THE INHIBITORY EFFECTS OF CHOLESTEROL AND RELATED STEROLS ON HAEMOLYSIS BY STREPTOLYSIN O.

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THE well-established observation that aqueous emulsions of cholesterol can protect mammalian red cells from lysis by certain bacterial and steroid haemolysins (van Heyningen, 1950) has suggested that the action of these toxic substances may depend on their forming some disruptive combination with the cholesterol of the cell envelope. For some bile acids, and especially for lithocholic acid, the mode of inhibition of haemolytic action by sterol compounds has been clarified by the work of Berliner and Schoenheimer (1938), who found that among those they examined, protective power was characteristic of sterols possessing a hydroxyl radicle at position 3 *cis* (or β-) to the methyl group at position 10 and accompanied by either a *trans* fusion of rings A and B or a double bond between the 5 and 6 carbon atoms. It appeared from this study that the neutralization of this type of haemolysin by its sterol inhibitors involved interactions of a closely-defined stereochemical nature. The present investigation has been made to determine, firstly, whether the action of the bacterial toxin streptolysin O can also be inhibited by sterols other than cholesterol, and secondly, if so, whether this property is associated with any comparably specific configuration of the molecules of these substances.

Before describing this work, it is necessary to point out that we have departed significantly from the techniques used by earlier workers in that the potentially inhibitory sterols are not presented to the streptolysin in a finely suspended particulate form but in an orientated manner as a film at a ligroin-water interface. There are two reasons for this modification: to lessen the likelihood that any recorded inhibition might result from some non-specific behaviour of the lysin on the surface of the particles in the emulsion, and to bring the hydrophilic radicles of the sterol molecules—notably the hydroxyl group at position 3—most intimately into relation with the solutes present in the aqueous phase. It was hoped that were neutralization of the streptolysin under these conditions found to be associated with some particular configuration of the hydrophilic radicles of the sterols examined, it would advance our knowledge of the mode of action of this important toxin.

MATERIALS AND METHODS.

Definition of a "Haemolytic Unit."—A solution of streptolysin O was regarded as containing one haemolytic unit (H.U.)/ml. when it produced 50 per cent haemolysis in an equal volume of a 2·5 per cent suspension of rabbit's red cells in 30 min. at 37°. Thus, for example, if this degree of haemolysis was produced by a 1 : 100 dilution of the lysin, the preparation would be regarded as containing 100 H.U./ml.

Preparation of streptolysin O.—This toxin was produced by growing the "Lambert" strain of *Streptococcus pyogenes* (Group A) in the semi-synthetic medium described by Bernheimer, Gillman, Hottle and Pappenheimer (1942). After sterilization according to their technique, 15 l. of the medium were distributed in four 5 l. conical flasks and inoculated with the saline-resuspended deposit from a 6 hr. culture of the organism grown at 37° in glucose broth. The flasks were incubated at 37°, and after the first 15 hr. of growth the increasing acidity of the cultures was neutralized by the frequent addition with gentle agitation of small amounts of 7·5 M NaOH.

After 28–30 hr. incubation, 1/10th of their volume of anaesthetic ether was added to the flasks to kill the organisms, and the cultures kept at 2° overnight. The following day the bacteria were removed in a Laval centrifugal separator. At this stage the streptolysin titres of the cultures varied slightly, the average being 20 H.U./ml. For further concentration the clarified supernatant was suspended in cellophane sacs (Visking transparent cellulose tubing), against which an air current was directed. After reduction in bulk from 1/5th to 1/8th, the preparation was dialysed overnight against running tap-water. After concentrating thus, the titres of the various batches varied from 75 to 160 H.U./ml. Merthiolate 1 : 10,000 was added as a preservative, and the sterility of the preparations checked at intervals. Through the kindness of Dr. Humphrey and Mr. Ward, of the National Institute for Medical Research, some of the preparations were freeze-dried and stored *in vacuo*. It was found, later, however, that concentrated solutions of the streptolysin were stable for at least 4 months when kept at 2°.

Estimation of haemolytic titres.—(a) *Reducing agent:* Sodium thioglycollate was used for the re-activation of the streptolysin O after its reversible oxidation, 0·1 ml. of a 90 per cent solution of thioglycollic acid being diluted with 2 ml. distilled water, a few drops of B.D.H. Universal Indicator added, and 7·5 M NaOH added drop by drop until the solution turned blue-green (pH 6–7). When made up to 5 ml. with saline-phosphate buffer (see below), this gave an approximately 0·2 M solution. (b) *Saline-phosphate buffer:* All haemolysis estimations were made in the isotonic saline-phosphate buffer described by Herbert (1941). (c) *Red cell suspensions:* Fresh rabbit's blood was collected in 3 per cent sodium citrate: the deposited cells were washed three times in the saline-phosphate buffer before being made up into a 2·5 per cent suspension in the same solution. The suspension was always freshly prepared on the day of use. (d) *Haemolysis:* Estimations were made in 15 ml. conical centrifuge tubes. Five ml. of the reducing solution was added to 20 ml. of the streptolysin solution, and the mixture allowed to stand at room temperature for 10–15 min. before use. For titration, falling dilutions of the lysin were prepared. To 1·5 ml. of each dilution, 1·5 ml. of the buffer and 3 ml. of the red cell suspension were added, and the tubes immersed in a water bath at 37° for 30 min. The tubes were then centrifuged, and the haemoglobin concentrations of the supernatants determined in a "Spekker" photo-electric absorptiometer, for which a calibration curve for haemoglobin solutions had previously been prepared. To exclude the possible presence of any oxygen-stable haemolysin (streptolysin S), initial titrations of the haemolysin solutions were carried out after the addition of potassium persulphate (about 0·5 ml. of an 0·1 M solution).

Presentation of the sterols to streptolysin O.—The sterols were obtained through the kindness of various donors and were used in the pure state. Berliner and Schoenheimer (1938) found that the unsaturated sterols, especially cholesterol, readily undergo oxidation at the double bond, giving highly heaemolytic substances which on re-crystallisation revert to their former protective form. In the present experiments all the cholesterol used was recrystallised from absolute alcohol on the day of use. The solvent used to carry the sterols and steroids was ligroin (B.D.H., b.p. 90–120°).

The solution of the sterol or steroid in ligroin (0·1 per cent) was presented to the streptolysin solution in its aqueous buffer in the apparatus depicted in Fig. 1. This apparatus consisted of a U-tube with a reservoir at the top of both limbs. A tap about midway down the right-hand limb controlled the rate of flow of the ligroin solution. The wider left-hand limb had a fine nozzle 5 cm. from its lower end, through which small drops of the ligroin solution issued and began their slow ascent through the column of the streptolysin solution. Before use the apparatus was washed with ligroin. The sterol-ligroin solution was then introduced into the right-hand limb and allowed to flow through the apparatus until it reached the nozzle. The tap was then closed, and the lysin-containing limb washed out, first with saline-buffer and then with 1 ml. of the activated streptolysin solution. Five ml. of the streptolysin solution was run into this limb, and the control tap then opened until

the ligroin-sterol solution entered through the nozzle at the rate of 1 to 4 drops/sec. The ligroin-sterol solution collecting above the lysin column was removed at intervals, and fresh solution added to the reservoir at the top of the right-hand limb to maintain a continuous flow. After 50 ml. of the ligroin-sterol solution had been allowed to pass in this way, the streptolysin solution was removed for titration of its residual haemolytic activity. In every experiment, the haemolytic activity of a control solution of activated streptolysin which had stood for the same time at room temperature was also estimated, and the degree of inhibition of the perfused lysin expressed in relation to this control.

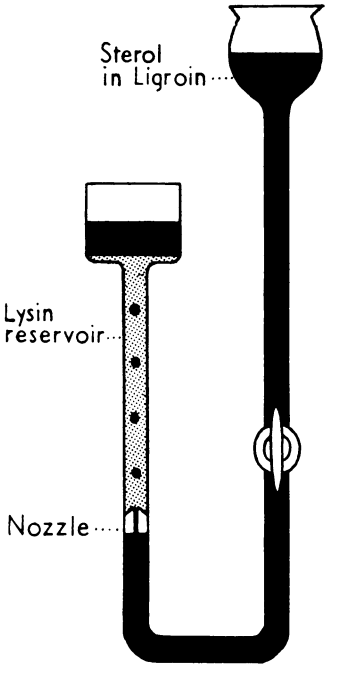


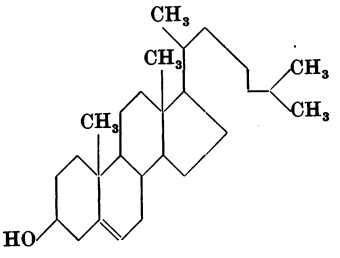
FIG. 1.—Diagram showing apparatus for passing ligroin-sterol solutions through the streptolysin reservoir.

Since it was possible that some loss of the streptolysin might occur in consequence of adsorption of the protein to the glass wall of the apparatus, the activity of a column of the lysin was determined after it had stood in the left-hand limb at room temperature for 1 hr.; no decrease in haemolytic activity was found. The specific character of the neutralization was further tested by passing ligroin alone through a column of the streptolysin; no fall in titre was found to follow.

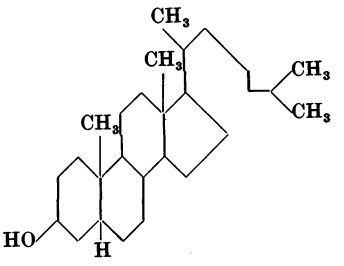
Benzene and chloroform were also considered as solvents for the sterols: the former was unsatisfactory in failing to dissolve all the compounds examined, while the higher specific gravity of the latter precluded its use in this type of apparatus.

RESULTS.

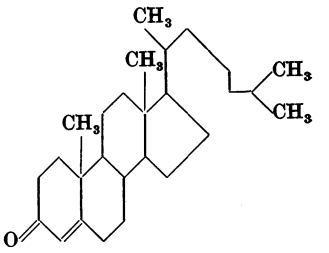
A list of the sterols and steroids examined together with their chemical configuration and their neutralizing capacities for streptolysin O are given in Fig. 2. With the exception of dehydroisoandrosterone, all the sterols with a β-hydroxyl group at carbon atom 3 were potent inhibitors of the lysin. Cholesterol, stigmasterol and cholestanol were all strongly inhibitory, ergosterol and



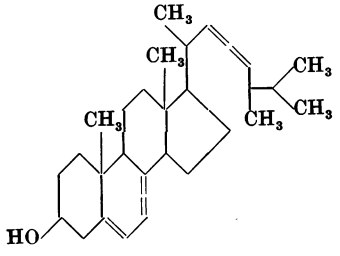
I. Cholesterol (β). +++.



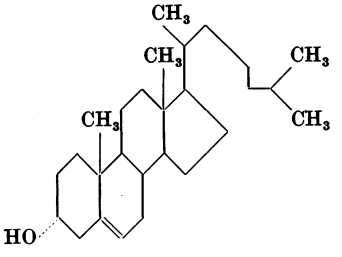
V. Coprostanol (β). ++ (Coprosterol).



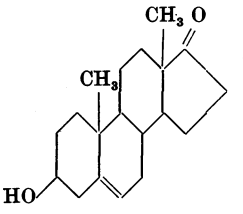
VIII. Cholest-4-en-3-one (keto). —.



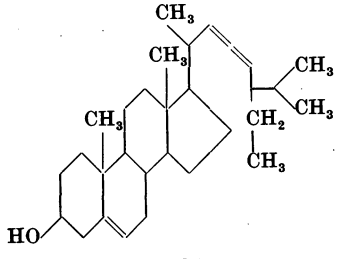
II. Ergosterol (β). ++.



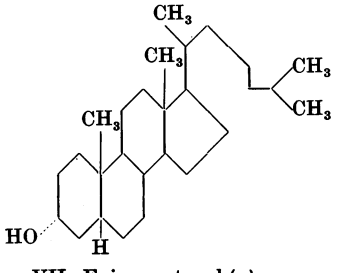
VI. Epicholesterol (α). —.



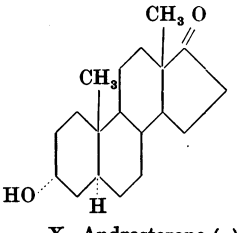
IX. Dehydroisoandrosterone (β). —.



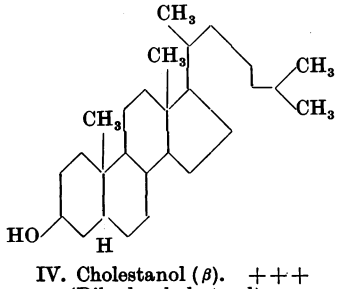
III. Stigmasterol (β). +++.



VII. Epicoprostanol (α). —. (Epicoprosterol).



X. Androsterone (α). —.



IV. Cholestanol (β). +++ (Dihydrocholesterol).

FIG. 2.—Names and structural formulas of the various sterols and steroids, together with the arrangement of the hydroxyl group at carbon atom 3 (α or β) and degree of inhibition of streptolysin O (—, no inhibition; ++, 50–75 per cent; and +++, > 75 per cent inhibition).

coprostanol rather less so. In contradistinction to these, none of the sterols that possess an α-hydroxyl group at carbon atom 3 effected any inhibition. The steroid cholest-4-en-3-one was included in the series to test the effect of a keto instead of an hydroxy group at carbon atom 3: this substance caused no inhibition.

A lecithin solution (0·1 per cent of egg lecithin in ligroin) also failed to inhibit. It was found, however, that a mixture of lecithin and cholesterol dissolved in ligroin was inhibitory, though less so than cholesterol alone.

DISCUSSION.

The ability of cholesterol and other kindred sterols to inhibit the haemolytic activity of a solution of streptolysin O when brought into relation with it across an oil-water interface appears to depend upon certain distinctive features in the steric configuration of their molecules. The inhibitory sterols are cholesterol, stigmasterol, cholestanol, ergosterol and coprostanol, all of which resemble one another in possessing a β-hydroxyl group at carbon atom 3 (C 3) of the cyclopentanephenanthrene nucleus (*i.e.*, a hydrophilic polar group) and a hydrophobic group attached to carbon atom 17 (C 17) at the other extremity of the molecule. The other sterols and steroids studied, all of which failed to neutralize the streptolysin, differed from this steric arrangement in various respects. Epicholesterol and epicoprostanol both possess the same hydrophilic hydroxyl and hydrophobic groups attached at the same sites in the molecule, but both of them have the α-arrangement, a steric configuration which seems to preclude its presentation across the interface to couple with the reactive groups of the lysin molecule. Cholest-4-en-3-one possesses a keto group at C 3—a polar group that has widely differing properties from those of an hydroxyl group. Dehydroisoandrosterone possesses a β-hydroxyl group at C 3, and in this respect resembles cholesterol and the other inhibitory sterols, but the hydrophobic group at C 17 that is typical of these compounds is replaced by a hydrophilic polar group whose presence would greatly modify the orientation of the molecule at such an interface. Androsterone fulfils neither of the above conditions that seem to be required for complex formation with the streptolysin, since the hydrophilic hydroxyl group at C 3 has the α-arrangement and the molecule possesses no hydrophobic group at C 17.

That the ability of certain sterols to inhibit the haemolytic action of streptolysin O depends upon the steric arrangement is supported by some observations made with other haemolysins. Cohen, Shwachman and Perkins (1937) found that the activity of the related oxygen-labile pneumococcal haemolysin is inhibited by much smaller concentrations of the digitonin-precipitable sterols, cholesterol, cholestanol and, to a lesser degree, coprostanol than are required for the digitonin-non-precipitable sterols. Ponder (1945), in a study of haemolysis produced by digitonin and saponin, found that the inhibition exerted by emulsions of cholesterol and its related sterols depended primarily upon a normal (or β-) as opposed to an epi- (or α) configuration of the hydroxyl group at C 3. It would seem, therefore, from these studies, as well as from that of Berliner and Schoenheimer already mentioned, that all these bacterial and glucosidal haemolysins are capable of neutralization by sterols that possess the same distinctive steric configuration at this site in the molecule. From this common characteristic,

it would seem likely that they possess some common mode of attack on the red cell envelope.

Although details of the structure of the red cell envelope are still obscure, it is now known to be composed of closely associated molecules of cholesterol, phosphatides and protein (Frey-Wyssling, 1948; Ponder, 1945). According to Winkler and Bungenberg De Jong (1940–41) and Booij (1949), the most superficial layer is a palisade-like film of orientated phosphatides and cholesterol, the molecules of the latter lying between and stabilizing the more highly dissociated and mutually repulsive phosphatide molecules. Were this so, the cholesterol in the envelope would be freely accessible to lysins in the surrounding medium, and the disruption of the envelope could be accomplished by penetration of its surface film. This conception of the mechanism of haemolysis by lysins of the type here considered is supported by observations of Schulman and Rideal (1937) on interactions between saponin and stable cholesterol-protein monolayers. They found that the factors which determine the penetration of such films are the same as those that determine complex-formation in these mixed monolayers: first, the polar heads of the two types of molecule must mutually interact, and second, there must be a hydrophobic tail to the film-forming molecule which must adhere or interact by van der Waal's forces with some hydrophobic group in the penetrating molecule. When only the polar interaction between the two substances occurs, no penetration of the film follows. It can be seen that this concept of interaction defines criteria that accord with our findings upon the characteristics required in the sterols studied for inhibition of streptolysin and by others for the inhibition of kinetic lytic agents, *viz.*, a specific hydrophilic hydroxyl group configuration at one end of the sterol so arranged as to be able to couple with the lysin and a hydrophobic group at the other.

SUMMARY.

The inhibition of the haemolytic activity of streptolysin O by cholesterol and closely related sterols has been studied by presenting the sterol to the lysin at a ligroin-water interface.

Inhibition of activity was effected by sterols which possessed a hydrophilic hydroxyl group at position 3 of the sterol nucleus in *cis* (or β-) position to the methyl group at position 10 and a hydrophobic group attached at position 17 (cholesterol, ergosterol, stigmasterol, cholestanol and coprostanol). Other sterols in which these criteria were not fulfilled caused no inhibition of haemolytic activity.

It is suggested that the inhibition of haemolysis is due to complex formation between the lysin and the sterol molecules when the latter are orientated at the interface, and that the lysis of red cells by streptolysin O is dependent on complex formation between the lysin and the cholesterol of their envelope with resultant penetration of the surface film.

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