Relation of sterol structure to utilization in pleuropneumonia-like organisms

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SUMMARY Six sterols, Δ^5-cholesten-3β-ol (cholesterol), Δ^5-cholesten-3α-ol, cholestan-3β-ol, cholestan-3α-ol, coprostan-3β-ol, and coprostan-3α-ol, selected to represent variations in the configuration of the A and B rings and of the 3-hydroxyl group, were tested for physiological activity. Only Δ^5-cholesten-3β-ol and cholestan-3β-ol supported growth of the pleuropneumonia-like organisms investigated, indicating the absolute requirement for a planar molecule with an equatorial 3-hydroxyl group. All six sterols were incorporated. The acetate esters of Δ^5-cholestenols were less readily hydrolyzed than those of cholestanols, indicating that unsaturation in the B ring impedes the esterase. With a *cis*-fused A/B ring, esters of the axial 3-hydroxyl radical are more easily hydrolyzed than those of the equatorial 3-hydroxyl, while in the cholestanols (A/B *trans*) there is no such difference between epimers. The differences in effects of varying configurations of the sterol molecule are explained on the basis of steric hindrance of movement in the cell membrane.

Sterols, which support the growth of pleuropneumonia-like organisms (PPLO), must possess a hydroxyl group at C_3 and a hydrocarbon side chain at C_{17} (1). Similar results have been reported for insects and protozoa (2–8). The necessity for such a molecular configuration could reflect the requirements for association by hydrogen bonding of the sterol molecule with phospholipid molecules in a lipoprotein membrane (9). In the case of insects, the molecular configuration required has been explained as necessary for proper fit into the space available and for proper bonding to the charges present in the lipoprotein (10). With protozoa, the configurational requirements also imply that a proper fit into the cell membrane must be made (6). However, studies on the uptake of sterols by resting cells of PPLO indicate that the side chain at C_{17} is also necessary if the sterol molecule is to be incorporated into the cells (11).

All strains of PPLO examined contain a sterol esterase associated with the cell membrane (12). A membrane-associated glucosidase has been detected (P. F. Smith and C. V. Henrikson, unpublished observations) only in strains capable of glucose utilization. In conformity with the presence of these enzymatic activities, the sterol fractions of PPLO comprise the unesterified sterol in which the organisms were grown (13, 14), sterol ester (15), and, in organisms capable of glucose utilization, sterol glucoside (13).

The utilization of the 3-hydroxyl group by enzymes normally occurring in PPLO led to the assumption that the sterol, which is found almost exclusively in cell membrane fragments (11), is involved not only in maintenance of membrane structure but also in permeability processes (12). Additional roles for sterols in insects and protozoa are postulated to be: as precursors for metabolic transformations to steroid hormones, bile acids, and vitamin D (3, 6); and in transport of high-energy phosphate groupings (16, 17). If the configurational requirements, exclusive of the side chain, of the sterol molecule capable of supporting growth are necessary for some physiological function other than maintenance of the integrity of the cell membrane, it might be possible to relate enzymatic activity to the capacity to support growth. Since PPLO do not synthesize or degrade sterols, the only enzymatic activity directed against the molecule involves reactions with the 3-hydroxyl group (12). Six sterols, Δ^5-cholesten-3β-ol (cholesterol), Δ^5-cholesten-3α-ol, cholestan-3β-ol, cholestan-3α-ol, coprostan-3β-ol, and coprostan-3α-ol, were selected to represent variations in the configuration of the A and B rings and of the 3-hydroxyl group. As shown in Table 1, these sterols vary in the configuration of rings A and B (*cis*, *trans*, or Δ^5) and the orientation (axial or equatorial) of the 3-hydroxyl groups. Each was as-

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sayed for its ability to support growth, to be incorporated into the cells and to participate as a substrate for the sterol esterase.

METHODS

Preparation of Sterols. The nature of this study necessitated the use of sterols with a high degree of purity. Commercial cholesterol (Nutritional Biochemicals Corp., Cleveland, Ohio) was purified by formation of the dibromide, followed by debromination (18) and two crystallizations from anhydrous methanol. The melting point of this material was 149° (compare reference 19). Fifteen microcuries of cholesterol-4-C^{14} (New England Nuclear Corp., Boston, Mass.) with a specific activity of 56 μc/mg was added to 1 g of cholesterol and the mixture was purified by bromination and debromination followed by column chromatography on 20 g silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.) packed in a column 2 cm in diameter. The sterol was added to the column in 40 ml petroleum ether (bp 30–60°). An additional 200 ml of petroleum ether was passed through the column followed by 1,000 ml benzene–petroleum ether 5:1 (all solvent ratios v/v), 10-ml fractions being collected. Cholesterol appeared as a single peak in the fraction eluted with 250–450 ml of benzene–petroleum ether 5:1. After two crystallizations from methanol, this material melted at 149° and had a specific activity of 3300 cpm/mg.

Δ^5-Cholesten-3α-ol (epicholesterol) was prepared by oxidation of 5α,6β-dibromocholestan-3β-ol by sodium dichromate, followed by debromination to yield Δ^5-cholesten-3-one (18) which was subsequently reduced (20) with hydrogen in the presence of Raney nickel (21). One gram of cholesterol yielded about 500 mg of crystalline material, which gave no precipitate with digitonin and had a melting point of 141° (19). Epicholesterol-4-C^{14} was prepared in similar fashion except that the starting material was cholesterol-4-C^{14} with a specific activity of approximately 3300 cpm/mg. The reaction products were placed on a column of 10 g silicic acid with 15 ml of petroleum ether and eluted with 100 ml petroleum ether, 500 ml petroleum ether–benzene 4:1, 250 ml petroleum ether–benzene 1:1, 500 ml petroleum ether–benzene 2:3, 150 ml petroleum ether–benzene 1:4, and 100 ml benzene. Ten-milliliter fractions were collected and monitored for radioactivity. Each solvent mixture was passed through the column until no further radioactivity was eluted. The fraction eluted with petroleum ether–benzene 1:1, following crystallization from methanol, gave no precipitate with digitonin and had a melting point of 141°. The specific activity of this material was approximately 1700 cpm/mg.

Cholestan-3β-ol (Nutritional Biochemicals Corp.), crystallized twice from anhydrous methanol, had a melting point of 142°, which agreed with the value in the literature (19), and gave no precipitate with bromine. Five microcuries of cholestan-3β-ol-4-C^{14} (New England Nuclear Corp.) with a specific activity of 13 μc/mg was added to 1 g of unlabeled cholestanol and purified by column chromatography on 20 g silicic acid using the same solvent system as for purification of cholesterol. Cholestanol was eluted with petroleum ether–benzene 1:1. This material, after two crystallizations from anhydrous methanol, was precipitable with digitonin, melted at 142°, and had a specific activity of 1280 cpm/mg.

Cholestan-3α-ol (epicholestanol) (Chemed, Inc., Odenton, Md.) was recrystallized twice from anhydrous methanol. This material gave no precipitate with digitonin and melted at 183° as compared with the reported value of 182° (22). Epicholestanol-4-C^{14} was prepared by refluxing 500 mg of the cholestanol-4-C^{14} described above with metallic sodium dissolved in *n*-amyl alcohol according to the method of Windaus and Uibrig (23). Purification was achieved by column chromatography on 20 g silicic acid, employing the solvent system used for purification of epicholesterol. Two radioactive peaks were obtained. The first, eluted with petroleum ether–benzene 5:1, upon recrystallization from anhydrous methanol, melted at 181°, gave no precipitate with digitonin, and had a specific activity of 1,300 cpm/mg. This fraction represented about 10% of the total radioactivity. The second fraction, eluted with petroleum ether–benzene 1:1, was unchanged cholestanol-4-C^{14}.

Coprostan-3β-ol (Steraloids, Inc., Queens, N.Y.) was crystallized twice from anhydrous methanol and then had a melting point of 101°, in agreement with the published value (19). Coprostanol-4-C^{14} was prepared biosynthetically by transformation of cholesterol-4-C^{14} (specific activity 3,300 cpm/mg) by rat intestinal microflora according to the method of Coleman and Baumann (24). The nonsaponifiable lipid fraction following incubation was chromatographed on a column of 20 g silicic acid, employing the solvent system used for purification of epicholesterol. Two major fractions based on radioactivity were obtained with petroleum ether–benzene 1:5. Both were dried and recrystallized from anhydrous methanol. The fraction eluted with 150–230 ml melted at 101°, identical with coprostan-3β-ol. It had a specific activity of 3,500 cpm/mg and represented about 20% of the total nonsaponifiable lipid. The fraction eluted with 250–450 ml petroleum ether–benzene 1:5 melted at 149°, identical with cholesterol. It had a specific activity of 3,300 cpm/gm. Elution of coprostanol and cholesterol from the silicic acid column occurred in the order given by Neher (25).

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Coprostan-3α-ol (epicoprostanol) (Chemed, Inc., Odenton, Md.) recrystallized from methanol exhibited a melting point of 115° as compared with the reported value of 117° (22). Coprostan-3α-ol-4-C^{14} was prepared by refluxing 25 mg of the coprostan-3β-ol-4-C^{14} described above in metallic sodium dissolved in *n*-amyl alcohol (26). Purification was effected by column chromatography on 10 g silicic acid, employing the solvent system used for purification of epicholesterol. The major fraction, representing >90% of total radioactivity, was eluted with petroleum ether–benzene 3:2. Crystallization of the material from this fraction yielded a product nonprecipitable with digitonin and melting at 115°, identical with coprostan-3α-ol. Its specific activity was 3,300 cpm/mg. Epicoprostanol is known to precede coprostanol upon elution from silicic acid columns (25). The second fraction, representing the remainder of the radioactivity, was eluted with petroleum ether–benzene 1:1. This small fraction was discarded.

Preparation of Sterol Acetates. Acetate esters of the six sterols employed in this study were prepared according to the method of Kellie, Smith, and Wade (27), starting with 100 mg sterol. Sterol acetates were separated from unreacted sterol by chromatography on 2 g alumina, Brockmann grade II (28), packed in a column 5 mm in diameter. Sterol acetates were eluted with 50 ml petroleum ether. The melting points of the sterol acetates following recrystallization from anhydrous methanol were as follows, with reported values (22) in parentheses: Δ^5-cholesten-3β-ol acetate, 114° (114°); Δ^5-cholesten-3α-ol acetate, 85° (85°); cholestan-3β-ol acetate, 110° (110°); cholestan-3α-ol acetate, 96° (96°); coprostan-3β-ol acetate, 89° (89°); coprostan-3α-ol acetate, 86° (88°).

Strain 07 of PPLO was used as the test organism throughout since its lipid physiology had been studied the most thoroughly. Other experimental procedures used in this study have been described in several previous reports (1, 11–14).

RESULTS

Effects of Sterol on Growth. Four concentrations (0.001, 0.005, 0.01, and 0.05 mg/ml) of each sterol were assayed for ability to elicit a growth response. A maximum yield of 10^9 organisms/ml was obtained with the optimal concentration of the most effective sterol. Comparison of the growth response among the various sterols was made by determination of the ratio of number of organisms per milliliter at the optimal level of sterol to the number of organisms per milliliter in the absence of sterol. Table 1 presents these data. Concentrations of sterol greater or less than those listed resulted in a poorer growth response. Coprostan-3α-ol at all concentrations actually prevented the survival apparent in the controls containing no sterol. It is evident that for growth to occur the sterol must possess an equatorial 3-hydroxyl group and either a *trans* A/B ring or Δ^5 double bond. Models indicate a closer similarity of shape of the Δ^5-cholestenols to the cholestanol series than to the coprostanol series.

Table 1 Growth Response of Strain 07 toward Six Selected Sterols

Sterol	Configuration of 3-hydroxy	Conformation of A/B Ring	Concentration*	Growth Responses†
Δ^5-Cholesten-3β-ol (cholesterol)	e‡	—	0.01–0.05	1,620
Δ^5-Cholesten-3α-ol	a	—	0.001	72
Cholestan-3β-ol	e	*trans*	0.01–0.05	2,390
Cholestan-3α-ol	a	*trans*	0.001	5
Coprostan-3β-ol	a	*cis*	0.001	3
Coprostan-3α-ol	e	*cis*	0.001–0.05	<0.1

* Concentration or range of concentration giving optimal growth response.
† Relative growth compared with absence of sterol.
‡ e, equatorial; a, axial.

All six sterols employed possess identical side chains necessary for incorporation into the organisms (11). Since the four sterols failing to support growth exhibited an inhibitory effect, particularly on increasing the concentration, these sterols might be expected to compete with cholesterol and cholestanol. The results obtained from growth experiments in which the concentration of active and inactive sterols were varied support this assumption (Tables 2 and 3). High levels of cholesterol were somewhat inhibitory in the presence of epicholesterol and epicholestanol. High levels of cholesterol as the sole sterol present have been shown to have some inhibitory effect on the bovine pleuropneumonia organisms (29).

Uptake of Sterols. Table 4 shows that all six sterols are incorporated into the cells and compares their rates of uptake. Corrections have been made to equalize the specific activities of the sterols.

There was no significant effect on the uptake of cholesterol by resting cells by the other five sterols used (Table 5). A similar lack of competition has been demonstrated previously with cholesterol and Δ^4-cholesten-3-one (11); presumably the cells do not even approach saturation with sterol over the period of the experiment.

Enzymatic Hydrolysis of Acetate Esters of Sterols. Strain 07 has been shown to be capable of hydrolyzing cholesterol esters (12). Acetate esters of the six sterols studied were incubated at a level of 5 μmole with resting cells equivalent to 1 mg cellular nitrogen at pH 6.5 in the presence of 10 μmole sodium cholate. Following 6-hr incubation at 37°, analyses were performed as previously

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described (12). The degree of hydrolysis of each sterol ester was calculated on the basis of production of unesterified sterol equated to standard cholesterol. Corrections were made for any hydrolysis that occurred in controls run for each sterol acetate in the absence of cells. The data are presented in Table 6.

Enzymatic hydrolysis of the acetate esters did not follow an immediately discernible pattern, as contrasted with growth response. The axial configuration at C_3 prevented hydrolysis in the Δ^5-cholestenols, but only reduced it in the case of cholestanols. The greater degree of hydrolysis of cholestan-3β-ol corresponds to the greater growth response to this sterol than to cholesterol (Table 1). It would appear that Δ^5-unsaturation slightly impedes the action of the sterol esterase; otherwise the cholesterols behave similarly to the cholestanols (A/B *trans*). *Cis*-fused A and B rings give rise to opposite effects with regard to effectiveness of hydrolysis of esters. Axial configuration at C_3 permitted hydrolysis of coprostanol-3β-ol acetate, whereas hydrolysis of coprostan-3α-ol acetate (C_3 equatorial) was negligible.

TABLE 2 INHIBITORY EFFECT OF DIFFERENT STEROLS ON CHOLESTEROL-SUPPORTED GROWTH OF STRAIN 07

Inhibitory Sterol	Concentration	Growth Response*
	mg/ml	
None†	—	1.00
Δ^5-Cholesten-3α-ol	0.005	0.70
	0.025	0.61
Cholestan-3α-ol	0.005	0.70
	0.025	0.18
Coprostan-3β-ol	0.005	0.85
	0.025	<0.00001
Coprostan-3α-ol	0.005	0.16
	0.025	0.002

* Relative growth compared with cholesterol alone.
† Basal medium contained 0.005 mg/ml of cholesterol.

TABLE 3 REVERSAL BY CHOLESTEROL OF GROWTH INHIBITION OF STRAIN 07 BY OTHER STEROLS

Inhibitory Sterol*	Cholesterol Added	Growth Response†
	mg/ml	
None	0.01	1.00
Δ^5-Cholesten-3α-ol	None	0.12
	0.005	0.59
	0.025	0.28
Cholestan-3α-ol	None	0.012
	0.005	0.63
	0.025	0.24
Coprostan-3β-ol	None	0.00003
	0.005	0.002
	0.025	0.16
Coprostan-3α-ol	None	0.00002
	0.005	0.0014

* Concentration of inhibitory sterol, 0.01 mg/ml.
† Relative growth compared with cholesterol.

DISCUSSION

This study shows that only sterols having an equatorial 3-hydroxyl group and the A/B rings either in the *trans* configuration or held planar by a Δ^5 double bond support growth. There was no such requirement for a planar molecule in order for *incorporation* to occur. A planar molecule might be needed to undergo rotational movements in the membrane in the course of permeation processes (30). No absolute correlation was obtained between the specificity of the esterase activity and the utilization of sterols for growth, but esterase activity for, and growth response to, cholestanol were both greater than for cholesterol. Steric factors appear to be

TABLE 4 UPTAKE OF SIX SELECTED STEROLS BY STRAIN 07

Sterol*	Time† (min) 15	30	60	180	Uncorrected Value at Zero Time
	cpm/mg dry wt				*cpm/mg dry wt*
Δ^5-Cholesten-3β-ol	8	12	20	88	5
Δ^5-Cholesten-3α-ol	27	38	59	80	4
Cholestan-3β-ol	3	17	33	88	3
Cholestan-3α-ol	8	22	33	90	5
Coprostan-3β-ol	12	24	39	56	9
Coprostan-3α-ol	16	24	36	39	5

* Final concentration of sterol, 0.01 mg/ml; corrected for individual specific activities.
† Corrected for value at "zero time" (last column).

TABLE 5 EFFECT OF VARIOUS STEROLS ON UPTAKE OF CHOLESTEROL-4-C^{14} BY STRAIN 07

Sterol Added	Time (min) 15	30	60	180
	cpm/mg dry wt			
None*	796	1307	1588	2687
Δ^5-Cholesten-3α-ol†	656	1027	1505	2005
Cholestan-3β-ol	779	1119	1405	2545
Cholestan-3α-ol	746	—	1645	2070
Coprostan-3β-ol	780	1152	1436	2318
Coprostan-3α-ol	812	1272	1629	2015

* Final concentration of cholesterol, 0.01 mg/ml.
† Final concentration of added sterol, 0.02 mg/ml.

TABLE 6 HYDROLYSIS OF ACETATE ESTERS OF SIX SELECTED STEROLS BY STRAIN 07

Acetate of	Total Sterol as Cholesterol	Free Sterol Formed per mg N in 6 hr	Extent of Hydrolysis
	μmole	*μmole*	%
Δ^5-Cholesten-3β-ol	5.35	0.91	17 (15.3–26.7)
Δ^5-Cholesten-3α-ol	4.00	0.04	1 (0 – 2)
Cholestan-3β-ol	1.60	0.64	40 (26.8–61.7)
Cholestan-3α-ol	1.76	0.44	25 (15.9–31.7)
Coprostan-3β-ol	2.52	0.53	21 (11.2–27.1)
Coprostan-3α-ol	1.03	0	0 (0)

Values represent average of 3 or 4 experiments.

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of importance, and this may be interpreted in terms of the "fit" of the sterol molecule in the appropriate space in the lipoprotein of the cell membrane of PPLO.

The structural specificity of sterols required to support growth of PPLO have an analogy in the sterol requirements of insects and protozoa. Clark and Bloch (3) originally hypothesized that cholesterol in the hide beetle, *Dermestes vulpinus*, functioned in two ways, namely, as a structural entity and as a precursor for bile acids, steroid hormones, and vitamin D. In support of this hypothesis, they found that the cholesterol requirement could be partially substituted by other sterols. This finding was suggestive of a metabolic role for cholesterol and a structural role for the sparing sterols. In an extension of these studies, Clayton and Edwards (31) noted that the minimal quantity of cholesterol required was not completely metabolized but was incorporated intact in preference to the sparing sterol, cholestanol. Recently, Clayton and Bloch (10) presented evidence for the structural role of cholesterol-sparing sterols by showing that sparing sterols must possess a planar shape, a side chain of the cholestane type, and a β (equatorially) oriented 3-hydroxyl group. In addition, double bonds, if present, must be appropriately situated. These requirements were considered necessary for the sparing sterol to fit into the functional spaces normally occupied by cholesterol. The absorption of sterols by *Dermestes* has not been studied, but in the roach, *Eurycotis floridana*, no correlation between efficiency of intestinal absorption of sterols and ability to support growth was obtained (10). This may be compared with the incorporation by PPLO of sterols, which do not support growth (Tables 1 and 4).

Structural requirements for sterols to support growth of protozoa are less well defined. The 3-hydroxy group in β configuration and a hydrocarbon side chain are required by *Paramecium aurelia* (4), *Tetrahymena* (5, 6, 7), and *Trichomonas* (8). An additional function other than maintenance of cell membrane structure has been postulated by Conner and coworkers (16, 17) for sterols in *Tetrahymena pyriformis*, namely the generation or transport of high-energy phosphate groupings (16). Thus sterol involvement in transport activities is also likely in protozoa as well as PPLO.

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