Interaction of Streptolysin-O with Natural and Artificial Membranes *

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1. Kinetic studies on the binding of ^{125}I-Streptolysin-O exhibited immediate fixation of activated toxin to natural and artificial membranes. Once fixed to the membrane no release of Streptolysin-O or Streptolysin-O-lipid-complexes has been observed.

2. In contrast to activated toxin (free SH-groups!), oxidized Streptolysin-O was shown to become also fixed to membranes, however, with different binding kinetics. The binding of oxidized material was clearly dependent on temperature and time. When the toxin was oxidized twice the amount of labelled material was bound as compared with the hemolytically active Streptolysin-O. This suggests that oxidized Streptolysin-O, too, possesses a "binding site" within the molecule, though free SH-groups were expected to be essential for toxin fixation at the membrane. It has been shown that oxidized (inactive) and reduced (active) Streptolysin-O forms stable "complexes" with liposomes in aqueous solution, which could be separated by chromatography on Sepharose gels.

3. The binding of ^{125}I-toxin to membranes and liposomes was specific since specific antisera against Streptolysin-O inhibited fixation of toxin completely.

4. Hydrolysis of phospholipids and release of lysophosphatides by Streptolysin-O esterase (EC 2.1.1.2) has not been observed, thus providing no evidence for an enzymatic concept of membrane damage.

Introduction

Streptococci of Group A, C and G strains produce cytolytic active substances which catalyse hemolysis and cytolysis of a broad variety of mammalian cells [1–3].

The pantropic cytolysin of β-hemolytic Streptococci, "Streptolysin-O", is characterized by its instability to oxygen [4]. Streptolysin-O is chemically and antigenically related to the oxygen labile, pantropic cytolysin of *D. pneumoniae, B. cereus, L. monocytogenes, C. perfringens and C. tetani* [5]. Besides the toxin of *C. perfringens* [6, 7] mainly Streptolysin-O was used as a "model substance" in studying the lytic mechanism of the pantropic, SH-dependent hemolysins [6, 8–11].

Although considerable progress has been made in the purification of Streptolysin-O [12–14], a molecular homogeneous product is not yet available. Recent work has shown that Streptolysin-O, Group C material, purified either by column chromatography [12] or by isoelectric focusing [15, 16] still contained esterase activity, generating hydrolysis of 4-nitrophenylesters [17].

The toxin is known to interact with cholesterol in membranes, though, the mode of action leading to cell lysis is still obscure. Recent studies suggested on the grounds of electron microscopic data [7, 18, 19] that Streptolysin-O produced membrane alterations only in membranes containing cholesterol. It has been shown [7, 18, 19] that formation of "ring"- or "C"-shaped structures occured in natural and artificial membranes treated with Streptolysin-O.

Since the value of electron microscopy in the elucidation of dynamic processes is limited it was the aim of this work to study the kinetics of toxin fixation as well as a possible release of membrane/toxin complexes during membrane damage.

Although it appears that an enzymatic concept of hemolysis [20, 21] is not supported by recent studies [19] it seemed essential to investigate whether the esterase associated with [15–17] Streptolysin-O catalyses the

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Abbreviations: SLO, Streptolysin-O; RBC, red blood cells; SRBC, sheep red blood cells; HU, hemolytic units; TLC, thin layer chromatography.

Enzymes: NAD-glycohydrolase, NADase, (EC 3.2.2.5); Streptolysin-O-esterase, arcl-ester hydrolase (EC 3.1.1.2).

breakdown of phospholipids by formation of lysophospholipids, also in the lights of recent work on different bacterial phospholipases [7, 22–27]. Accumulation of lysophosphatides in the erythrocyte membrane is known to induce hemolysis [28–31].

Moreover, besides an enzymatic concept of cytolysis surface activity of certain polypeptides [32] may play an important role in cytolysis. Thus it was of interest to obtain a more detailed knowledge on the cytolytic action of Streptolysin-O especially in respect to a possible "enzymatic"- or "detergent"-effect of this pantropic cytolysin.

Material and Methods

Palmitoyl-propandiol-(1.3)-phosphorylcholine was prepared as described earlier [33].

Purification of SLO

Purification of Streptolysin-O *, from Group C Streptococcus (H 46 A) was achieved by isoelectric focusing, according to the procedure of Smyth and Fehrenbach [15]. SLO, twice purified by isoelectric focusing, major form [15], pool fractions pI = 5.5 – 6.5, had a spec.act. of 1.8×10^3 HU/mg prot. The preparation possessed esterase activity, 3.2×10^2 U/mg prot. [17], and NAD-glycohydrolase activity 1.9×10^2 U/mg prot. The enzyme activities of NADase and esterase were measured according to Kaplan, Colowick and Ciotti [34] and Fehrenbach [17] respectively.

Estimation of hemolytic activity

Hemolytic activity of SLO was estimated by the method of Alouf and Raynaud [10] using one day old sheep red blood cells. SLO was activated for 20 min at 20 °C by diluting the toxin with isotonic NaCl solution containing cysteine, 10^{-2} M. SLO was inactivated by dialysis for 4 h in 0.1 M Tris/HCl buffer, pH 8.0, containing 1 mM EDTA and 1 mM H_2O_2. Subsequently H_2O_2 was removed by dialysis against 0.1 M Tris/HCl, pH 8.0 containing 1 mM EDTA.

Protein estimation

Protein was estimated by the method of Folin and Ciocalteau [35], in the modification of Lowry [36].

Labelling of SLO

Purified SLO was labelled according to the procedure of McConahey and Dixon [37], with the following modifications. The protein was thoroughly dialysed before labelling in 0.1 M phosphate buffer (pH 7.0), 1 mM in H_2O_2. Oxidation of SH-groups remarkably increased iodination, but reduced the hemolytic activity of SLO after reactivation by about 50%. The reaction mixture, total vol. 5.8 ml, contained 5.4 ml of SLO (1.3 – 1.9 mg prot./ml), 0.1 ml of Na ^{125}I solution (≙ 1 m Ci), 0.1 ml Chloramin-T (2.5 mg/ml) and was incubated at 4 °C for 10 min. The reaction was stopped by the addition of 0.2 ml of $Na_2S_2O_5$ solution (2.5 mg/ml). Separation of labelled protein from free iodate was achieved by chromatography on Sephadex G-25 (column: 1 × 15 cm) in the appropriate buffer. The specific radioactivity of the labelled material varied from $0.5 - 1.0 \times 10^5$ cpm/μg prot.

Preparation of liposomes

Liposomes were prepared by sonication of a lipid suspension in the appropriate buffer in a 1.5 ml water-jacketed vessel, kept at 39 °C.

Pure lipids or lipid mixtures, containing $1 \times 10^{-1} - 1 \times 10^{-4}$ mol phospholipid/ml, estimated by determining inorganic phosphate, (see lipid analysis), were dissolved in chloroform/methanol, 9 : 1 (v/v) and transfered to the sonication vessel. The organic solvent was subsequently removed by drying the lipids under reduced pressure. After the addition of 1.0 ml of buffer the suspension was sonicated for 10 – 20 min, using a Branson Sonifer, B 12, micro tip, at an energy of 80 W.

Lipid extraction of erythrocytes

RBC were separated from 300 ml of blood. The cells were suspended in 10 ml of isotonic sodium chloride solution and extracted by the addition of 12 ml methanol and 10 ml chloroform. The mixture was shaken and the lower phase was collected. The extraction was repeated with 10 ml chloroform. The combined chloroform extracts were filtered through glass wool and taken to dryness in a rotatory evaporator. The lipids were dissolved in a mixture of chloroform/methanol 9 : 1 (v/v) and stored under nitrogen at 4 °C. Erythrocyte ghosts were prepared from RBC according to Maddy [38].

Lipid analysis

Phospholipids were separated on microslides according to Peifer [39]. The plates were sprayed with sulphuric acid, 7.2 N, heated to 300 °C. The dark spots on the plates were scraped off into test tubes and treated with 0.5 ml of 7.2 N sulphuric acid at 300 °C to convert organic phosphate esters into free

* Streptolysin-O, crude material, was a kind gift of Behringwerke, Marburg/Lahn.

phosphate [40]. Distilled water, 5.5 ml, were added to the test tubes and the precipitated silica gel was removed by centrifugation. The clear supernatant was used to determine phosphate by adding 0.5 ml of a mixture of ammonium molybdate and Triton-X-100 [41]. The phosphate values were corrected for blanks with silica gel only. Alternatively, dilutions of the extracted phospholipids were transfered to test tubes. The organic solvent was removed by evaporation with N_2. Organic bound phosphate was converted to inorganic phosphate by treatment with 7.2 N sulfuric acid at 300 °C. Free inorganic phosphate and esterified fatty acids were determined as described by Eibl and Lands [40].

Detergent effect of Streptolysin-O

Sonicated erythrocyte membranes (180 nmol of phosphate/ml) were treated with increasing amounts of SLO, 10 – 100 nM, (calculated from mol.wt. 55000 for Streptolysin-O) to investigate a possible detergent activity of the cytolysin.

Reduced and oxidized SLO, obtained by dialysis against 0.1 M Tris/HCl buffer, pH 8.0, containing either 1 mM of cysteine or 1 mM of H_2O_2, were used in order to study hemolytically active and inactive toxin.

These experiments were compared with those, where sodium dodecylsulphate and palmitoyl-propandiol-(1.3)-phosphorylcholine (palmitoyl-2-desoxy-lysolecithin), 0.1 – 1.0 mM, were added to the membranes. Finally, ghosts were pretreated with increasing amounts of active SLO and the sodium dodecylsulfate added to demonstrate a possible solubilization effect of the cytolysin.

Binding of [^{125}I]SLO to membranes and liposomes

SRBC used for binding experiments were washed 3 times in isotonic NaCl- or a suitable buffer solution. The cell number of a given SRBC suspension was estimated photometrically according to Alouf and Raynaud [10], assuming that the standardized erythrocyte suspension contained on the average 3.0×10^8 erythrocytes/ml.

For binding studies a reaction vessel, 6.0 ml total vol., equipped with a magnetic stirrer, kept at constant temperature, was used. At zero time 2.0 ml of SRBC, 6.1×10^8 cells/ml in isotonic NaCl solution, were mixed with 2.0 ml of an isotonic NaCl-solution of [^{125}I]SLO, 16.0 HU/ml, 10.3 μg prot./ml, 1.0×10^5 cpm/μg prot. At intervals 0.2 ml volumes of the mixture were withdrawn, added to 1.0 ml of an ice cold isotonic NaCl solution and centrifuged immediately in the cold for 10 min at $10000 \times g$ in an Eppendorf Type 3200 centrifuge. The supernatant was discarded and the pellet washed twice. SRBC or the ghosts were solubilized by the addition of 1.0 ml SDS, 10 mg/ml, and transfered to a Packard Auto-Gamma counter, Type 5120.

Binding assays at different temperatures ranging from 4 – 20 °C with labelled, oxidized or reduced SLO were performed by incubating 3.0 ml SLO, (16.0 HU/ml; 10.3 μg prot./ml; 1.0×10^5 cpm/μg prot.) with 3.0 ml of SRBC (24.4×10^8 cells/ml, in isotonic solution) ranging from 4 – 20 °C. SRBC were removed from the incubation mixture after the appropriate time intervals by centrifugation, solubilized after three repeated washings and measured for radioactivity.

Alternatively for binding studies with liposomes 0.2 ml of [^{125}I]SLO, 16.0 HU/ml, 10.3 μg prot./ml, 1.0×10^5 cpm/μg prot., were mixed with 0.2 ml isotonic NaCl solution and 0.2 ml of the liposome suspension. The liposome suspension was standardized by measuring the concentration of phospholipids, based on inorganic phosphate determination, according to the method of Eibl and Lands [40]. In terms of inorganic phosphate, total lipid concentration in the suspension varied from $10^{-1} - 10^{-4}$ mol phospholipid/ml. Serial assays and controls, where the lipid was omitted, were performed and the binding of toxin to liposomes was demonstrated by separation of labelled lipid protein complex from free [^{125}I]toxin by gel filtration on Sepharose 4B [42], (column: 1×20 cm; 3.5 ml/h). Fractions of 2.0 ml were collected and measured for radioactivity. The phospholipid content was estimated after extraction with chloroform/methanol.

Inhibition of binding of Streptolysin-O by antibodies

Inhibition of binding of SLO – activity was studied by incubation of 0.7 ml of serial twofold dilutions of pooled human γ-globulin or antitetanolysin with 0.5 ml of an isotonic NaCl solution of labelled SLO (13.8 μg prot./ml = 8.8 HU/ml) at 37 °C for 30 min. The anti-SLO titer of the starting γ-globulin dilution was 1120 units/mg prot. *. In parallel 0.5 ml of iodinated SLO in isotonic NaCl solution (27.7 μg prot./ml = 17.7 HU/ml), were mixed with 0.7 ml of serial dilutions of anti-tetanolysin as mentioned above. The anti-SLO titer of the starting serum dilution of antitetanolysin was 1440 anti-SLO units/ml **.

The anti-Streptosylin-O determination was carried out with the micro anti-Streptolysin-O test [43].

* Pooled human γ-globulin, OP Nr. 3324, was kindly supplied by Behringwerke, Marburg/Lahn.

** Anti-tetanolysin was a kind gift of Commonwealth, State Laboratory, Mass. 02130.

Esterase activity of SLO and degradation of membrane lipids

Purified SLO from Group C-Streptococcus (H 46 A) has been shown to contain esterase activity [15–17]. A possible specificity of this enzyme for phospholipid substrates was therefore investigated by incubating the Streptolysin-O, with substrates, such as lecithin, cephalin and SRBC membranes. Lecithin and cephalin (6 μmol) were suspended in 1.8 ml of 0.1 M NaOH/glycin buffer, pH 7.3, 1 mM in $CaCl_2$ and $MgCl_2$. After the addition of 0.2 ml of SLO (1.8×10^3 U/ml esterase, spec.act. 1.4×10^3) or phospholipase A_2 (18 U/ml, spec.act. 9.0) the mixture was shaken for 4 hours at 34 °C. The lipids were extracted with chloroform/methanol and determined as mentioned above. When SRBC membranes were used, membrane suspension, 5 μmol of phospholipid were incubated with Streptolysin-O, (2.6×10^3 HU, spec.act. 1.5×10^3), in 0.1 M NaOH/glycine buffer, pH 7.3, 1 mM in cysteine in a final volume of 1 ml. The reaction was terminated after 3 hours shaking at 34 °C by the addition of chloroform/methanol. Furthermore the cholesterol-ester fatty acid fraction, (3 μmol of cholesterol)), was incubated with SLO, (1.3×10^3 HU; spec.act. 1.5×10^3), in 0.1 M NaOH/glycin, pH 7.3, 1 mM in cysteine, for 4 hours at 34 °C, in a final volume of 2 ml. The lipid extract was analysed for degradation product by thin-layer and gaschromatography.

Results and Discussion

Thin-layer chromatography of the total lipid extract from erythrocyte membranes resulted in the separation of 4 fractions, Fig. 1 a *. Organic bound phosphate was detected in 3 fractions by spraying with molybdate. The fractions were identified as sphingomyelin I, lecithin II, and cephalin III (molar ratio – 43 : 12 : 45) by respective reference compounds. Fraction IV was resolved by thin-layer chromatography into a cholesterol V and cholesterol-ester/fatty acid fraction VI, Fig. 1 b.

Since partially purified preparations of SLO [15–17] were shown to contain esterase activity, which hydrolyses 4-nitrophenyl-esters, it seemed plausible to assume that membrane lipids might serve as natural substrates for the esterase. However, as shown by the experiment, phospholipids were not generated by SLO-esterase, though, under identical conditions, phospholipase A_2 liberated free fatty acids from lecithin and cephalin with the formation of the respective lysophospholipids (Table I). This was also true when ghost membranes were incubated with the esterase, indicating that the natural arrangement of phospholipids in the membrane cannot provide the essential substrate properties for the esterase.

Table I. Comparison of hydrolytic activities of phospholipase A_2 and Streptolysin-O esterase with phospholipids as substrates (+ indicates hydrolysis).

Substrate	Phospholipase A_2	Streptolysin-O esterase
sphingomyelin	–	–
lecithin	+	–
cephalin	+	–
cholesterolester	–	–
lipid total extract	+	–
erythrocyte membranes	+	–

As demonstrated by thin-layer and quantitative gas chromatography no hydrolysis of cholesterol-esters was observed when incubated with SLO-esterase. Therefore, the experiments lend no support to an "enzymatic concept" of hemolysis proposed earlier [20, 21].

From studies with staphylococcus α-toxin Buckelew and Colaccico [32] have suggested that the polypeptide itself may exhibit surface active properties. Extending this hypothesis to Streptolysin-O, we found that this model is not suited for the toxin, so far investigated. As shown in Fig. 2, treatment of ghosts with both sodium dodecylsulphate and palmitoyl-propandiol-(1.3)-phosphorylcholine, 0.1 – 1.0 mM, decreased the extinction at 660 nm, thus indicating a solubilization of the membranes.

Neither the reduced (active) nor the oxidized (inactive) SLO showed comparable effects. Furthermore, no supporting solubilization effect has been detected when ghosts were treated with SLO prior to the addition of sodiumdodecylsulphate. As shown by the diagram the SLO concentration ranged from 10 – 100 nM. Calculated in terms of hemolytic units [10] the applied SLO concentration was sufficient to hemolyse 100 times more erythrocytes than used in the above experiment. Thus, the experiments lend no support to the view that the SLO-preparations may possess surface activity.

Kinetic experiments

Although the mechanism of toxin binding to biomembranes has been investigated in the past on an experimental [6, 10, 11] and theoretical level [6, 44], it

* Fig. 1 see Plate on page 104 a.

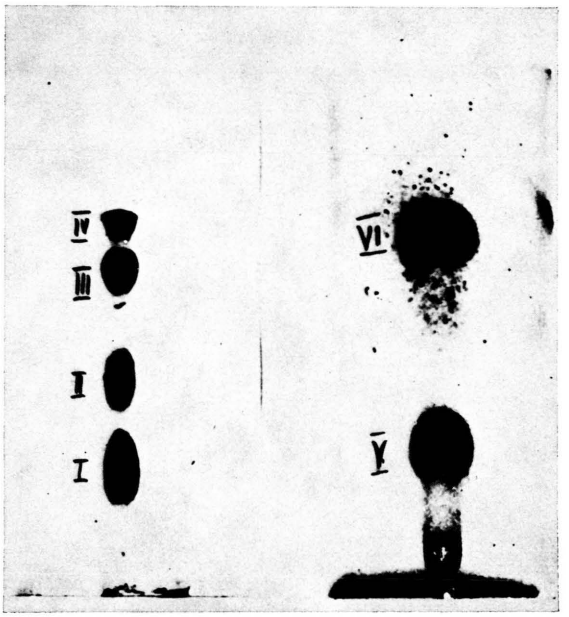

Fig. 1. a) Analysis of total lipid extract from ox erythrocyte membranes by thin layer chromatography; sphingomyelin (I), lecithin (II), cephalin (III) and cholesterol/cholesterolester/fatty acid fraction (IV). Solventsystem: $CHCl_3$/CH_3OH/HCOOH (10% in H_2O) 65:30:3 (v/v/v); amount of phospholipids applied = 15 nM PO_4^{2-}. b) Further separation of fraction (IV), 1 a into a cholesterol (V)- and cholesterolester/fatty acid (VI) fraction. Solventsystem: Diisopropylether/hexane 1:1 (v/v).

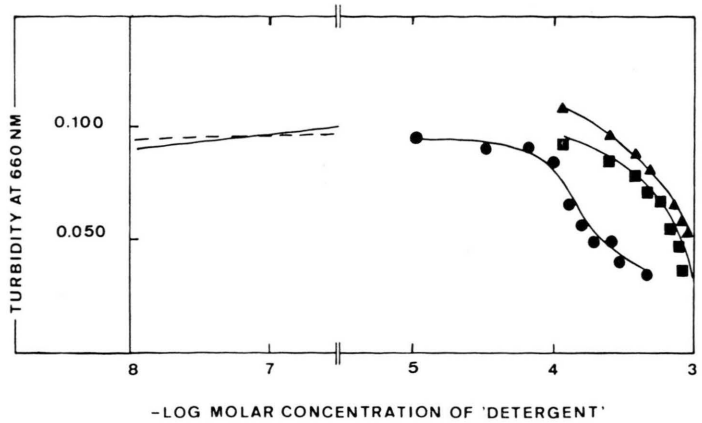

Fig. 2. Solubilization of membrane particles by Streptolysin-O, activated toxin (– – –), inactivated toxin (———), palmitoyl-propandiol-(1.3)-phosphoryl-choline (●–●–●), sodiumdodecylsulfate (■–■–■) and sodiumdodecylsulfate after pretreatment with Streptolysin-O (▲–▲–▲).

seemed essential to reinvestigate this process with artificial membranes in more detail. Incubation of SRBC with reduced [^{125}I]SLO at 20 °C resulted in prompt absorption of labelled material. Thus, as obvious from the curve, Fig. 3 a, equilibrium has been achieved in less than 1 min. Since the toxin concentration is highly lytic (16.0 HU/ml), hemolysis occurs immediately and release or additional

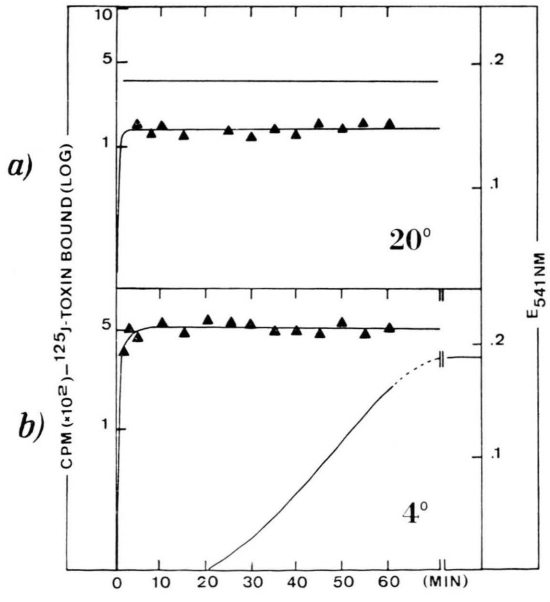

Fig. 3. a) Binding of activated [^{125}I]Streptolysin-O, cpm × 10^2 (▲–▲–▲) to SRBC in isotonic NaCl solution at 20 °C as a function of time. Hemolysis of SRBC was measured by the increase of extinction at $\lambda = 541$ nm (———). b) Binding of activated [^{125}I]Streptolysin-O, cpm × 10^2 (▲–▲–▲) to SRBC in isotonic NaCl solution, at 4 °C, as a function of time. Hemolysis (———), increase of extinction at $\lambda =$ 541 nm at 4 °C, starts first after 20 min. The curve shows that binding of radioactivity (▲–▲–▲) is irreversible, since the amount of toxin bound is uneffected by hemolysis.

binding of toxin during the lytic phase cannot be detected from the graph. Therefore, the kinetics of binding of SLO were reinvestigated at 4 °C. The diagram shows, Fig. 3 b, that an almost identical absorption behaviour was observed exhibiting again, prompt binding of SLO. It can be seen that hemolysis now starts after 20 min. This is expected, since earlier studies revealed a marked dependence of cytolysis on temperature [6, 10] and osmolarity [6, 21].

At 4 °C, binding and cytolysis are clearly separated into two distinct steps. Our result are therefore in agreement with those reported earlier by Alouf and Raynaud [10].

The diagram shows also that substantial loss of membrane bound [^{125}I]SLO with hemolysis is not observed. Although, under given conditions, the supernatant is still hemolytically active, additional binding of free SLO after the onset of hemolysis does not occur. This may indicate that the toxin has reached its partition equilibrium before lysis starts. The experiment suggests that membrane damage by SLO is not followed by the concomitant release of toxin or toxin-lipid-complexes from membrane structures. It is of interest that Staph. α-toxin exhibits a quite different and more complex binding behaviour [45, 46]. With the onset of hemolysis a considerable amount of α-toxin is released from the cells. However, in comparing the binding kinetics of SLO and α-toxin, the different mechanism of action as well as the different experimental conditions should be noticed [45].

Binding of reduced and oxidized [^{125}I]Streptolysin-O

Incubation of SRBC with reduced [^{125}I]SLO for 90 min at temperatures from 4 – 20 °C, resulted in

Table II. Binding of reduced and oxidized [^{125}I]Streptolysin (SLO) to SRBC at different temperatures (± indicates slight hemolysis).

Temperature [°C]	Reduced [^{125}I]SLO % of total radioactivity bound after 90 min	Lysis	Oxidized [^{125}I]SLO % of total radioactivity bound after 90 min	Lysis
4	9.3	−	12.6	−
10	7.3	+	14.4	±
20	8.0	+	13.6	±

the binding of $7.3-9.3\pm0.6\%$ of total radiactivity (Table II). With oxidized [^{125}I]SLO the values were found to be significantly higher ranging from $12.6-14.4\pm0.6\%$. Furthermore kinetic studies of binding of oxidized material revealed a considerable dependence on temperature and time. As compared with the binding of reduced SLO, the fixation rate of oxidized SLO becomes greater with increasing temperature, Fig. 4. Hence, for both, the

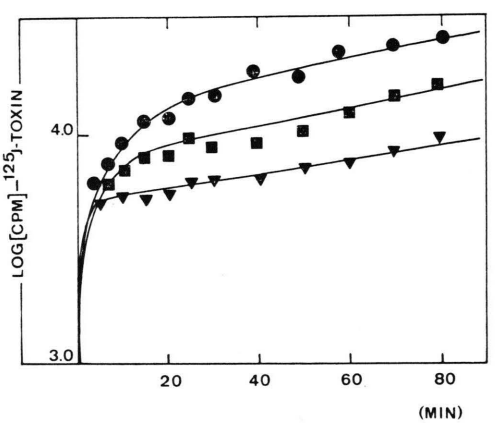

Fig. 4. Binding of oxidized Streptolysin-O, log cpm, to SRBC in isotonic NaCl solution, at 4 °C (▲-▲-▲), 10 °C (■-■-■) and at 20 °C (●-●-●) respectively as function of time. SLO applied: 16 HU/ml, 10.3 μg prot./ml, spec. radioactiv. 1.0×10^5 cqm/μg.

cytolytically active and inactive Streptolysin-O, binding and lysis were recognized as two different steps. One may object that binding of oxidized material involves primary reduction of inactive toxin at the surface of SRBC. However, further experiments revealed that oxidized toxin was also bound to liposomes, in which case one would not expect to get toxin activation. The binding behaviour of the reduced (active) toxin thus differs from the oxidized toxin in that it gains an immediate access to the membrane (Fig. 3), whereas the fixation of oxidized SLO is a much slower process (Fig. 4). On the other hand, the oxidized toxin-molecule, too, has a "binding region" whose function is retained also in the absence of free SH-groups. The results on the fixation of oxidized material are not necessarily conflicting with those of Alouf and Raynaud [10] and Prigent, Alouf, and Raynaud [11]. These authors used rabbit instead of sheep red blood cells and shorter incubating periods for binding assays with oxidized material.

It is well established that cholesterol and homologueous sterols inhibit the hemolytic activity of SLO and related SH-dependent cytolysins [47–50].

Therefore, liposomes prepared from total lipid extract of SRBC might serve as an ideal tool in studying the interaction of the toxin with lipids. When SRBC were mixed with SLO and liposomes, only $0.8\pm0.1\%$ of total radioactivity was bound to the erythrocytes (Table III). Since the amount

Table III. Binding of [^{125}I]Streptolysin to SRBC in the presence and the absence of liposomes. Competition of SRBC and liposomes for Streptolysin-O was demonstrated in a set of parallel assays by mixing 0.2 ml SRBC ($=1.2\times10^8$ cells/ml), 0.4 ml of a liposome suspension (0.4 ml ≙ 0.1 M phospholipid; total lipid extract from SRBC) and 0.2 ml SLO (16 HU/ml; 10.3 μg prot./ml; spec. radioactivity: 1.0×10^5 cpm/μg prot.) in that sequence.

Experiment No.	[^{125}I]Streptolysin-O bound [cbm] in the presence of liposomes	in the absence of liposomes
1	9634	98517
2	11509	87446
3	8693	93642
4	10313	102205
% of total radioactivity bound	$0.8\pm0.1\%$	$7.3\pm0.6\%$

of phospholipid in the liposomes, based on determination of phosphate [40], exceeded that of membrane phospholipid in SRBC by 10^3, complete inhibition of hemolysis has been observed. In contrast, in the absence of liposomes $7.3\pm0.6\%$ of total radioactivity were fixed to SRBC (Table III). The experiments revealed furthermore that inhibition of toxin fixation to SRBC of both, reduced and oxidized SLO (the latter being not included in the Table) has been observed in the presence of liposomes.

These findings suggest that liposomes, possibly by their cholesterol constituent, compete with SRBC for the toxin. Binding of [^{125}I]SLO to liposomes is therefore suggested, though no direct proof of binding can be obtained from the above experiments. One may infer, however, that liposomes interact with SRBC in such a way as to block the access of toxin to the membranes. The hypothesis [11], that the toxin first binds to a lipid carrier, *i.e.* lipid aggregates or liposomes, and subsequently attaches to the membranes as a complex is not supported by our findings. For the demonstration of free liposome/toxin interaction further experiments were needed. Therefore, liposomes and liposome-SLO mixtures were studied in column chromatography on Sepharose-4B.

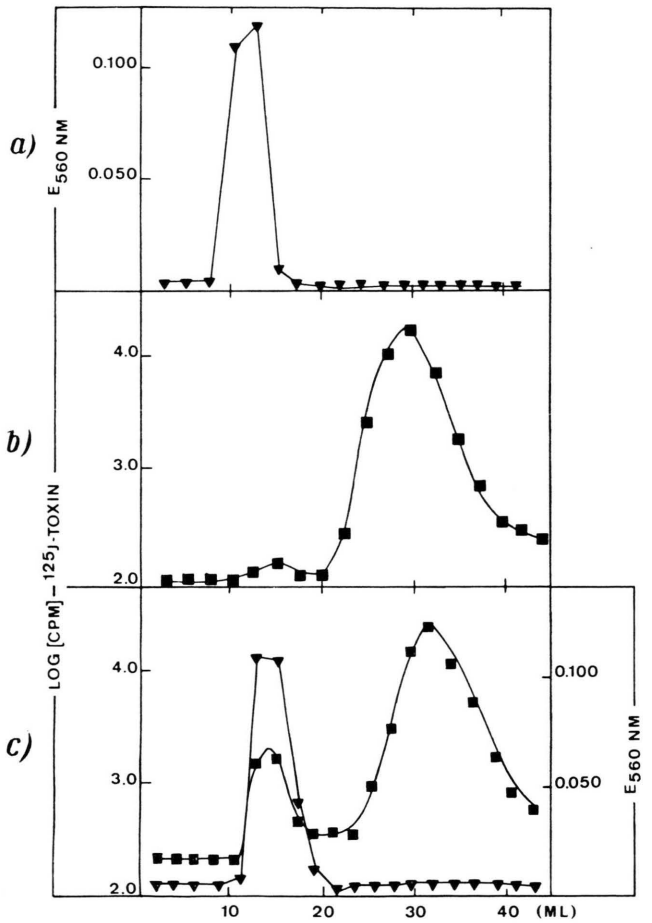

Fig. 5. Column chromatography on Sepharose 4B: a) Elution profile of liposomes (▼–▼–▼), turbidity at $\lambda = 560$ nm. b) Elution profile of activated [^{125}I]Streptolysin-O, (■–■–■), log cpm. c) Elution profile of the liposome [^{125}I]Streptolysin-O mixture. The liposome-peak (▼–▼–▼) is again localized as shown in Fig. 5 b. Streptolysin-O (■–■–■), log cpm, is distributed into two peaks, indicating that a considerable amount of radioactive material is now eluted with the liposome fraction.

Chromatography of a liposome suspension on a calibrated Sepharose-4B column resulted in the elution of a single peak with an elution volume of $V_e = 12.4 \pm 0.5$ ml (Fig. 5 a). When liposomes preincubated with reduced [^{125}I]SLO were filtered through the column, the same elution volume, $V_e = 12.9 \pm 0.05$ ml, and profile has been observed (5 c). However, the elution profile of the [^{125}I]SLO labelled material shows, that considerable activity was found to be associated with the liposome fraction, Fig. 5 c, indicating binding of radioactive material to liposomes. As a control, the labelled SLO was filtered through the column in the absence of liposomes. Fig. 5 b, shows a broad peak of iodinated material with $V_e = 30.3 \pm 0.5$ ml. When liposomes from the supernatant of a competition assay, where SRBC, liposomes and toxin have been incubated together (Table II) or those incubated with oxidized [^{125}I]SLO, were filtered through the column, the elution curve was identical with that depicted in Fig. 5 c. The radioactive material is again distributed into two distinct peaks with $V_e = 12.4 \pm 0.5$ ml and $V_e = 30.3 \pm 0.5$ ml. Hence the experiments reveals that oxidized and reduced SLO were bound to natural and artificial membranes.

Specifity of binding

The possibility of unspecific binding of toxin to liposomes and membranes was investigated by repeated absorption of [^{125}I]SLO with ghost membranes. The toxin free supernatant was then mixed with liposomes in order to demonstrate possible binding of labelled material other than toxin. Liposomes were separated from free iodinated materials as mentioned above. The experiment showed, that only $1.8 \pm 0.1\%$ of total radioactivity was found to be associated with liposomes after absorption with ghost membranes, whereas the value of the unabsorbed control amounted to $7.3 \pm 0.6\%$. Additional data on the binding of [^{125}I]SLO to SRBC or liposomes has been obtained by inhibition studies with specific SLO-antisera. Following incubation of SLO with either pooled human γ-globulin or anti-tetanolysin, which is known to inhibit effectively SLO induced hemolysis [14, 48], a decrease in the binding rate of [^{125}I]SLO to SRBC with increasing antiserum concentration has been observed (Fig. 6).

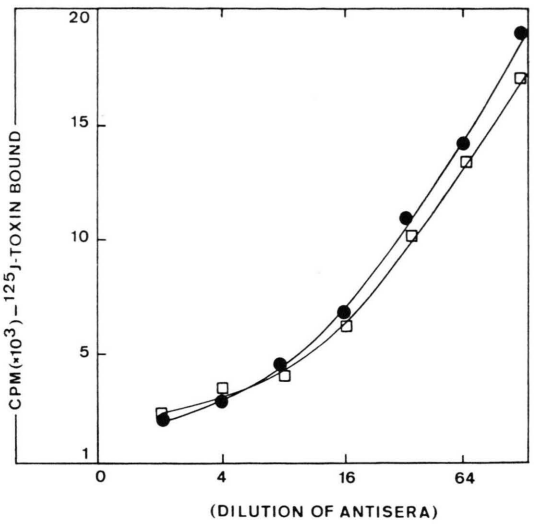

Fig. 6. Inhibition of binding of [^{125}I]Streptolysin-O to SRBC by specific antisera. Amount of toxin bound after treatment of SLO with different concentrations of γ-globulin (□–□–□) (2240 anti-SLO units/ml) or anti-tetanolysin respectively (●–●–●) (5760 anti-SLO units/ml). Abscissa: Dilution of antisera. Ordinate: Amount of [^{125}I]SLO fixed to SRBC (cpm).

At high antibody concentration both antisera were shown to inhibit binding of iodinated material. Compared with controls binding was reduced from 7.3 to 0.5% of total radioactivity. Therefore the experiment shows that labelled material usually bound to SRBC or liposomes exhibits SLO-antigenicity. Moreover, the antigen-antibody-complex is not only hemolytically inactive but is also unable to become fixed to membranes or liposomes.

Inhibition of binding of [^{125}I]SLO is not due to a possible interaction with contaminating serum cholesterol introduced with the antiserum. This has been ascertained by extraction of both antisera with chloroform/methanol and subsequent TLC analysis of the extract. Considering the lower limit of detectability of cholesterol by the applied TLC-method (see Material and Methods), the concentration of cholesterol in the undiluted serum was less than 5.5×10^{-8} M. Since the binding of radioactive material was dependent on the oxidation – or reduction – state of the protein, as well as on temperature, specific binding of a protein, sharing all the physico-chemical and antigenic properties with Streptoysin-O must be assumed. Moreover, binding of [^{125}I]SLO to SRBC and liposomes could be prevented by addition of an excess of highly purified, hemolytically active, unlabelled ("cold") SLO. Besides the selectivity of binding, considerable stability of the lipid-toxin complex in aqueous solution has been demonstrated by gel filtration chromatography on Sepharose-4B.

There is strong evidence from earlier work [18, 19], that liposomes prepared from a mixture of total membrane lipids, which, besides cholesterol, contain fatty acids, cholesterol-esters and phospholipids, combine with the toxin only by their cholesterol constituent. The fact that Streptolysin-O accumulates in the lipid/cholesterol phase by binding to both liposomes and SRBC-membranes, shows that the toxin is distributed between two phases. Generally spoken, the interaction of SLO with membranes is a partition problem considering the water- and membrane phase. Cholesterol, in one of two phases will strongly shift the partition coefficients of the toxin and may explains why the toxin is concentrated in a cellular or artificial membrane.

Consequently the general designation of cholesterol as an "inhibitor", used throughout the literature [49–53], is misleading and should be omitted. It is consistent with our findings that cholesterol in membranes facilitates the binding of SLO to liposomes or membranes. Therefore, cholesterol is neither an inhibitor of toxin fixation nor of hemolysis, since hemolysis starts only after binding of the toxin.

The binding studies with artificial membranes have shown furthermore that the toxin was bound to liposomes which were devoid of any protein. It thus seems plausible that the protein in the natural membrane does not necessarily participate in toxin fixation.

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