evidence that the higher plant sterol consumption, the lesser microbial cholesterol metabolism. At the higher dose (0.2% by weight of the diet), plant sterols were effective in reducing cholesterol absorption in the intestine as proved by increased excretion of untransformed cholesterol, decreased total and non-HDL blood cholesterol, decreased liver cholesterol, and reduction of the atherosclerotic plaque by more than 50% [33]. Since the plant sterols had no effect on gene expression of the different transporters and enzymes involved in intestinal cholesterol absorption, it was concluded and in vitro experienced that plant sterols could displace cholesterol from micelles and therefore inhibit its intestinal absorption [33].

In pigs, high cholesterol intake led to a quadrupled neutral sterols stool excretion, but the ratio of coprostanol to cholesterol in stools was shifted from more than ten to less than one. This provides evidence that the efficiency of cholesterol to coprostanol reduction was limited in this animal model [34]. In parallel, total and LDL blood cholesterol markedly increased when moving from the low to the high cholesterol diet. In the same study, the addition of a cholesterol sequestering agent, β -cyclodextrin, to the hypercholesterolemic diet, brought back cholesterolemia to normal values, while still increasing excretion of neutral sterols in stools, mainly in the form of untransformed cholesterol [34]. Limited bio-reduction of cholesterol to coprostanol when dietary cholesterol supply increased was also observed in rats [35]. At last, in three healthy male volunteers, exclusive consumption of a liquid diet for 10 days was accompanied by a drastic drop in fecal excretion of neutral steroids and conversion of cholesterol to coprostanol [36].

Clearly, many, if not all, dietary ingredients have an impact on the biotransformation of cholesterol in the hindgut, but mechanisms are far from all being elucidated. In particular, these changes in cholesterol biotransformation have never been put in perspective with structural and functional modulations of the gut microbiota.

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