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Sterol Structural Requirements for Inhibition of Streptolysin O Activity

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Reduced streptolysin O, a toxin produced by certain β-haemolytic streptococci, lyses human erythrocytes. The reaction is inhibited by cholesterol at concentrations of about 1.0 μg/ml. Other sterols inhibit the lysin and there is a specific requirement for a 3β-hydroxyl group. Inhibition was obtained with 3β-hydroxychol-5-en-24-oic acid, containing a hydrophilic group at C-24. The mode of inhibition is likely to involve attachment to the fixation site of the lysin which attaches the molecule to cell membranes, probably to membrane cholesterol. A second streptolysin site, concerned in the final haemolytic event, may also be involved. Inhibitors of the latter site have not been characterized, other than antibody with specificity for the site.

Certain β-haemolytic streptococci can produce two haemolysins, (a) an O_2-labile antigenic toxin, streptolysin O, which causes erythrocyte lysis only when in the reduced form, and (b) an O_2-stable non-antigenic toxin, streptolysin S.

Attachment of streptolysin O to erythrocytes is believed to result from binding to cell-membrane cholesterol (Van Heyningen, 1950). Other bacterial lysins, e.g. pneumolysin, tetanolysin and listeriolysin, as well as saponin and digitonin, appear to act in the same way (Cohen *et al.*, 1937; Ponder, 1945). Petersen *et al.* (1966) found that delipidation of erythrocytes decreased their ability to adsorb streptolysin O and Bernheimer (1966) noted that the lysin had no effect on membranes of organisms such as bacteria that lack membrane cholesterol. Cholesterol in aqueous emulsion inhibits the haemolytic activity of streptolysin O (Van Heyningen, 1950), and the work of Alouf & Raynaud (1968) suggests that cholesterol attaches to a fixation site, consisting of two cysteine residues, on the lysin molecule which attaches the molecule to the cell. Alouf & Raynaud (1968) have also shown that the final haemolytic event is due to activity of a second site on the lysin molecule, and that cells adsorbing lysin at 0°C do not undergo haemolysis since the site is inactive at low temperatures. Their evidence also indicates that the fixation site is the one blocked by oxidation and that antisera against streptolysin O may contain two types of antibody with specificity for one or other site. Either antibody would thus be capable of neutralizing the haemolytic activity of the lysin. Further evidence favouring lysin attachment to membrane cholesterol is the failure of cholesterol in aqueous emulsion to inhibit lysis of erythrocytes after binding of lysin to membranes (Alouf & Raynaud, 1968).

Other sterols will inhibit streptolysin O activity. Howard *et al.* (1953) suggested that the structural requirements of such inhibitors were a 3β-hydroxyl group and a hydrophobic side chain attached to C-17. Inhibitory compounds fulfilling these criteria included cholesterol, ergosterol, stigmasterol, cholestanol (5α-cholestan-3β-ol) and coprostanol (5β-cholestan-3β-ol). Their observations were based on the concept of the mechanism of interaction between saponin and cholesterol–protein monolayers postulated by Schulman & Rideal (1937).

The present study embraces a wider range of sterols, including bile acids, with results suggesting that spatial orientation of inhibitory molecules may not always be determined by a hydrophobic side chain at C-17. The test system is extremely sensitive, cholesterol inhibition of lysis being detected at concentrations of about 1.0 μg/ml (Watson *et al.*, 1972).

Materials

Sterols

Sterol compounds were dissolved in ethanol in 1.0% concentration. From these, stock solutions were prepared by adding 1.0 ml to 4.0 ml of phosphate-buffered saline (0.2 M, pH 6.5, containing NaCl, 4.25 g, $NaH_2PO_4,2H_2O$, 8.42 g and $Na_2HPO_4,12H_2O$, 8.95 g/litre). With some sterols it was necessary to add 0.01 ml of Tween 80 to ensure stable colloidal emulsions. This concentration of Tween 80 had no effect on either streptolysin O or erythroctyes. Stock solutions were stored at 4°C. Since unsaturated sterols may undergo oxidation to haemolytic compounds, recrystallization from ethanol was carried out if necessary, as a preliminary to preparing stock solutions (Berliner & Schoenheimer, 1938).

Buffer

The standard phosphate buffer described above was used as in the antistreptolysin titration method of Rantz & Randall (1945).

Streptolysin O

Commercially available reduced streptolysin O was used (Burroughs Wellcome Ltd., Beckenham, Kent, U.K.), 20ml of water being added to a vial containing 40 units.

Methods

Standard test procedure

Stock solutions were diluted serially in buffer in 1.0ml amounts in 75mm×12mm tubes. Freshly prepared streptolysin O was added (0.5ml containing 1.0 unit). Tubes were incubated at 37°C for 15min. Three-times-washed human group O erythrocytes were added in 0.5ml amounts of a 5.0% (v/v) suspension. The tubes were then re-incubated for a further 45min. End points were read visually as 50% haemolysis compared with standard control preparations showing haemolysis varying between 0% and 100%. With practice accurate reading of end points is easily attained and more sophisticated colorimetric procedures were not necessary.

Light-petroleum method

A number of tests were done with the method described in detail by Howard *et al.* (1953). Here the sterol is dissolved in light petroleum (BDH; b.p. 90°–120°C), in an attempt to minimize any non-specific interactions between sterol and streptolysin and also to present the sterol to the lysin in an orientated manner at a water–petroleum interface. Briefly, the method uses a U-tube fitted with a fine nozzle in one arm, a tap in the other and a reservoir on top of each limb. The sterol–petroleum solution is placed in the limb with the tap and allowed to flow until it reaches the nozzle. A 5.0ml volume of lysin containing 2.0 units/ml is then run into the other limb above the nozzle. By opening the tap the sterol solution is then allowed to ascend through the lysin at the rate of 2–3 drops/s. After 50ml has passed and been periodically removed, the lysin is aspirated and assayed for haemolytic activity with human erythrocytes, against a control lysin preparation. Various concentrations of sterols were used, but a comparison of the methods, with cholesterol as inhibitor, showed no advantage for the second method, both methods giving inhibitory end points of the order of 0.8–1.0μg/ml. Consequently most of the tests were done by the standard method.

Results

Altogether 38 compounds were tested. Those with inhibitor activity are illustrated. The concentrations required for 50% inhibition of lysis are given in Table 1.

Table 1. *Comparative efficiency of compounds inhibiting streptolysin O*

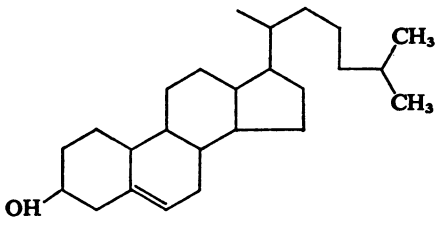
Compound	Mean concentration showing 50% haemolytic end point with 1.0 unit of streptolysin activity (μg/ml)
Cholesterol	1.5
Cholestanol (5α-cholestan-3β-ol)	1.5
Desmosterol (5,24-cholestadien-3β-ol)	0.5
Fucosterol	0.5
Sitosterol	1.5
3β-Hydroxychol-5-en-24-oic acid	9.0

1. Differences in solubility in ethanol of inhibiting sterols did not appear to contribute significantly to differences in inhibiting end points since the sterols at near-end-point dilutions appear to be entirely in emulsion form.

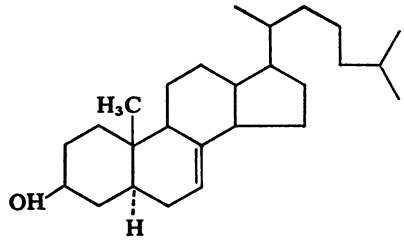
Compounds that were not active included the following: 5α-cholestane; stigmasterol; lanosterol; cholesteryl acetate; dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one); pregnenolone (3β-hydroxypregn-5-en-20-one); 3β-hydroxychol-5-en-24-oic acid methyl ester; androst-5-en-3β-ol; pregn-5-en-3β-ol; cholest-4-en-3-one; 25-hydroxycholesterol; 26-hydroxycholesterol; 1-deoxycorticosterone acetate (pregn-4-ene-3,20-dione-21-ylacetate); prednisone (17,21-dihydroxypregna-1,4-diene-3,11,20-trione); prednisolone (11β,17,21-trihydroxypregna-1,4-diene-3,20-dione); cortisone acetate; cortisol; decamethasone (9α-fluoro-16α-methylprednisolone); progesterone; ethynyloestradiol (17α-ethynyloestra-1,3,5(10)-triene-3,17β-diol); taurocholic acid; taurodeoxycholic acid: taurochenodeoxycholic acid; taurolithocholic acid; glycocholic acid; glycodeoxycholic acid; glycolithocholic acid; cholic acid; lithocholic acid; chenocholic acid; deoxycholic acid.

Discussion

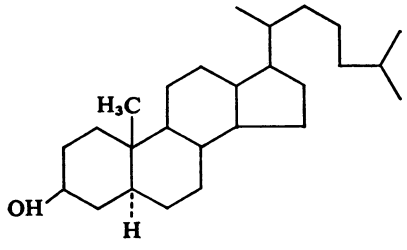
The results clearly confirm that sterol inhibitors of streptolysin O require a 3β-hydroxyl group. The presence of such a group is in itself not sufficient, as shown by the structures of some of the non-inhibitory compounds. In some, failure of inhibition appears to be due to lack of a side chain of suitable size at C-17, e.g. androst-5-en-3β-ol, 3β-hydroxypregn-5-en-20-one and 3β-hydroxyandrost-5-en-17-one, or possibly, in the case of lanosterol, because of steric-hindrance effects associated with side chains at C-4 interfering with the C-3 hydroxyl group or the very large size of the side chain at C-17. Compounds with side chains at C-17 similar to that of cholesterol



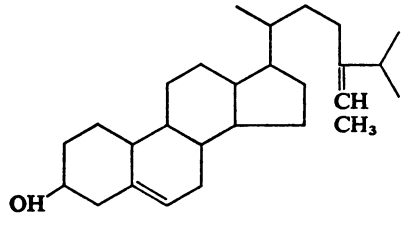
Cholesterol



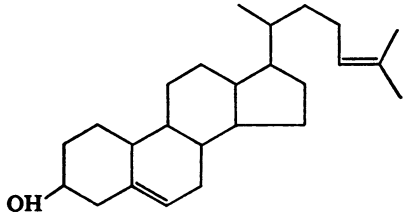
Lathosterol



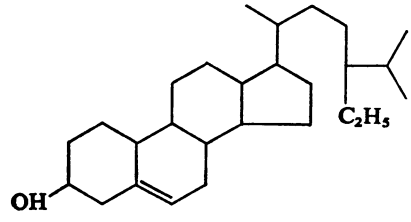
Cholestanol



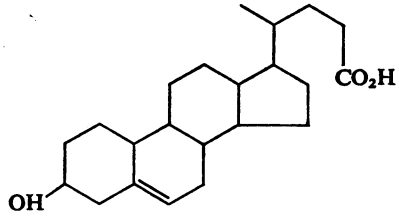
Fucosterol



Desmosterol



Sitosterol



3β-Hydroxychol-5-en-24-oic acid

but with groups other than hydroxyl at C-3 were also inactive, e.g. cholesterol acetate, and cholest-4-en-3-one. Howard *et al.* (1953) found that compounds with 3α-hydroxyl groups were ineffective, e.g. 3-epicholesterol, 3α-cholestan-5β-ol and androsterone.

The structure of 3β-hydroxychol-5-en-24-oic acid, a compound with a polar group at C-24 which was an inhibitor is shown. This indicates that the requirement for a non-polar side chain at this part of the molecule, as previously suggested, is no longer tenable. It should be noted that the methyl ester of 3β-hydroxychol-5-en-24-oic acid was not active.

Toxicity of streptolysin O for erythrocytes and other tissues, e.g. myocardial cells, appears to result from adsorption to membrane cholesterol. Apart from evidence cited above, the lysin has no effect on bacterial protoplasts which lack cholesterol, and bacterial toxins which do affect protoplasts, such as staphylococcal α toxin and streptolysin S, are unaffected by cholesterol. However, formal proof of the

chemical mechanisms involved in streptolysin O attachment is lacking. Attachment of hydrophobic side chains of inhibitors is probably by Van der Waal-type forces reacting with corresponding regions of the streptolysin molecules. With 3β-hydroxychol-5-en-24-oic acid inhibition may be due to Coulomb forces between the C-17 side chain and ionized groups on the streptolysin. Clearly spatial orientation is important since compounds structurally very similar to cholesterol, such as 25-hydroxycholesterol and 26-hydroxycholesterol, were ineffective.

It is likely that cholesterol and related inhibitors with non-polar side chains at C-17 inhibit streptolysin O activity by attachment to the fixation site of the molecule as shown for cholesterol by Alouf & Raynaud (1968). Further investigation is needed to see if 3β-hydroxychol-5-en-24-oic acid acts here or whether it may interfere with the haemolytic site. So far inhibitors of the latter site have not been characterized apart from the demonstration by Alouf & Raynaud (1968) that the site is antigenic and that antibody directed against the site will inhibit streptolysin O activity. The chemical nature of the site is unknown but the presence of ionized groups in amino acid residues such as lysine and histidine may provide an explanation for inhibition by 3β-hydroxychol-5-en-24-oic acid.

Our failure to demonstrate inhibition with stigmasterol is unexplained. Its structure suggests that it should be active and it was found to be so by Howard *et al.* (1953). However, repeated attempts with different batches and by using both methods of interaction with lysin were unsuccessful. Oxidation of sterols at the double bond between C-5 and C-6 can give rise to haemolytic compounds as mentioned above, but recrystallization of stigmasterol from ethanolic solutions failed to yield an active product and t.l.c. preparations of the stigmasterol did not reveal any other substances that might have affected its action. Sitosterol, however, was fully inhibitory although differing from stigmasterol only in the absence of the double bond between C-22 and C-23.

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