127. PURIFICATION AND PROPERTIES OF A HAEMOLYSIN PRODUCED BY GROUP A HAEMOLYTIC STREPTOCOCCI (STREPTOLYSIN O)

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IT has long been known that many strains of haemolytic streptococci produce filterable haemolysins. Work on this subject has been complicated by the facts that (1) either or both of two different haemolysins may be produced according to the conditions of growth and the bacterial strain, (2) one of these haemolysins is only active in the presence of certain reducing agents such as sodium hydrosulphite, cysteine and other —SH compounds.

Neill & Mallory [1926] first made the important observation that the haemolytic activity of fresh broth filtrates in which haemolytic streptococci have grown, rapidly disappeared on standing in air, though less rapidly in the absence of oxygen. If reducing agents such as hydrosulphite were then added, the haemolytic activity was restored to the original level or even higher.

These observations were confirmed and extended by Todd [1932] who has called this haemolysin 'streptolysin O' [Todd, 1938] to indicate susceptibility to oxidation and reduction. Todd [1939] has also shown that streptolysin O is produced by most strains of group A streptococci when grown in a serum-free broth such as the glucose-bicarbonate-phosphate broth of Todd & Hewitt [1932]; it is also produced by group C strains from human infections and by group G strains, but not by streptococci of other groups. It is antigenic, and potent antisera may be made by injecting such broth filtrates into horses or rabbits. The streptolysins produced by all types and strains of group A streptococci are serologically identical, and the antibody to any one of them will neutralize all of them to the same extent.

If haemolytic streptococci are grown in media containing serum a second haemolysin is also produced, whose activity is not affected by oxidation and reduction. Todd [1938] named this 'streptolysin S' to indicate that it is only produced in the presence of serum, and has shown that streptolysins O and S are two entirely distinct haemolysins and are neutralized by different antibodies.

So far these haemolysins have been studied mainly from a serological standpoint, and little chemical work has been carried out, apart from a single paper by Smythe & Harris [1940]. These authors were able to purify streptolysin O to a considerable extent but the details of procedure, yields etc., given in their paper are so scanty that it is impossible to repeat their methods. Their purified products were non-dialysable and appeared to be protein in nature, though they also contained *ca.* 60% carbohydrate.

The object of the present work was to isolate streptolysin O in as pure a state as possible with a view to making a detailed study of its chemical properties.

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Besides the two streptolysins already mentioned, haemolytic streptococci produce a number of other toxins, e.g. the scarlet fever toxin, a leucocidin, a 'diffusing factor' etc., and it is at present difficult to say whether these various toxins are the same or different, and to what extent any of them are associated with virulence. This is a problem which can only be settled by isolation of the different toxins in a pure state.

Experimental

(1) *Methods*

(*a*) *Determination of haemolytic activity.* The haemolytic activity of streptolysin O solutions was determined by the accurate colorimetric method described in the preceding paper [Herbert, 1941]. The standard conditions therein described were used throughout this work except where otherwise stated, and the same haemolytic unit (H.U.) is meant in both papers. (It should be pointed out that this is different from the M.H.D. used by Todd in previous work on streptolysin O. A solution containing 100 Todd M.H.D./ml. has about 250 H.U./ml.)

As previously mentioned, streptolysin O has almost no haemolytic activity except in the presence of certain reducing agents, and these must always be added before testing. Thiolacetic acid was used routinely. 0·1 ml. of B.D.H. 90% thiolacetic acid is added to *ca.* 2 ml. of distilled water, neutralized to *p*H 6·5 with *N* NaOH and diluted to 5 ml.; this gives an approx. *M*/5 solution. 0·5 ml. of this was added to 2 ml. of the streptolysin solution to be tested; after 10 min. at room temperature it was diluted with isotonic phosphate-NaCl; appropriate amounts were measured out, and incubated with red cells in the usual way.

Neutralized *M*/5 solutions of cysteine or Na_2SO_3 were occasionally used instead. Any of the above reagents is more convenient than sodium hydrosulphite which has been commonly used up to now. The great speed with which the latter is oxidized in air, and the fact that its solutions become extremely acid on oxidation, renders it less convenient than the other reagents mentioned.

(*b*) *Dry weights.* Throughout this work, the purity of any streptolysin O preparation is expressed in terms of H.U./mg. dry weight. Dry weights were determined by dialysing a known amount of solution until salt-free, measuring an aliquot into a tared glass dish, freezing at $-10°$, drying from the frozen state *in vacuo* over H_2SO_4 and reweighing.

(*c*) *Chemical methods.* Total carbohydrate in protein preparations was determined by the orcin method of Pirie [1936]. Phosphorus was determined by the method of Fiske and Subbarow as modified by Horecker *et al.* [1940]. Arginine was determined according to Weber [1930]. Sulphur was determined by the method of Denis & Reed [1926] as modified by Dr E. M. Crook (private communication).

(2) *Method of purification of streptolysin O*

The starting material was filtrates of cultures of group A streptococci grown in the broth of Todd & Hewitt [1932].

This broth was found to contain *ca.* 30 g./l. of total solids and about 10 g./l. of non-dialysable material, almost all of which is precipitated by full saturation with $(NH_4)_2SO_4$. These figures are not greatly changed after the organism has grown in the broth for 16 hr. From the point of view of purification, this high protein content constitutes a disadvantage which is, however, outweighed by the fact that this broth is the best so far evolved for the production of streptolysin O.

Except where otherwise stated the well-known 'Richards' strain (group A, type 3) was used. Stock cultures were preserved in dried form and inoculated

into broth tubes which were incubated 6 hr.; 2 l. batches of broth in Winchester bottles were inoculated with *ca.* 2 ml. of the 6 hr. culture, and incubated 16 hr. at 37°, tested for contamination and, if uncontaminated, filtered through Pasteur-Chamberland F candles.

The method of purification adopted involves fractionation with $(NH_4)_2SO_4$, adsorption and elution from calcium phosphate gel, followed by adsorption and elution from alumina Cγ. Full details of a typical preparation are given below.

The starting material was 4190 ml. of broth, containing 167 H.U./ml., or 700,000 H.U. in all.

Stage 1. 1510 g. solid $(NH_4)_2SO_4$ were added, making the solution 0·57 saturated. The bulky, flocculent precipitate was filtered on large Büchner funnels and sucked as dry as possible; the filtrate was discarded. The precipitate was dissolved in 400 ml. of *M*/15 phosphate *p*H 7·2, giving 480 ml. of a rather brown, sticky solution, containing 1110 H.U./ml., or 534,000 H.U. in all.

Stage 2. To the above solution, which was 0·14 saturated with $(NH_4)_2SO_4$, were added 212 ml. of saturated $(NH_4)_2SO_4$, slowly and with good stirring: final concentration 0·37 saturated. The dark brown, very sticky precipitate was centrifuged off and discarded, and the water-clear, only slightly coloured, solution dialysed overnight against running water: final volume 915 ml., containing 356 H.U./ml., or 326,000 H.U. in all.

Stage 3. To the above solution were added 30 ml. *M*/2 phosphate buffer *p*H 7·2, followed by 12·5 ml. *M* calcium acetate. After 20 min. the calcium phosphate precipitate was centrifuged, and the supernatant, which contained only 3 H.U./ml., discarded. The streptolysin was all adsorbed on the calcium precipitate, and was eluted therefrom with three successive 25 ml. portions of 0·25 saturated $(NH_4)_2SO_4$. The combined eluates were dialysed against distilled water: final volume 112 ml. containing 2560 H.U./ml., or 287,000 H.U. in all.

Stage 4. Adsorption and elution from calcium phosphate was now repeated. 10 ml. *M*/2 phosphate *p*H 7·2 were added to the above solution followed by 4 ml. *M* calcium acetate. (The *p*H if necessary was then adjusted to 6·5 with dilute acetic acid.) After 20 min. the calcium phosphate precipitate was centrifuged and eluted 5 times with successive portions of 0·25 saturated $(NH_4)_2SO_4$; the combined eluates were dialysed: final volume 31·5 ml., containing 6000 H.U./ml., or 189,000 H.U. in all.

Stage 5. To the above solution were added 5 ml. of an alumina Cγ gel containing 14·2 mg. Al_2O_3/ml., and the *p*H adjusted to 6·5 with dilute acetic acid. The alumina was centrifuged down, the supernatant discarded and the alumina was eluted twice with 4 ml. portions of ammoniacal $(NH_4)_2SO_4$ (97·5 ml. saturated $(NH_4)_2SO_4$, 2·5 ml. ammonia sp. gr. 0·880, diluted to 400 ml.) and the combined eluates were exhaustively dialysed: final volume 10 ml., containing 12,100 H.U./ml., or 121,000 H.U. in all.

The yields and relative purity of the streptolysin at the different stages of purification are shown in Table 1.

The starting material contained altogether 700,000 H.U. the relative purity of which, on a dry weight basis was 18·4 H.U./mg. The final product, 121,000 H.U. in all, had a relative purity of 3050 H.U./mg. dry weight. The degree of purification was therefore 166-fold, with an overall yield of streptolysin O of 17·3 %.

The final product is undoubtedly not completely pure; however, the small quantity of material available (39·7 mg. of purified streptolysin from 4 l. of broth) made further purification impracticable.

Table 1. *Purification of streptolysin O*

Stage	Volume ml.	H.U./ml.	Total H.U.	Dry weight mg./ml.	Total dry weight	H.U./mg.
Broth	4190	167	700,000	9·08	38·1 g.	18·4
1	480	1,110	534,000	34·2	16·4 g.	33·2
2	915	356	326,000	7·06	6·45 g.	50·5
3	112	2,560	287,000	3·5	392 mg.	732
4	31·5	6,000	189,000	3·1	97·6 mg.	1935
5	10	12,100	121,000	3·97	39·7 mg.	3050

(3) *Properties of the purified streptolysin O*

(*a*) *Analysis.* The final product from the preparation described above, which had been dialysed against many changes of glass-distilled water, was dried from the frozen state *in vacuo* over H_2SO_4. (Found: C, 46·7; H, 6·8; N, 16·8; S, 2·34; P, 0·053; ash, 3·6; total carbohydrate, 2·6 %.) The C, H, and N figures are micro-analyses by Dr Weiler, Oxford. The frozen-dried substance lost a further 6·35 % of H_2O on drying to constant weight *in vacuo* at 100°; the above figures are corrected for this.

Other preparations gave closely similar results. The ash content was usually lower than that of this particular preparation, e.g. 1·0–1·5 %. The above are typical analytical figures of a protein, and all evidence favours the view that streptolysin O is in fact a protein. Purified preparations gave all the usual colour reactions for proteins, e.g. the biuret reaction and tests for tyrosine and tryptophan. They also gave a strong Sakaguchi reaction, and quantitative analysis by the Weber method showed the above preparation to contain 4·7 % of arginine.

The protein gave a negative nitroprusside reaction before, and a positive reaction after, treatment with KCN; i.e. it presumably contains —S—S— linkages which can be reduced to —SH groups.

The preparation described above was from a 'Richards' strain. In one case, broth from a culture of strain 0·89 Matt Attenuated was put through the first four stages of the purification process described above. It behaved similarly in all respects, and a highly purified product was obtained, which gave similar analytical figures to the above and behaved in the same way. While the matter has not been closely investigated, it seems probable that the streptolysins O produced by all types of group A streptococci are very closely similar, if not identical, chemically as well as immunologically.

(*b*) *General properties.* Purified streptolysin O preparations on drying from the frozen state form a white fluffy powder. It is readily soluble in distilled water or dilute salt solutions; the exact solubility is unknown but 5 % solutions can easily be obtained. Such solutions are crystal clear and usually completely colourless, though sometimes faintly yellow. It is precipitated by trichloroacetic acid and the usual protein precipitants, by high concentrations of $(NH_4)_2SO_4$, and by alcohol and acetone. It is coagulated on boiling.

Fairly concentrated solutions are perfectly stable for weeks at 0°, though they lose an appreciable part of their activity in two months. Very weak solutions (0·001 mg./ml.) are less stable, and may be irreversibly inactivated in a few hours at room temperature or even less at 37°, presumably owing to surface denaturation, since the purified material is much less stable than crude streptolysin-broth in this respect in weak solutions, though equally stable in strong solutions. At all stages of purity, boiling for 2 min. causes complete and irreversible inactivation; so also does treatment with strong acids and alkalis. Frozen-dried preparations appear to retain their activity indefinitely, and the

drying process itself causes no loss of activity. Some data on relative stabilities of purified preparations under different conditions may be found also in Table 2 of the preceding paper.

(c) *Activation of streptolysin O by reducing agents.* As already stated, the haemolytic activity of streptolysin broth filtrates was extremely small if tested in the absence of cysteine or some other reducing agent. The haemolytic activity after activation with cysteine was usually 20–40 times that of the unactivated streptolysin broth, e.g.

	Haemolytic activity (H.U./ml.)	
Broth batch no.	Tested without activator	With *M*/25 cysteine
64	3	125
68	4	149
69	3	84

The same also applies to purified streptolysin O at all stages of purification.

The activating effect of a large number of substances was tested with results shown in Table 2. In each series of experiments, one with cysteine was included; the relative degrees of activation brought about by the various substances tested are then expressed as percentages of the activity with cysteine, which is given an arbitrary value of 100%.

Table 2. *Activation of streptolysin O by various reducing agents*

In all cases, 2 ml. of a streptolysin O solution, containing *ca.* 1 mg. of purified haemolysin, was added to 0·5 ml. of a neutralized *M*/5 solution* of the reducing or oxidizing agent to be tested. After 10 min. at room temperature, the mixture was diluted with saline-phosphate *p*H 6·5 to the appropriate strength, and its haemolytic activity measured in the usual way.

Controls were done on all the substances tested, by adding 1 ml. of a *M*/25 solution to 1 ml. red cells; in no case was any haemolysis observed.

The three experiments were made with different streptolysin preparations.

Oxidizing or reducing agent	Haemolytic activity H.U./ml.	Relative activity (cysteine = 100)
	Exp. 1	
Cysteine	1850	100
Thiolacetic acid	1840	99·5
β-Thiolpropionic acid	1760	95·2
Reduced glutathione	1745	94·5
Na_2SO_3	1940	104
None	97	5·2
	Exp. 2	
Cysteine	890	100
o-Thiolbenzoic acid	934	105
H_2S	800–1000†	90–110†
$Na_2S_2O_4$	890	100
KCN	132	15
$Na_2S_2O_3$	77	8·6
$K_4Fe(CN)_6$	33	3·7
Ascorbic acid	32	3·6
None	32	3·6
	Exp. 3	
Cysteine	856	100
Cystine	6	0·7
Oxidized glutathione	3	0·35
Dithiodiacetic acid	8	0·94
H_2O_2	5	0·58
None	18·6	2·2

* In the case of H_2S, a saturated solution was used; in the case of cystine a *M*/5 suspension.

† When H_2S is used, the formation of sulphaemoglobin and deposition of colloidal S make accurate colorimetric readings impossible; these figures are approximate.

The substances tested can be divided into three groups so far as their activating effects are concerned:

(1) All the —SH compounds tested[1] (cysteine, reduced glutathione etc.), and also H_2S, $Na_2S_2O_4$ and Na_2SO_3 have the same maximum activating effect (within the limits of error of the methods used).

(2) The —S—S— compounds corresponding to the above —SH compounds (cystine, oxidized glutathione, dithiodiacetic acid etc.) and also ascorbic acid and ferrocyanide have no activating effect at all; the former group of compounds in fact decrease the small blank activity.

(3) KCN and $Na_2S_2O_3$ occupy an intermediate position. They bring about a definite activation, quite outside the limits of experimental error (haemolytic activity being increased 2–5 times above the control), but much smaller than that brought about by substances of group (1) which is *ca.* 30 times greater than that of the control.

The activation of streptolysin O by cysteine etc. is a very rapid process at room temperature; e.g. if 0·5 ml. *M*/5 cysteine is added to 2 ml. streptolysin and then diluted and tested immediately, the haemolytic activity found is the same as when the streptolysin is kept 30 min. with the cysteine before testing.

No detailed study was made of the amount of cysteine (or other reducing agent) required for the activation of a given amount of streptolysin. The method adopted in practice was to keep 2 ml. of streptolysin solution containing *ca.* 200–2000 H.U. for a short time with 0·5 ml. of a *M*/5 solution of the reducing agent; the mixture was then diluted until it contained *ca.* 1 H.U./ml. The final concentration of reducing agent is therefore very small; however, if the dilution is made instead with saline-phosphate containing *M*/25 cysteine, thiolacetate or Na_2SO_3, exactly the same results are obtained.

The activation produced by reducing agents is completely reversible; this can be shown in the following way. If cysteine is added to a streptolysin solution, the initially low haemolytic activity is of course greatly increased. If the streptolysin-cysteine solution is now dialysed until the cysteine is all removed, the haemolytic activity falls again to its original low level; but if cysteine is once more added, the haemolytic activity is again increased as much as before. The following figures illustrate such an experiment:

	Haemolytic activity H.U./ml.
(1) Unactivated streptolysin O solution	6·7
(2) The same after adding cysteine to a final concentration of *M*/25	422
(3) Streptolysin-cysteine solution after 24 hr. dialysis (nitroprusside test negative)	9·0
(4) Dialysed solution after adding cysteine (*M*/25)	400

(N.B. the values in (3) and (4) are corrected for the volume increase occurring on dialysis.) Another portion of the streptolysin-cysteine solution, instead of dialysing, was kept for 24 hr. at room temperature; the haemolytic activity had not decreased. Some of the cysteine had been oxidized to cystine, but there was still sufficient to give a most intense nitroprusside reaction.

[1] It is interesting that the aromatic —SH compound *o*-thiolbenzoic acid, in which the —SH group is directly attached to the benzene ring, has exactly the same activating effect as the aliphatic —SH compounds tested.

The most obvious hypothesis to explain all the above facts is that streptolysin O is a protein containing —S—S— linkages, which can be reversibly reduced to —SH groups. The —S—S— form of the protein may be assumed to be haemolytically inactive while the —SH form is active. The activation of inactive streptolysin by cystine, reduced glutathione, Na_2SO_3 etc., is then to be interpreted as a reduction of the protein —S—S— groups to —SH by the above compounds:

$$\underset{\text{(inactive)}}{\text{Streptolysin}\begin{matrix}\text{S}\\|\\\text{S}\end{matrix}} + 2R\text{SH} \rightleftharpoons \underset{\text{(active)}}{\text{Streptolysin}\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix}} + R\text{SS}R$$

The —SH groups of the inactive haemolysin must be assumed to be, like most —SH groups, autoxidizable in air. This oxidation cannot take place when large amounts of cysteine (or other reducing agent) are present, since the cysteine —SH groups, so long as they are present in excess, will keep the sulphur linkages of the protein in the reduced state by mass action effect, and will be oxidized instead. When the cysteine is removed, by dialysis or any other means, the protein —SH groups are slowly oxidized by atmospheric oxygen.

The unactivated preparations of streptolysin always possessed a small haemolytic activity. This, however, was relatively diminished in the course of purification; thus in the preparation described in § 2, the following figures were found:

	Haemolytic activity (H.U./ml.)		
	With thiolacetic acid	No activator	Ratio
Streptolysin broth	167	4·2	40/1
Purified streptolysin, stage 3	2,560	33	78/1
Purified streptolysin, stage 5	11,100	101	111/1

This suggests that the small haemolytic activity of the unactivated streptolysin is due to the presence of minute traces of activating compounds, derived from the broth or the growth of the organism, which are only removed with difficulty.

An alternative explanation might be that, while most of the streptolysin —SH groups are comparatively rapidly autoxidizable, a remainder oxidize only very slowly. The relatively smaller 'residual' activity of the purified haemolysin might simply indicate more complete oxidation due to the amount of manipulation it has undergone in purification. This argument is strengthened by the fact that oxidizing agents such as —S—S— compounds and H_2O_2 greatly diminish this 'residual' activity as shown in Table 2.

Yet a third explanation, that the residual haemolysis is due to another, different, haemolysin, does not seem probable, since it is completely abolished by anti-streptolysin O and by the θ antiserum to *Cl. welchii*.

The results so far described are mostly in good agreement with the thiol-disulphide hypothesis outlined above; in spite of its attractive nature, however, this hypothesis can by no means be taken for granted, as later results will show.

(*d*) *Effect of* p*H*. Fig. 1 shows the effect of carrying out tests of haemolytic activity in a series of isotonic buffers of varying *p*H. The haemolytic activity is greatest at *p*H 6·5 and falls off markedly on both sides of this 'optimum *p*H'; the curve, in fact, resembles the *p*H-activity curve of an enzyme. The two results at *p*H 8·0 and 8·05 show that there is no significant difference between borate and phosphate buffers of the same *p*H.

It is of course impossible to say whether the effect of *p*H is on the haemolysin or the red cells or both. These experiments do show, however, the necessity of conducting haemolysis tests in buffered media if accurate results are to be obtained.

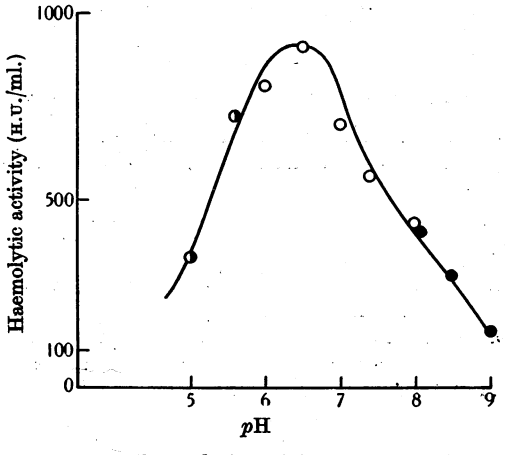


Fig. 1. Effect of *p*H on haemolytic activity. ◑ phosphate-citrate buffer. ○ phosphate buffer. ● borate buffer.

(*e*) *Effect of temperature.* The haemolytic activity of the same streptolysin solution was tested at 0, 18 and 38°. The results are shown in Table 3. The haemolytic activity is very much reduced at 0°, being only 3 % of the activity

Table 3. *Effect of temperature*

Purified streptolysin activated with thiolacetic acid was used. Varying amounts were measured into small tubes, brought to 1 ml. and these and the red cell suspension separately brought to the required temperature before mixing. Incubated 30 min. in each case.

Temp.	Haemolytic activity (H.U./ml.)	Relative activity %
0°	19	3·2
18°	234	39·9
38°	588	100

at 38°; the activity at 18°, however, is 40 % of the activity at 38°. In other words the haemolytic process appears to have a temperature coefficient which decreases with temperature.

Certain bacterial haemolysins, e.g. the α-haemolysin of *Cl. welchii* [van Heyningen, 1941] and staphylococcus β-haemolysin, display the phenomenon of so-called 'hot-cold' lysis; that is, if an appropriate amount of haemolysin is incubated with red cells at 38°, little or no haemolysis is observed, but on subsequently cooling, the red cells are haemolysed. It may be worth recording that streptolysin O does not display this interesting though somewhat obscure phenomenon.

(*f*) *Adsorption of the haemolysin on the red cell surface.* As shown above, when moderate amounts of streptolysin are mixed with red cells at 0°, no haemolysis occurs in half an hour. If the red cells are then centrifuged, all the haemolysin has disappeared from the supernatant, as can be seen by adding fresh red cells

to it and incubating at 38°, when no haemolysis occurs. The haemolysin has all been adsorbed on the original red cells, as can be shown by suspending them in fresh saline and incubating at 38°, when they are rapidly haemolysed. Small amounts of streptolysin are all adsorbed on the red cells as described; with larger amounts the red cell surface becomes 'saturated' and some haemolysin remains unabsorbed (Table 4).

Table 4. *Adsorption of streptolysin O on red cells at 0°*

Varying amounts of a streptolysin O solution activated with $Na_2S_2O_4$ were incubated with 1 ml. portions of red cell suspension for 30 min. at 38°, in a total volume of 2 ml. The following results were obtained:

Streptolysin (ml.)	0·2	0·1	0·05	0·02	0·01
Haemolysis (%)	100	100	100	60	10

A similar series of tubes of the same streptolysin solution was set up at 0° (lysin and red cells being separately cooled to 0° before mixing), kept at 0° for half an hour and centrifuged. There was no haemolysis in any of the tubes. The supernatants were then drawn off and the red cells suspended in fresh saline; the supernatants were added to fresh red cells. Both series of tubes were incubated 30 min. at 38°, with the following results:

Streptolysin (ml.)	0·2	0·1	0·05	0·02	0·01
Supernatants + fresh red cells, % haemolysis	15	5	0	0	0
Red cells + fresh saline, % haemolysis	100	100	100	60	10

(N.B. The values for percentage haemolysis were obtained by visual comparison with standards consisting of varying amounts of red cells laked by saponin; they are approximate only.)

These results show that the adsorption of streptolysin on the red cell surface is a different reaction from the actual process of lysis, in which a chemical process is probably involved, since it is inhibited, while adsorption processes are known to be increased, by low temperatures.

If an experiment similar to that in Table 4 is carried out with unactivated streptolysin, this is not adsorbed on the red cells; it all remains in the supernatant where its presence can be detected by adding cysteine followed by fresh red cells. A similar phenomenon was observed by Cohen & Shwachman [1937] with pneumolysin, which is similar to streptolysin O in many respects.

(*g*) *The effect of varying the red cell concentration.* In the course of working out the method used for the quantitative estimation of haemolysis described in the preceding paper, experiments were made with red cell suspensions of varying strengths. The results are shown in Fig. 2. The somewhat surprising phenomenon was observed that when small amounts of haemolysin were added, so that only a small percentage of the red cells present were haemolysed, the amount of haemolysis was greater the smaller the number of red cells present. A tentative explanation of this phenomenon is that (*a*) once streptolysin is adsorbed on the red cell surface it is not easily released, (*b*) for each individual red cell, a certain critical amount of streptolysin must be adsorbed on its surface to cause haemolysis in a given time, and if less than this amount is present no haemolysis takes place (*vide* the preceding paper). Hence with a large amount of red cells and a small amount of haemolysin, the latter may be so 'spread out' over the total red cell surface that there is insufficient on any one red cell to cause haemolysis. It may be for this reason that, when measuring haemolysis, irregular results are

obtained if the haemolysin and red cells are not properly mixed at the moment of addition.

However that may be, these results show that haemolysis tests can only be accurate if red cell suspensions of exactly known strength are employed.

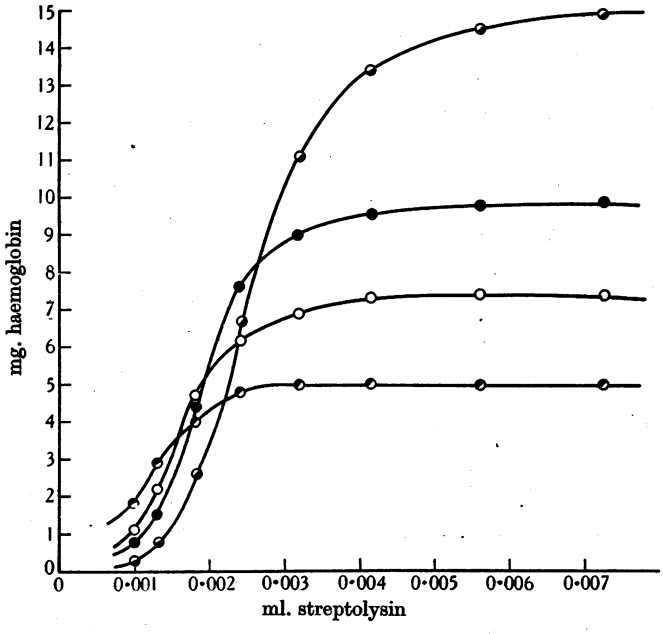


Fig. 2. Effect of varying red cell concentration. The four curves represent four series of measurements with red cell suspensions containing 15, 10, 7·5 and 5 mg. haemoglobin per ml.

(*h*) *The effect of various substances on the haemolysin.* The α-haemolysin (or α-toxin) of group A *Cl. welchii* has been shown by Macfarlane, R. G. *et al.* [1941] to be activated by calcium and to be inhibited by substances which combine with ionic calcium such as phosphate, citrate etc. Experiments were conducted to determine whether the same holds for streptolysin O.

It has already been shown that phosphate has no inhibiting action, since the haemolytic activity is the same in phosphate and borate buffers of the same *p*H (Fig. 1). Table 5 shows that fluoride, oxalate and citrate also have no effect. Likewise the addition of calcium to streptolysin preparations (exhaustively dialysed against glass-distilled water and presumably calcium-free) causes no increase in haemolytic activity, but a small decrease. The addition of magnesium also has no effect.

It has been claimed that sulphanilamide inhibits streptolysin. To test this, 20 mg. sulphanilamide were added to 2 ml. of a streptolysin O solution, followed after 20 min. by 0·5 ml. of *M*/25 thiolacetate; the mixture was then diluted and tested in the usual way, a control being run simultaneously, with the following results:

	H.U./ml.	Inhibition %
Control	182	—
Sulphanilamide	159	13

The result can hardly be considered significant.

Table 5. *Effect of calcium, etc.*

Streptolysin O activated with thiolacetic acid was used. The red cells were suspended in 0·9% NaCl and all dilutions of the streptolysin were made in 0·9% NaCl containing the various substances adjusted to *p*H 6·5, instead of the usual saline-phosphate.

Substance added	Haemolytic activity H.U./ml.	Inhibition %
Exp. 1		
None (control)	801	—
M/1000 $CaCl_2$	596	25
M/50 NaF	775	3
M/100 potassium oxalate	800	0
M/100 sodium citrate	790	2
Exp. 2		
None (control)	575	—
M/100 $CaCl_2$	319	45
M/100 $MgCl_2$	578	0

N.B. The final concentrations of the various substances are given; i.e. after the streptolysin and red cells have been mixed. The two experiments were carried out with different streptolysin preparations.

The effect of iodoacetic acid and iodoacetamide, was considered interesting, since many enzymes are known which are activated by —SH compounds (papain, cathepsin, succinic dehydrogenase etc.) in a similar way to streptolysin O, and all such enzymes are strongly and irreversibly inhibited by iodoacetic acid or its amide, often in concentrations as low as *M*/1000. However, Table 6

Table 6. *Iodoacetic acid and iodoacetamide*

Exp. I. 1·0 ml. streptolysin O + 1·0 ml. iodoacetic acid (*M*/25) kept 30 min. at room temperature; 1·0 ml. *M*/5 thiolacetic acid then added and after 10 min., diluted and measured haemolytic activity.

	Haemolytic activity H.U./ml.
Control	851
Iodoacetic acid	862

Exp. II. As exp. I, but the streptolysin O was incubated with the iodoacetic acid for 20 min. at 38°; similarly with iodoacetamide.

	Haemolytic activity H.U./ml.	Inhibition %
Control	524	—
Iodoacetic acid	406	22
Iodoacetamide	207	60

shows that even *M*/50 iodoacetic acid has no effect on streptolysin O when kept with it for 20 min. at room temperature and only a slight effect at 38°. Even *M*/50 iodoacetamide, which is considered to react with protein —SH groups more readily than iodoacetic acid, only causes partial inhibition. These results are in complete agreement with those of Symthe & Harris [1940], and as these authors state, are not in agreement with the hypothesis that streptolysin O activity depends on a thiol-disulphide system.

Nevertheless, it might be argued for this hypothesis that in these experiments iodoacetic acid was added to the inactive streptolysin presumably in the disulphide state, and that iodoacetic does not attack —S—S— links.

To test this, excess of iodoacetic acid was allowed to act upon streptolysin activated with thiolacetic acid and hence presumably in the —SH state. Twice as much iodoacetic acid was added as was needed to combine with all the thiolacetic acid present. On testing again, it was found that the haemolytic activity had disappeared. This inactivation was partly reversible, however, and on adding a further excess of thiolacetic acid, the haemolytic activity was restored to the extent of 40 %. The actual figures were as follows:

	Haemolytic activity H.U./ml.
(1) Unactivated streptolysin	18
(2) Streptolysin + thiolacetic acid (*M*/50)	870
(3) Streptolysin-thiolacetic acid + iodoacetic acid (*M*/25) for 30 min.	23
(4) Above + thiolacetic acid (*M*/10)	360

This seems to indicate either that the activity of streptolysin O is not dependent upon reduced —SH groups, or that the —SH groups of this compound react very slowly with iodoacetic acid. These results also are in agreement with those of Smythe & Harris.

Many other substances that oxidize or combine with —SH groups will bring about reversible inhibition of activated streptolysin, e.g.

	Haemolytic activity H.U./ml.
(1) Unactivated streptolysin	19
(2) Streptolysin + thiolacetic acid (*M*/50)	866
(3) Streptolysin-thiolacetic acid + H_2O_2 (*M*/50) for 30 min.	4
(4) Above + thiolacetic acid (*M*/25)	790

(*i*) *Serological properties.* Todd [1934] showed that tetanolysin and pneumolysin were both neutralized by anti-streptolysin O, though to a less extent than streptolysin O. The conclusion is that these three haemolysins are chemically very similar substances, so much so that they are all neutralized by the antiserum to any one of them. The work of Shwachman *et al.* [1934] confirms this view since it shows pneumolysin to resemble streptolysin O in many ways, including the phenomenon of activation by —SH compounds. Todd [1941] and van Heyningen [1941] have shown that one of the haemolysins of *Cl. welchii*, the θ-haemolysin, also resembles streptolysin O in being activated by $Na_2S_2O_4$, —SH compounds etc., and Todd has shown that here also the phenomenon of cross-neutralization occurs, *welchii* θ-haemolysin being neutralized by anti-streptolysin and *vice versa*.

Table 7 shows that purified preparations of streptolysin O are neutralized by anti-streptolysin O to the same extent as crude preparations; also that they are neutralized by antisera to *Cl. welchii* θ-haemolysin, but not by antisera to *welchii* α-haemolysin. The fact that crude and purified preparations are neutralized to the same extent shows that little conversion into toxoid occurs during the purification process (if, indeed, streptolysin O can exist in a toxoid form, which is not known).

The α-haemolysin of *Cl. welchii* is probably a lecithinase [Macfarlane, M. G. & Knight, 1941] and has the property of producing a flocculent precipitate when added to a solution of lecitho-vitellin prepared from egg-yolk [Macfarlane, R. G. *et al.* 1941]. Streptolysin O does not give this reaction, no trace of opalescence being observed after incubating a strong solution of streptolysin (1850 H.U./ml.) in 0·9 % NaCl for 24 hr. at 38° with the egg solution.

Table 7. *Neutralization of streptolysin by anti-streptolysin O and by antisera to* Cl. welchii *toxins*

The streptolysin O solutions activated with thiolacetic acid were titrated, and amounts corresponding to exactly 10 H.U. measured out into a series of tubes containing varying amounts of anti-sera, made up to 1 ml. After 15 min. at room temperature 1 ml. of red cell suspension was added and the tubes incubated for 30 min. at 38°. Below are given the amounts of the various sera required for complete neutralization of 10 H.U. The streptolysin O broth used had 15·3 H.U./mg. dry weight; the purified preparation, which was made from the same sample of broth, had 780 H.U./mg.

Serum	Amount of serum required to neutralize 10 H.U. of streptolysin O: Purified streptolysin ml.	Crude streptolysin broth ml.
(1) Anti-streptolysin O	0·0056	0·0049
(2) *Cl. welchii* θ anti-toxin	0·00049	0·00042
(3) *Cl. welchii* α anti-toxin	0·27	0·21

Serum (2) containing 100 θ and 0·2 α units per ml., and serum (3) containing 100 α and 0·3 θ units per ml., were kindly provided by Dr Glenny.

(*j*) *Lethal effect on mice.* Purified streptolysin O preparations are toxic to mice on intravenous injection. The minimum lethal dose was about 135 H.U. or 0·044 mg. of the purest preparation. The streptolysin O has a much greater lethal effect when injected in an activated form, e.g. together with cysteine, although the cysteine alone has no harmful effect (Table 8).

This suggests that *in vivo* its action is similar to that *in vitro*; it also suggests that the lethal effect is due to streptolysin O itself and not to some impurity.

Table 8. *Lethal effect of streptolysin O*

Purified streptolysin O solutions in 0·9% NaCl were injected into the tail veins of albino mice (*ca.* 20 g.). The volume injected was always made up to 0·5 ml.

	Streptolysin injected mg.	Total H.U. injected	No. mice injected	Deaths	Interval till death
		Exp. 1			
(*a*) Streptolysin activated with cysteine	0·176	540	2	2	30 sec.; 30 sec.
	0·088	270	2	2	30 sec.; 3 min.
	0·044	135	2	1	40 min.
	0·022	67·5	2	0	Survived
(*b*) Unactivated streptolysin	0·352	1080	2	1	Died overnight
	0·176	540	2	1	,,
	0·088	270	2	0	Survived
	0·044	135	2	0	,,
		Exp. 2			
(*a*) Streptolysin activated with cysteine	0·242	740	2	2	10 sec.; 10 sec.
	0·098	296	2	2	15 sec.; 20 sec.
	0·048	148	2	1	7 min.
	0·024	74	2	0	Survived
(*b*) Unactivated streptolysin	0·240	740	2	0	,,
	0·098	296	2	0	,,

As a control experiment, 3 mice were injected with 0·5 ml. *M*/25 cysteine (neutralized) in 0·9% NaCl. All survived with no apparent harmful effects.

(*k*) *Streptolysin O and other toxins of group A streptococci.* Group A haemolytic streptococci are stated in the literature to produce the following toxic substances, (*a*) the erythrogenic or scarlet fever toxin, (*b*) streptolysin O,

(*c*) streptolysin S, (*d*) a leucocidin, (*e*) a fibrinolysin, (*f*) a diffusing factor. The relations between these factors have hitherto remained obscure.

The non-identity of streptolysins O and S has already been demonstrated by Todd, and further demonstrated in the course of this work and work in progress on streptolysin S.

It may also be concluded that streptolysin O and the erythrogenic toxin are two different substances. Barron *et al.* [1941] have obtained highly purified erythrogenic toxin, and have shown it to be a protein of low molecular weight and stable to heat; its activity in the skin test is not affected by oxidizing and reducing agents; comparison of the properties of their product and ours makes it certain that the two substances are different. Todd *et al.* [1933] have already shown that the antibodies to these two toxins are different.

Dr McClean has kindly tested purified streptolysin O preparations for diffusing factor and hyaluronidase activities, and found these to be completely absent. Streptolysin O and diffusing factor are therefore different substances.

Discussion

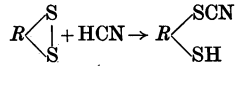
As a result of this work streptolysin O has been shown to be a protein, and highly purified preparations of it have been obtained. This protein haemolyses red blood corpuscles, but only after it has been activated with hydrosulphite, cysteine, or a number of other reducing agents. In this behaviour it resembles a number of enzymes which are similarly affected by oxidation and reduction (urease, arginase, papain, cathepsin, succinic dehydrogenase, triosephosphate dehydrogenase and several others). All these enzymes are strongly inhibited by low concentrations of iodoacetic acid.

The current theory is that these enzymes possess —SH groups capable of reversible oxidation and reduction which are essential for enzymic activity. This explains many facts including their inactivation by iodoacetic acid which is known to combine with —SH groups. Does this theory cover streptolysin O?

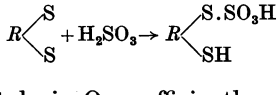
We have seen that the theory explains many of the observed facts. Moreover, purified streptolysin O preparations do contain —S—S— linkages, or at least linkages which can be converted into —SH groups by treatment with reducing agents such as KCN.

Inactive streptolysin O is not adsorbed by red blood cells, while the active form is, and it is tempting to suppose that the —SH groups are necessary to attach the haemolysin to the red cell.

There are, however, a number of facts which are not in accord with this hypothesis. One is the very feeble activation of streptolysin O by KCN, which strongly activates many of the above enzymes. This might be ascribed to the fact that the reaction of KCN with —S—S— links only releases half the available —SH groups:



Na_2SO_3, however, behaves in an entirely analogous way:



yet Na_2SO_3 activates streptolysin O as efficiently as cysteine or reduced glutathione.

The resistance of streptolysin O to inactivation by iodoacetic acid is also difficult to reconcile with the above theory.

The '—SH theory' is much in vogue at the present time, yet even in the case of certain enzymes there are many facts that conflict with it. Fruton & Bergmann [1940] completely reject this theory; they suggest that in the activation of papain, for example, cysteine, HCN and reduced glutathione do not act by reducing the —S—S— groups of papain to —SH, but act rather as 'coenzymes', forming a dissociable cysteine-papain complex, a dissociable KCN-papain complex, etc.

In the case of streptolysin O, this theory explains the observed facts at least as well as the other. It explains, for example, the inactivation of streptolysin-cysteine on dialysis and the apparent anomaly in the action of iodoacetic acid. On the theory of Fruton & Bergmann, iodoacetic acid, H_2O_2 and other oxidizing agents bring about reversible inactivation of streptolysin-cysteine, not by oxidizing the —SH groups of the streptolysin, but simply by removing the cysteine. It does not explain so well the fact that the eight different compounds mentioned in Table 2 all activate streptolysin O to exactly the same extent.

At present it is not possible to decide whether either of these theories adequately explains the activation of streptolysin O by reducing agents.

Another problem of major importance is the manner in which activated streptolysin O brings about the haemolysis of red cells. The simplest explanation is that it is an enzyme which attacks some constituent of the red cell membrane. Its protein nature, its activation by —SH compounds and many of its other properties all suggest this. So far, however, no enzymic function has been found for it; the difficulty of course lies in finding the appropriate substrate, which can only be a matter of trial and error. The exceedingly small doses in which all bacterial toxins work suggest that their action must be catalytic. Two bacterial toxins have already been identified as enzymes, namely the α toxin of *Cl. welchii*, which M. G. Macfarlane & Knight [1941] have shown to be probably a lecithinase, and the 'diffusing factor' of the same organism, and of streptococci which McClean & Hale [1941] and McClean [1941] have shown to be probably hyaluronidases. The possibility exists that all bacterial toxins are in fact enzymes, and this would seem to be a fruitful line of approach at the present time.

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REFERENCES

Barron, Dick & Lyman (1941). *J. biol. Chem.* **137**, 267.
Cohen & Shwachman (1937). *J. Bact.* **33**, 67.
Denis & Reed (1926). *J. biol. Chem.* **71**, 191.
Fruton & Bergmann (1940). *J. biol. Chem.* **133**, 154.
Herbert (1941). *Biochem. J.* **35**, 1116.
van Heyningen (1941). *Biochem. J.* **35**, 1257.
Horecker, Ma & Haas (1940). *J. biol. Chem.* **136**, 775.
McClean (1941). *J. Path. Bact.* **53**, 13.
—— & Hale (1941). *Biochem. J.* **35**, 159.
Macfarlane, M. G. & Knight (1941). *Biochem. J.* **35**, 884.
Macfarlane, R. G., Oakley & Anderson (1941). *J. Path. Bact.* **52**, 99.
Neill & Mallory (1926). *J. exp. Med.* **44**, 241.

Pirie (1936). *Brit. J. exp. Path.* **17**, 269.
Shwachman, Hellerman & Cohen (1934). *J. biol. Chem.* **107**, 257.
Smythe & Harris (1940). *J. Immunol.* **38**, 283.
Todd (1932). *J. exp. Med.* **55**, 267.
—— (1934). *J. Path. Bact.* **39**, 299.
—— (1938). *J. Path. Bact.* **47**, 423.
—— (1939). *J. Hyg., Camb.*, **39**, 1.
—— (1941). *Brit. J. exp. Path.* **22**, 172.
—— & Hewitt (1932). *J. Path. Bact.* **35**, 973.
—— Laurent & Hill (1933). *J. Path. Bact.* **36**, 201.
Weber (1930). *J. biol. Chem.* **86**, 216.