

# Some factors influencing the effect of cholesterol on streptolysin O activity

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**SYNOPSIS** Inhibition of streptolysin O activity by cholesterol depends on the latter being in free form. The normal esterified and protein-bound cholesterol fractions in serum do not influence streptolysin O activity. However, high cholesterol levels in rabbits fed cholesterol with cholic acid were associated with an increased antistreptolysin O effect. It is suggested that this occurs when all available protein sites are saturated and where a true 'free' cholesterol fraction is present.

Splitting the esterified cholesterol fraction of human sera with raised cholesterol levels, by means of pancreatin, produced an increased antistreptolysin O effect, again presumably because of saturation of protein-binding sites. Similarly, removal of non-esterified cholesterol from sera of cholesterol-fed rabbits, by means of digitonin, reduced antistreptolysin O activity of the sera.

Evidence is presented that combination of bovine serum albumin and streptolysin O has a steric hindrance effect on attachment of cholesterol to streptolysin O.

The method described for the estimation of free cholesterol is extremely sensitive, being capable of detecting concentrations of less than 1.0 µg/ml.

Confirmation of group A streptococcal infection by estimation of antistreptolysin activity remains one of the most useful of serological tests in terms of reproducibility, clarity of end-point, high percentage of positive results, and relative absence of non-specific reactions. Non-specific positive reactions are known to be obtained with sera contaminated with bacteria. Strains of the genus *Pseudomonas* appear to be especially involved (Watson, Rose, and Kerr, 1971). Additionally it is known that cholesterol can inhibit reduced streptolysin 'O' (SLO) activity (Hewitt and Todd, 1939). The mechanism of the latter has been studied by Alouf and Raynaud (1968), who have shown that cholesterol acts by combining with the reduced SLO and prevents its attachment to cell membranes. Haemolysis by saponins is also inhibited by cholesterol but the mechanism of action appears different.

The present investigation was undertaken to determine the effect of variations in serum cholesterol levels on streptolysin O activity and also some of the factors influencing cholesterol inhibition of streptolysin O.

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## Materials and Methods

### ANTISTREPTOLYSIN ESTIMATIONS

These were done by the standard method of Rantz and Randall (1945). Briefly, heat inactivated serum (56° for 30 minutes) is diluted in phosphate-buffered saline (pH 6.7) from three master dilutions (1/10, 1/100, and 1/500). To each 1.0 ml volume of serum dilution in 3 in. × ½ in. tubes is added 0.5 ml of reduced streptolysin O (containing 1.0 IU). Tubes are incubated at 37° for 15 minutes followed by the addition of 0.5 ml of a 5.0% suspension of washed human red cells. Tubes are then reincubated for a further 45 minutes at 37°. After centrifugation of the tubes the percentage haemolysis in the supernatant is estimated visually. Suitable streptolysin O and standard antistreptolysin serum controls are included. The 50% haemolytic end points are expressed as Todd units, representing the reciprocals of the serum dilutions.

### CHOLESTEROL SUSPENSIONS

Pure cholesterol powder was dissolved in absolute ethanol at 1.0% concentration. Further dilutions

were then made in antistreptolysin O phosphate buffer (pH 6.7). With some batches of cholesterol this procedure resulted in a stable colloidal suspension presenting maximum surface area of cholesterol to the streptolysin O. However, with some batches dilution in buffer produced a floccular precipitate with much reduced activity on streptolysin O. In this instance stable colloidal suspensions were obtained by the addition of 0.01 ml of Tween 80 to 5.0 ml volumes of the cholesterol suspension in buffer. At this concentration Tween 80 did not affect either the erythrocyte or the streptolysin O.

#### CHOLESTEROL-TREATED SERA

Many of the experiments performed were done with a single batch of sterile horse serum, stored within a few hours of collection in 10.0 ml aliquots at  $-20^{\circ}$ . Preliminary tests showed that it had a very low titre of antistreptolysin O activity due to antibody ( $< 125$ ). Cholesterol suspensions in buffer were added as described under Results.

#### BOVINE SERUM ALBUMIN

This was supplied as a 30% suspension (Sigma Chemicals). Suitable dilutions were prepared and treated with cholesterol as described under Results.

#### SERA WITH RAISED CHOLESTEROL LEVELS

Human sera with high total cholesterol levels were obtained from patients with essential hypercholesterolaemia and with the nephrotic syndrome. However, great difficulty was experienced in obtaining sera with levels in excess of 450 mg%. To overcome this, rabbits were fed a cholesterol-rich diet by simply adding cholesterol powder to solid feed along with powdered cholic acid. After some three weeks venous blood was collected from the ear veins. Cholesterol levels were measured by the method of Levine and Zak (1964).

#### PANCREATIN SOLUTION

Pancreatin was used to split the esterified cholesterol fraction of serum. Solutions were prepared at 10% concentration in 2N sodium carbonate by gentle heating to  $56^{\circ}\text{C}$ .

#### DIGITONIN SOLUTION

Digitonin powder (Analar grade BDH) was dissolved in 50% ethyl alcohol. Since digitonin is haemolytic preliminary experiments were done to find a suitable concentration which would precipitate the protein-bound non-esterified cholesterol fraction of serum without causing haemolysis of erythrocytes added subsequently. This corresponded to 0.08 ml of a 2.5% solution in 50% alcohol added to 2.0 ml volumes of serum together with 0.01 ml of 10% acetic acid.

## Results

#### EFFECT OF PHYSICAL STATE OF CHOLESTEROL

Two preparations of cholesterol were used. The first was prepared by dissolving 50 mg in 10 ml of absolute ethanol followed by dilution in buffer with Tween 80 as described. The second was made from the same batch of cholesterol with 50 mg added to 10 ml of buffer and shaken vigorously to obtain maximal dispersion. Serial dilutions in buffer were made in the same way. In these latter tubes most of the cholesterol remained on the surface leaving a relatively clear layer beneath. Streptolysin O and red cells were then added as described. The 50% haemolytic endpoints are shown in Table I.

Concentration of Cholesterol ( $\mu\text{g/ml}$ )	Percentage Haemolysis	
	Colloidal Suspension of Cholesterol	Flocculated Suspension of Cholesterol
125	0	0
62.5	0	25
31.25	0	40
15.62	0	70
7.81	0	80
3.9	10	90
1.95	20	100
0.97	35	100
0.48	55	100
0.24	80	100
Nil	100	100

Table I *Effect of physical state of cholesterol on streptolysin O activity*

The results show that free cholesterol in dispersed colloidal form could be detected by its antistreptolysin effect at a concentration of approximately  $0.4 \mu\text{g/ml}$ . A series of such investigations consistently gave values for 50% haemolysis corresponding to cholesterol levels of between 0.4 and  $1.0 \mu\text{g/ml}$ . This compares with a level of about 25 to  $30 \mu\text{g/ml}$  where the cholesterol was in a less dispersed physical state. In these studies it was essential to use separate pipettes for each dilution. Where the same pipette was used a marked 'tailing' effect on the 50% endpoint was observed due to the tendency of the cholesterol to adhere to the sides of the pipette.

#### EFFECT OF HIGH SERUM CHOLESTEROL LEVELS ON STREPTOLYSIN O ACTIVITY

Antistreptolysin activity was measured as described in a number of human and rabbit sera with raised total cholesterol levels. Results are shown in Table II.

In none of the human sera tested was there a rise of antistreptolysin O activity associated with raised total cholesterol levels at least as far as 450 mg.

Sera	Total Cholesterol Level (mg%)	Serum Antistreptolysin Level (Todd units)
1 Human	334	< 125
2 Human	336	< 125
3 Human	344	166
4 Human	400	< 125
5 Human	450	< 125
6 Human	450	125
7a Rabbit	40	< 125
7b Rabbit	90	< 125
8a Rabbit	45	< 125
8b Rabbit	215	500
9a Rabbit	40	< 125
9b Rabbit	390	2500
10a Rabbit	30	< 125
10b Rabbit	175	833

Table II Serum cholesterol and antistreptolysin activity

7a, 8a, 9a, 10a: before cholesterol diet.  
7b, 8b, 9b, 10b: after cholesterol diet.

However, three of the four rabbits fed cholesterol had significantly raised antistreptolysin activity. The mean serum cholesterol level in the normal rabbit is about  $45 \pm 18$  mg% as compared with  $181 \pm 22$  mg% in human sera. In addition about 50% of rabbit serum cholesterol is esterified as compared with about 33% in the human. The remaining fraction, often referred to as 'free' cholesterol, is in fact protein bound, mainly to lipoprotein moieties. Neither the esterified nor protein-bound fractions of normal sera are available to interfere with streptolysin O activity and raising total cholesterol in human serum two to three fold does not produce an antistreptolysin effect, presumably because the additional cholesterol is protein bound or esterified. Where the total cholesterol is raised fivefold or greater, as in the case of the rabbit sera in Table II, an antistreptolysin effect is apparent. In view of the high sensitivity of the method in detecting free cholesterol (between 0.4 and 1.0  $\mu\text{g}/\text{ml}$ ) the findings suggest that there is a limit to the amount of cholesterol which can be esterified or will saturate lipoprotein binding sites and that beyond this some of the cholesterol exists in fact in true 'free' form.

#### EFFECT OF ADDING CHOLESTEROL TO NORMAL SERUM

Alcoholic cholesterol solutions containing varying amounts of cholesterol were added in 0.1 ml amounts to 1.0 ml volumes of sterile horse serum. The initial cholesterol level of the serum was 120 mg%. Antistreptolysin levels were then determined as before. Results are shown in Table III.

These results show that up to 400  $\mu\text{g}$  of cholesterol could be added to 1.0 ml of horse serum without any antistreptolysin effect becoming manifest. This is

Cholesterol Added to 1.0 ml of Serum <sup>1</sup> ( $\mu\text{g}$ )	Antistreptolysin Activity (Todd units)
1000	1280
700	833
600	500
400	< 125
200	< 125
100	< 125
1	< 125

Table III Addition of cholesterol to normal horse serum

<sup>1</sup>Serum cholesterol level—120 mg%.

approximately a 40% increase in the total amount of cholesterol. Beyond this level additional cholesterol can no longer be bound and is detectable by its antistreptolysin effect.

#### EFFECT OF PANCREATIN-TREATED SERA ON ANTISTREPTOLYSIN LEVELS

Pancreatin splits the ester linkage of bound cholesterol. It was therefore anticipated that sera with raised cholesterol levels, but which did not exhibit an increased antistreptolysin O effect, might do so if the ester moiety were split and if the cholesterol so released led to saturation of the lipoprotein fraction, which already contained an excess of cholesterol.

Pancreatin was dissolved in sodium carbonate as described. It was then added in 0.06 ml amounts to replicate tubes containing 1.0 ml amounts of human sera. Tubes were then incubated at 56° for three hours. Serial dilutions were made, streptolysin O and cells were then added as before and the antistreptolysin effect was measured. The sera used in these experiments were the human sera with raised total cholesterol levels shown in Table II. Suitable control tubes with pancreatin and sodium carbonate were included. Results are shown in Table IV.

Sera	Cholesterol Level (mg%)	Antistreptolysin Activity (Todd units)	
		Before Pancreatin	After Pancreatin
1	450	125	833
2	336	< 125	833
3	334	< 125	500
4	344	166	500
5	450	< 125	500
6	450	125	500

Table IV Effect of pancreatin on antistreptolysin levels of human sera

Table IV shows that after pancreatin treatment of the sera there was a marked rise in antistreptolysin O activity. No diminution of streptolysin O activity was noted with either sodium carbonate or pan-

creatin and the antistreptolysin O effect appears to be due to the liberation of free cholesterol, unable to be bound by lipoproteins.

#### REMOVAL OF PROTEIN-BOUND CHOLESTEROL WITH DIGITONIN

Digitonin solution was added to pools of sera from rabbits fed cholesterol (antistreptolysin O levels of 2500), as described under Methods. After mixing gently the tubes were kept at 4.0° for 72 hours. After centrifuging the supernatant fluids were removed from any precipitate which formed. The alcohol was then driven off by gentle heat at 55° in a water bath under vacuum. Streptolysin O and cells were added in the usual way and the antistreptolysin O activity remaining was determined. Results are detailed in Table V.

Pooled Sera	Cholesterol Level (mg%)	Antistreptolysin Titre	
		Before Digitonin	After Digitonin
1	390	2500	125
2	215	2500	125

Table V *Antistreptolysin levels of rabbit sera after removal of non-esterified cholesterol with digitonin*

Table V indicates clearly that removal of the non-esterified cholesterol fraction abolished the anti-streptolysin activity of the sera. Since these pooled sera had raised antistreptolysin levels (2500) due to cholesterol, it is likely that the non-esterified fraction consists of lipoprotein-bound cholesterol and a true 'free' cholesterol unable to bind because of saturation of lipoprotein sites.

#### EFFECT OF BOVINE ALBUMIN ON INTERACTION OF CHOLESTEROL WITH STREPTOLYSIN O

Lipoproteins constitute the main transporters of non-esterified cholesterol in serum. Specifically, serum albumin does not appear to play any part in cholesterol binding as shown by conventional chemical methods. However, in view of the sensitivity of the present method in detecting free cholesterol experiments were carried out to determine the effect of adding serum albumin to cholesterol and streptolysin.

Cholesterol in 1.0% alcoholic solution containing 6000 µg/ml was added in 0.4 ml (2400 µg) amounts to 0.6 ml volumes of varying concentrations of bovine serum albumin. After standing at room temperature for two hours, serial dilutions were prepared. Streptolysin was added and antistreptolysin activity detected as before.

Results are shown in Figure 1.

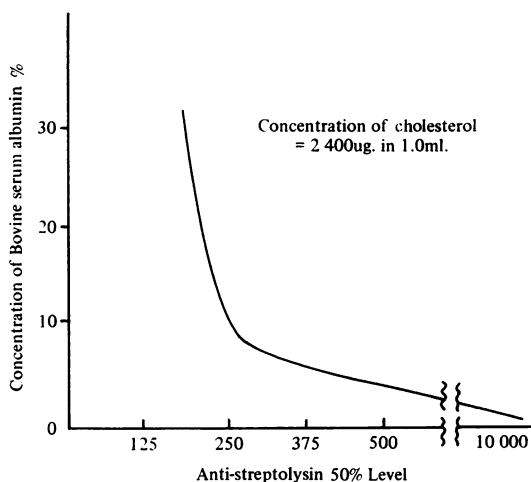


Fig. *Effect of bovine albumin on interaction of cholesterol with streptolysin O.*

The Figure shows that increasing the concentration of bovine serum albumin reduced the anti-streptolysin activity of the cholesterol. This was further investigated by varying the method of addition of the reagents as follows. An alcoholic solution of cholesterol containing 60 µg/ml was diluted in ASO buffer in 1.1 ml amounts in 3 in. × ½ in. tubes (dilution series, —12, 50, 100, 125, 166, 250, and 333). Four replicate sets of tubes were prepared. To each tube of set 1 was added 0.25 ml of a 4.0 unit/ml solution of streptolysin O and after 10 minutes at room temperature a further 0.25 ml of buffer solution was added. To set 2 was added 0.25 ml of 5.0% bovine serum albumin and after 10 minutes at room temperature 0.25 ml of streptolysin O. Set 3 was similar to set 2 except that the streptolysin O was added first and the bovine serum albumin solution after 10 minutes. To set 4 was added 0.5 ml of an aliquot mixture of streptolysin O and bovine serum albumin mixed immediately before addition to the cholesterol. All tubes were then incubated for a further 10 minutes at 37°. Cells were then added and 50% haemolytic endpoints noted as before.

Identical endpoints were obtained for each set of tubes containing bovine serum albumin, ie, the sequence of addition of streptolysin O and bovine serum albumin did not affect the reduced ability of the cholesterol to inhibit the streptolysin O in the presence of bovine serum albumin.

There appear to be four possible reasons for the albumin effect. (1) Albumin might combine with cholesterol and prevent it from combining with streptolysin O. However, when <sup>14</sup>C labelled cholesterol

was added to the serum albumin used in the present experiments and then subjected to electrophoresis on cellulose acetate all radioactivity was located at the point of origin and none was present in the albumin region. (2) Albumin might combine directly with the erythrocyte membrane and increase its sensitivity to the effect of streptolysin O. However, treatment of red cells with varying concentrations of albumin from 1.0% to 30.0% for periods up to 18 hours did not alter the 50% haemolytic dose of streptolysin O. (3) Albumin might combine with streptolysin O and increase its haemolytic activity, but again concentrations of albumin from 1.0% to 30.0% added to streptolysin O did not affect 50% haemolytic endpoints. (4) Albumin may combine with streptolysin O by some form of protein-protein interaction, possibly involving Van der Waal's forces, at a site on the streptolysin O molecule distant from either the site for fixation to the erythrocyte or the additional site shown to be responsible for haemolysis. Such combination might then exert a steric hindrance effect on the attachment of cholesterol to the membrane fixation site of the streptolysin O molecule. This last hypothesis is supported by the results obtained by adding varying concentrations of bovine serum albumin to a standard amount of streptolysin O (40 I-U/ml). Albumin itself moved with a mobility almost identical to that of streptolysin O. The mobility of the streptolysin O progressively decreased with increasing concentrations of bovine serum albumin, indicating complex formation.

#### EFFECT OF PANCREATIN ON ANTISTREPTOLYSIN ACTIVITY OF BOVINE SERUM ALBUMIN-CHOLESTEROL MIXTURES

A series of Universal containers was prepared containing reagents as set out in the left hand portion of Table VI. They were then incubated in a water bath at 56° for three hours. Serial dilutions in buffer were then made in 1.0 ml amounts in 3 in. × ½ in. tubes for each container. Streptolysin O and red cells were added and the tubes incubated as before.

The 50% haemolytic endpoints are shown in the right hand side of the table.

Neither pancreatin nor bovine serum albumin affected streptolysin O activity at the concentrations used. However, the interaction between bovine serum albumin and streptolysin O is prevented by pancreatin allowing cholesterol to exert its anti-streptolysin effect on the streptolysin O.

#### Discussion

Streptolysin is capable of attaching to lipoprotein membranes of erythrocytes and other cells including leucocytes, macrophages, heart muscle cells, and chick embryo cells. In this respect it resembles other oxygen labile haemolysins, eg, pneumolysin, tetanolysin, and listeriolysin. Reduced streptolysin O has been shown by Alouf and Raynaud (1968) to possess two biologically active sites: the (f) or fixation site attaches the streptolysin O molecule to cholesterol in the external lipoprotein layer of the erythrocyte membrane. Oxidized streptolysin O fails to attach. Binding appears to be thiol dependent and the disulphide bonds so formed are readily disrupted by sulphhydryl agents. The second site (t) is concerned with disruption of the cell membrane and release of haemoglobin. Whereas the binding stage is rapid and temperature independent, lysis is much slower and temperature dependent, being maximal at 37.0°C and almost absent at 4.0°C. Approximately 360 molecules of streptolysin O are required to lyse a single cell. The (f) site which appears to comprise two cysteine residues is blocked by oxidation, thiol-binding agents, and cholesterol. However cholesterol does not affect a stromal-bound streptolysin O. The activity of cholesterol appears to be dependent on possession of the beta-hydroxyl group at position 3 of the A ring and a hydrophobic side chain attached to position 17. The structural requirements of this side chain are at present under investigation in this laboratory.

Petersen, Nowak, Thiele, and Urbaschek (1966)

Container	Reactants (ml)				Antistreptolysin (50%) (Todd units)
	Bovine Serum Albumin (30%)	Cholesterol (20 mg/ml in alcohol)	Buffer	Pancreatin (10% in 2N Na <sub>2</sub> CO <sub>3</sub> )	
1	5.0	0.1	0.2	—	Serial dilution in buffer after incubation Streptolysin O and erythrocytes added
2	—	0.1	5.2	—	
3	5.0	—	0.3	—	
4	5.0	0.1	—	0.2	
5	—	0.1	5.0	0.2	
6	5.0	—	0.1	0.2	
7	—	—	5.1	0.2	

Table VI Effect of pancreatin on antistreptolysin activity of bovine serum albumin-cholesterol mixtures

<sup>1</sup>H = Complete haemolysis in all tubes.

have shown that delipidation of erythrocytes renders them unable to fix streptolysin O and the lipid extracts are strongly inhibitory. Kavanau (1965) has also shown that cholesterol is a constituent of cell membranes other than erythrocytes but it is not clear whether the mechanism of combination with streptolysin O is identical to that of erythrocytes.

The present results confirm the extreme degree of sensitivity of reduced streptolysin O to the inhibiting effect of free cholesterol, the method being capable of detecting less than 1000 ng/ml. They also show that raised levels of cholesterol in human sera were not associated with raised antistreptolysin O activity of the sera, at least up to 450 mg%, approximately two and a half times normal levels. However, in rabbits with levels of cholesterol some five times normal, increased antistreptolysin O activity was demonstrated. It is suggestive that this represents a cholesterol serum fraction which is neither esterified nor combined to lipoproteins, possibly because of saturation of binding sites on the latter. These findings are similar to those of Stollerman (1954) who also showed that addition of heparin to serum from cholesterol-fed rabbits increased the antistreptolysin O effect. Horse serum appears less capable of binding added cholesterol since increased antistreptolysin O activity was noted when the concentration was increased from the base level of 1200  $\mu\text{g/ml}$  to 1800  $\mu\text{g/ml}$  a rise of only 50%.

Treatment of human sera with raised total cholesterol levels with pancreatin released the esterified fraction resulting in a total concentration of non-esterified cholesterol sufficiently high to saturate lipoprotein-binding sites and leave 'free' cholesterol which showed an antistreptolysin O effect. Lecithinase derived from *Cl. welchii* has a similar effect (Stollerman, 1954). Conversely, removal of non-esterified cholesterol with digitonin before treatment with pancreatin did not increase antistreptolysin O activity of the serum since the cholesterol freed by splitting the ester linkage is presumably able to attach to available lipoprotein sites.

In the case of rabbits with raised antistreptolysin O activity and high serum cholesterol levels precipitation of the non-esterified fraction with digitonin reduced antistreptolysin O activity, again due to removal of what would appear to be a true 'free' cholesterol moiety. Badin and Barillec (1970) noted a similar effect of digitonin after removal of excess digitonin with transdehydroandrosterone.

The ability of bovine serum albumin to diminish the inhibiting effect of cholesterol is of interest. At first sight it was thought possible that a weak combination of bovine serum albumin with cholesterol might be taking place and was being detected in view

of the high sensitivity of the method. However, the failure of  $^{14}\text{C}$ -labelled cholesterol to move electrophoretically with bovine serum albumin in mixtures discounts this possibility, and evidence of complexing of bovine serum albumin and streptolysin O was obtained from cellulose acetate electrophoresis of mixtures of these. As shown in Table VI complexing was prevented by the addition of pancreatin to bovine serum albumin before addition of streptolysin O. As already mentioned it appears likely that the combination of bovine serum albumin and streptolysin O sterically prevents attachment of cholesterol to the latter.

The effect of bacterial contamination of sera in producing false high levels of antistreptolysin O activity has not been fully investigated. Preliminary studies in this laboratory suggest that two mechanisms might be involved, either a direct proteolytic effect on streptolysin O or release of enzymes capable of splitting the esterified cholesterol fraction and raising the non-esterified fraction to a level higher than is capable of being bound to lipoprotein sites. The latter mechanism is undoubtedly involved as far as *Cl. welchii* is concerned. Early studies suggest that *Pseudomonas* strains are especially capable of producing this effect but the mechanism is not clear.

False positive antistreptolysin results are not likely to be a problem with most human sera with raised cholesterol values. However it is known that high antistreptolysin values may be found in cholestatic liver disease (Badin, Cabau, Lévy, and Cachin, 1962) and nephrosis (Stollerman, 1954). Badin and Barillec (1970) have shown that these may be distinguished from true antibody-mediated antistreptolysin activity since digitonin will remove that activity in the former case but has no effect on antistreptolysin levels due to immunoglobulins.

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