EFFECT OF SOME ANTIMETABOLITES ON THE PRODUCTION OF STREPTOLYSIN S′[1]

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Streptolysin S has attracted the interest of many investigators owing to several unique properties. However, its nature and mechanism of formation remain to be elucidated.

Todd (1938) was the first to differentiate the hemolysins produced by *Streptococcus pyogenes* into streptolysin O (oxygen labile) and streptolysin S (serum extractable). He shared the view expressed by Weld (1934, 1935) and by Smith (1937) that serum containing media facilitated a physical extraction of a preformed intracellular hemolysin from hemolytic streptococci. Streptolysin S was partially purified by Herbert and Todd (1944) who felt that it exhibited lipoprotein properties. Okamoto (1939) discovered that yeast nucleic acid enhanced the formation of a potent hemolysin by hemolytic streptococci which he believed to be a polynucleotide. Bernheimer and Rodbart (1948) isolated an active fraction (AF)[2] from the nondialyzable core of ribonuclease treated yeast RNA which possessed approximately 100 times the streptolysin inducing capacity of the starting RNA. They showed that the hemolysin produced (referred to as S′) by resting cocci in a simple synthetic medium appeared to be identical with Todd's streptolysin S. Bernheimer (1949) also presented evidence that the production of the hemolysin under these conditions resulted from a *de novo* synthesis, and that a peptide or a protein moiety was an essential constituent for activity. Simultaneously, Ito *et al.* (1948) concluded that the increase in hemolytic activity was due to interaction of resting cells and RNA. They suggested that the hemotoxic nucleic acid molecule was somewhat larger than the original nonhemolytic nucleic acid and that in the course of transformation intramolecular dehydration occurred. Interest in the "preformation" theory was revived by recent observations reported by Schwab (1956). He showed that extracts of sonic disrupted streptococcal cells contain appreciable amounts of an intracellular hemolysin. The hemolytic activity could be increased either by prolonging the period of treatment in the sonic oscillator or by temporary alteration of pH. The hemolysin was similar to streptolysin S in some features.

It was anticipated that insight into the mechanism of streptolysin S′ production might be gained by inhibition studies. This communication deals mainly with the effect of some amino acid analogues and antibiotics on this process. The bearing of the findings on the theory of *de novo* synthesis of the hemolysin is discussed.

MATERIALS AND METHODS

The experimental conditions of Bernheimer (1949) with minor modifications were employed in this study.

Organism. Strain C203S, a type 3, group A hemolytic streptococcus was used. Stock inocula were prepared from an 8 hr culture. Two ml aliquots were apportioned, sealed in thermal death point tubes and rapidly frozen in dry ice-acetone mixtures. They were maintained at −50 C until needed.

Culture medium and cultivation technique. A tube of the stock culture was thawed immediately before use and the contents inoculated into 50 ml of peptone-beef heart broth containing 0.01 per cent thioglycolic acid. After incubation for 15 hr at 37 C, the culture was transferred into 500 ml of the same broth and allowed to grow at 37 C for 7 to 8 hr. Cocci from 10 ml cultures were centrifuged in the cold and washed twice with cold sodium phosphate saline solution.

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[2] The following abbreviations are used: AF, active fraction obtained from the ribonuclease treated yeast nucleic acid according to the method of Bernheimer and Rodbart (1948); RNA, yeast ribonucleic acid.

Solutions. (1) Sodium phosphate saline, 24.0 g $Na_2HPO_4 \cdot 12H_2O$ and 4.5 g NaCl, were dissolved in 800 ml water, adjusted to pH 7.0 with HCl and the volume brought to 1 L.

(2) Potassium phosphate saline, 15.5 g K_2HPO_4 and 5.5 g KCl, were dissolved in 800 ml water, adjusted to pH 7.0 with HCl, and the volume brought to 1 L.

(3) The suspension medium was made up with 10 ml 0.05 M glucosamine hydrochloride solution, 2.0 ml 2.0 per cent $MgSO_4 \cdot 7H_2O$ solution, 1.0 ml 20.0 per cent KH_2PO_4 (previously adjusted to pH 7.0 with 1 M NaOH) and the volume brought up to 60 ml with water.

(4) The stock solution of the active fraction contained 20 mg of AF per ml. This solution was stored in the refrigerator and diluted 1 to 50 with water before use.

(5) Schwab's phosphate buffer, 100 ml 0.2 M Na_2HPO_4 was mixed with 200 ml 0.2 M NaH_2PO_4, the pH adjusted to 6.5 and the volume brought to 1 L.

Mixture of amino acids. This was made up of 19 amino acids including L-arginine·HCl, L-cystine, glycine, L-histidine·HCl, DL-isoleucine, L-leucine, L-lysine·HCl, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, DL-valine, DL-alanine, L-asparagine, DL-aspartic, and L-glutamic. The mixture was prepared according to Slade *et al.* (1951).

Purine-pyrimidine mixture. This mixture contained equimolar amounts of uracil, thymine, cytosine, adenine sulfate, guanine hydrochloride, xanthine and hypoxanthine.

Production of streptolysin S′. Washed cocci from 10 ml of culture were suspended in 0.6 ml of suspension medium and 0.1 ml of diluted AF solution. The suspension was incubated at 37 C for 90 min with occasional shaking. When a compound was tested, it was dissolved in the suspension solution and the proper volume added to the incubation mixture at the expense of an equal volume of the suspension medium. After incubation, the cocci were centrifuged in the cold, and the clear supernatant was assayed for hemolytic activity.

Determination of hemolytic activity. A seven tenths (0.7) per cent (vol/vol) suspension of washed rabbit erythrocytes was used as substrate. Determination of hemolytic activity was carried out by incubating 1.0 ml of increasing dilutions of the solution to be assayed with 1.0 ml of the erythrocyte suspension at 37 C. After 30 min, the remaining erythrocytes were sedimented by centrifugation and the hemoglobin content of the supernatant was measured colorimetrically. The hemolytic unit was taken as the amount of hemoglobin released from 0.5 ml of the erythrocyte suspension (complete hemolysis was achieved by minimal amount of saponin), in a total volume of 2.0 ml. Potassium phosphate saline was used for erythrocyte washing, suspension and dilution.

The percentage inhibition of streptolysin S′ production was calculated according to the formula: $\left(\frac{A - B}{A}\right) 100$, where A = hemolytic activity per ml of the uninhibited system, and B = hemolytic activity per ml of the inhibited system.

Each experiment was performed in triplicate and the average reported. The standard deviation was not above 10 per cent of the result in any case. Hemolytic activity below 25 units per ml was considered negligible.

When a compound was found to have some effect on the system studied, its ability to influence the activity of known solutions of streptolysin S′ was tested. None of the active compounds were found to interfere with the hemolytic action of the toxin. Therefore, the results observed stemmed from an impairment of streptolysin production.

Analysis of the endogenous amino acid pool. Heating a suspension of cells in water at 100 C was found to be a simple and accurate procedure for extracting the endogenous amino acids (Spiegelman *et al.*, 1955).

Cocci from 80 ml of an 8 hr culture were centrifuged, washed, suspended and incubated under the conditions specified. After the termination of the incubation period, the cells were resedimented and washed twice with sodium phosphate saline. The washed cocci were then heated with 0.2 ml of distilled water by immersion in a boiling water bath for 10 min. The debris was sedimented and the clear supernatant which contained the endogenous amino acids was kept frozen till analyzed. Fifty μL was spotted on a Whatman no. 1 filter paper and unidimensional chromatographic analysis was made using ethylene glycol monobutyl ether:water (2:1) as an ascending solvent. The method of Fisher *et al.* (1948), measuring the area developed after treatment with ninhydrin,

was used as a semiquantitative assay of the amino acid spots.

RESULTS

The dependence of streptolysin S′ production on the concentration of AF and the time of incubation was determined as a guide for choosing the conditions which were employed in these studies. The results are illustrated in figure 1, and indicate that streptolysin production varied with concentrations of AF below 0.05 mg/ml. When levels of AF of 0.05 mg/ml or more were used the largest amount of streptolysin activity was obtained after 90 min of incubation.

Effect of some amino acid analogues. Amino acid analogues are generally believed to inhibit protein synthesis by competing with the corresponding natural free amino acids. Consequently, the production of streptolysin S′ by resting cells should be inhibited by the analogues of any free amino acids which might be participating in the process.

β-DL-Phenylserine was the most potent of 10 amino acid analogues tested. Fifty per cent inhibition was obtained at a concentration of 1×10^{-3} M (figure 2). As is also indicated, the inhibition of 5×10^{-3} M β-DL-phenylserine was completely reversed by 1×10^{-3} M DL-phenylalanine. Similar results were obtained with β-3-DL-thienylalanine, another phenylalanine analogue, when incorporated at a higher concentration (table 1).

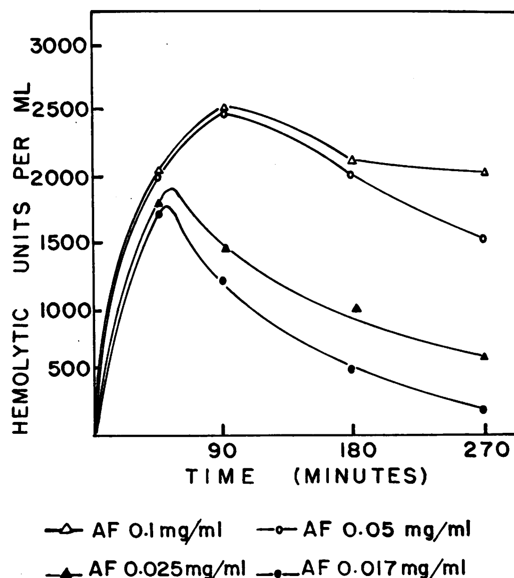

Figure 1. Production of streptolysin S′ at various concentrations of active fraction (AF) as a function of time.

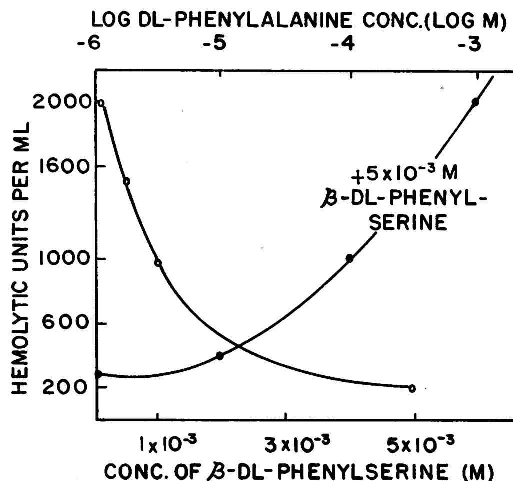

Figure 2. Inhibition of streptolysin S′ production by β-DL-phenylserine and its reversal with DL-phenylserine.

The following amino acid analogues were inactive, or brought about inhibition of 10 per cent or less at 1×10^{-3} M: DL-ethionine, 5-methyl-DL-tryptophan, β-2-DL-thienylalanine, ω-trifluoro-DL-threonine, DL-propargylglycine, *cis*-ω-chloro-DL-allylglycine, and γ-chloro-DL-allylglycine. At higher concentrations, DL-ethionine, and 5-methyl-DL-tryptophan caused appreciable inhibition of streptolysin S′ formation (table 1). With these analogues, however, addition of the corresponding natural amino acid failed to restore the production of hemolysin.

Effect of some purine and pyrimidine analogues. Woolley (1944) has shown that benzimidazole inhibits the growth of several yeasts and bacteria by virtue of being a purine antagonist. When tested in the present system, it caused 84 per cent inhibition of the streptolysin production at 1×10^{-2} M (table 1). However, the addition of adenine or guanine failed to restore the hemolytic activity, while DL-tryptophan or the 19 amino acid mixture caused only statistically marginal reversal.

The analogues 5-ethyluracil, 2-thiouracil, and azaguanine were without effect on the production of the hemolysin at 1×10^{-3} M.

Endogenous amino acid pool and the effect of starvation. As indicated by data in table 2, the

TABLE 1

Inhibition of streptolysin S′ production by amino acid analogues

Exp. No.	Addenda to Suspension Medium*	Hemolytic Activity	Inhibition
		units/ml	%
1	Uninhibited	2,000	50
	DL-Ethionine (2.5×10^{-2}M)	1,000	
	DL-Ethionine (2.5×10^{-2}M) + DL-methionine (1×10^{-2}M)	1,200	
	DL-Methionine (1×10^{-2}M)	1,800	
2	Uninhibited	2,000	60
	5-Methyl-DL-tryptophan (2.5×10^{-2}M)	800	
	5-Methyl-DL-tryptophan (2.5×10^{-2}M) + L-tryptophan (2.5×10^{-2}M)	150	
	5-Methyl-DL-tryptophan (2.5×10^{-2}M) + L-tryptophan (1×10^{-2}M)	400	
	L-Tryptophan (1×10^{-2}M)	800	
3	Uninhibited	2,000	50
	β-3-DL-Thienylalanine (1×10^{-2}M)	1,000	
	β-3-DL-Thienylalanine (1×10^{-2}M) + DL-phenylalanine (1×10^{-2}M)	2,000	
	DL-Phenylalanine (1×10^{-2}M)	2,000	
4	Uninhibited	2,500	84
	Benzimidazole (1×10^{-2}M)	400	
	Benzimidazole (1×10^{-2}M) + DL-tryptophan (1×10^{-2}M)	600	
	Benzimidazole (1×10^{-2}M) + 19 amino acid mixture (0.4%†)	550	
	Benzimidazole (1×10^{-2}M) + adenine sulfate (1×10^{-2}M)	350	
	Benzimidazole (1×10^{-2}M) + guanine-HCl (1×10^{-2}M)	400	

* Active fraction (AF) added to all tubes.
† Weight per volume.

optimum production of the hemolysin by resting cells is not dependent upon an exogenous nitrogen source although *Streptococcus pyogenes* is unable to synthesize 15 amino acids required for growth (Slade *et al.*, 1951). Analysis of the endogenous pool showed that cells grown under the conditions described contained approximately 1.5 per cent (dry weight) of intracellular ninhydrin reactive material. About half of this pool was utilized during the incubation of the resting cocci in the amino acid-free suspension medium for 2 hr, whether or not the AF was incorporated. Thus while the AF caused phenomenal enhancement of the hemolysin production, it did not bring about an appreciable change in the over-all rate of uptake of endogenous amino acids.

Halvorson and Spiegelman (1953) were able to demonstrate a relation between the enzyme synthesizing capacity of yeast cells and the level of their endogenous amino acid pool. This relationship was established by starvation and replenishment experiments. This approach was applied in a double incubation experiment to test the effect of preincubation under various conditions on the capacity of the cocci to produce streptolysin S′. After the initial incubation of 2 hr, the cells were sedimented by centrifugation, washed twice with sodium phosphate saline solution in the cold and suspended in a fresh supply of suspension medium for 90 min. The hemolytic activity in the supernatants of the first and second incubation media was assayed. The results are presented in table 3. It is apparent from the data of experiments 7 and 11 that while freshly harvested cells produced about 2,000 units of streptolysin S′ per ml, the yield from cocci preincubated in the amino acid-free suspension medium dropped to 400 units per ml. However, the drop does not seem to be due solely to the exhaustion of the intracellular amino acid supply. This is evident from the fact that the addition of the mixture of 19 amino acids caused, at best, statistically marginal restoration of the hemolysin production (experiments 8–10). It is interesting to note that cocci which produced 2,000 units per ml during the preincubation period in the

TABLE 2

Effect of amino acid mixture and purine-pyrimidine mixture on the production of streptolysin S′

Exp. No.	Addenda to Suspension Medium	Hemolytic Activity
		units/ml
5	None	Negligible
	AF (active fraction)	2,500
	Amino acid mixture*	Negligible
	Amino acid mixture* + AF	2,500
6	None	Negligible
	AF	2,500
	Purine-pyrimidine mixture†	Negligible
	Purine-pyrimidine mixture† + AF	2,000

* 0.2 per cent (w/vol) of the mixture.
† 5×10^{-4} M of each base.

presence of AF, still produced more hemolysin during the second incubation period than the cells preincubated in the absence of AF.

There is ample evidence in the literature that net protein formation is accompanied by a concomitant active nucleic acid synthesis. Thus nucleic acids or their precursors were found to stimulate enzyme formation (Gale and Folkes, 1955; Gale, 1956). In this system, however, a mixture of seven purines and pyrimidines failed to enhance the production of streptolysin S′ in the absence of AF, or augment the action of the latter when present (table 2).

Relative production of extracellular and intracellular streptolysins. Bernheimer (1949) has shown that only traces of preformed streptolysin exist in or upon the streptococcal cells. Recently, however, Schwab (1956) demonstrated the existence of appreciable amounts of an intracellular streptolysin which was difficult to differentiate from streptolysin S′.

The following experiment was performed to investigate any possible relation between the production of streptolysin S′ and the level of intracellular hemolytic activity. The current procedure was modified slightly to suit the conditions for optimum production of Schwab's intracellular hemolysin as well as of streptolysin S′.

Cocci from 100 ml of a 15 hr culture were washed twice with sodium phosphate saline solution in the cold, sedimented by centrifugation and then suspended in 6 ml of the suspension medium plus 1 ml of the dilute AF solution (or in 7 ml of the suspension medium in case the AF was eliminated). After incubating the mixture for 90 min at 37 C, the cocci were sedimented and the supernatant retained for the determination of the extracellular hemolytic activity. For the assay of the intracellular hemolysin, the washed cells from 100 ml culture were suspended in 10 ml of Schwab's phosphate buffer (pH 6.5) and placed in a Raytheon 9-kc sonic oscillator for 1 hr. The broken cell suspension was then centrifuged at 20,000 rpm for 30 min in the cold, the supernatant recovered and kept frozen until assayed. For the sake of comparison of data, Schwab's procedure for the determination of

TABLE 3

Effect of starvation on the capacity of Streptococcus pyogenes to produce streptolysin S′

	Experiment No.				
	7	8	9	10	11
First Incubation 2 hr					
Additions to suspension medium	None	None	+ Amino acid mixture*	+ Amino acid mixture*	+ AF†
Hemolytic activity (units/ml)	Negative	Negative	Negative	Negative	2,000
Second Incubation 90 min					
Additions to suspension medium	+AF	+ AF + amino acid mixture*	+ AF	AF + amino acid mixture*	+ AF
Hemolytic activity (units/ml)	400	400	450	450	1,000

* 0.2 per cent (wt/vol) of the mixture.
† Active fraction.

TABLE 4

Relative production of streptolysin S′ and intracellular hemolysin

Conditions of Incubation	Toxin	Hemolytic Activity	Total Yield of Toxin*
		units†/ml	*units†*
Unincubated	Intracellular	25	250
Incubated without active fraction	Intracellular	25	250
	Extracellular	10	70
Incubated with active fraction	Intracellular	50	500
	Extracellular	5,000	35,000

* From cocci from 100 ml culture.

† Schwab's hemolytic unit (Schwab, 1956).

hemolytic activity and his unit were adopted in this experiment.

The results obtained (table 4) indicate that in the presence of AF, the ratio of the yields of extracellular to intracellular hemolysin is equal to 70:1. It is also interesting to observe that the endogenous streptolysin of the cocci incubated in the presence of AF was double that of the cells incubated in the absence of AF. These data do not support a simple extraction mechanism for the appearance of the extracellular hemolytic activity but favor Bernheimer's thesis of active synthesis.

Effect of some antibiotics. Figure 3 illustrates the effect of various concentrations of streptomycin sulfate, chloramphenicol and aureomycin on the production of streptolysin S′ by resting cocci. Streptomycin was the weakest inhibitor of the three. The inhibition by chloramphenicol (at 1×10^{-4} M) was not reversed by DL-phenylalanine (1×10^{-3} M), indicating that the effect of the antibiotic is probably more complex than mere antagonism between the two substances as suggested by Woolley (1950). Gale and Folkes (1953) found that chloramphenicol inhibits protein synthesis without affecting nucleic acid formation.

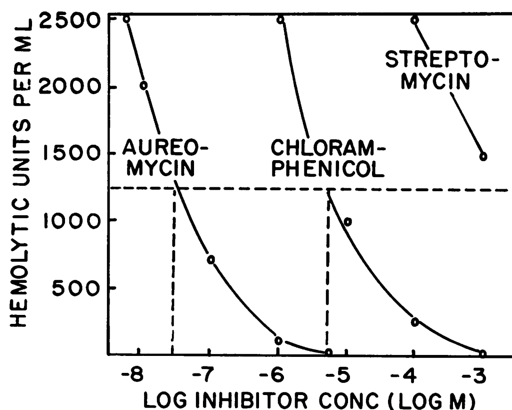

Figure 3. Effect of antibiotics on the production of streptolysin S′.

By far, the most potent inhibitor was aureomycin. Fifty per cent inhibition was observed at 5×10^{-8} M (0.02 μg/ml). It would seem to be active on a molecular level. Two other tetracyclines, namely terramycin and tetracycline itself, were similarly effective at the same concentration. Albert (1953) demonstrated the great avidity of the tetracyclines for several metallic cations. Preincubation of aureomycin (1×10^{-6} M) with Mg^{++}, Fe^{++}, Mn^{++} or sodium molybdate (5×10^{-5} M) did not reverse its inhibitory effect. Cu^{++} was found to cause almost complete inhibition of the production of streptolysin S′ at 5×10^{-5} M.

Penicillin G, although a potent bacteriostatic agent for growing streptococci, was completely inert up to a concentration of 1×10^{-3} M (373 μg/ml). Azaserine (30 μg/ml) was also inactive.

The clinical significance of these observations remains to be evaluated.

Further fractionation of AF. The possibility existed that the activity of AF is not due to its polynucleotide constituents but rather to a cofactor associated with it. In an effort to isolate such a factor, the concentrated solution of AF was extracted with *n*-butanol and with ethylacetate at pH 6.0, 7.0 and 8.0. In no case, however, was the activity extracted in the organic solvent.

Recently, Bendich *et al.* (1955) successfully fractionated deoxyribonucleic acid on a cellulose ion exchange adsorbent. The AF was successfully fractioned using this adsorbent. A detailed account of the distribution of the polynucleotide (as measured by 260 mμ absorption) and the hemolysin-inducing activity will be reported later.

DISCUSSION

The data presented here and the work of Bernheimer (1949) leave little doubt that the appearance of streptolysin S′ in the suspension medium of resting streptococci depends upon an active metabolic process rather than a physical extraction of a preformed intracellular hemolysin.

The presence in freshly harvested cocci of an appreciable intracellular free amino acid pool would readily explain the lack of dependence of streptolysin production on a source of exogenous amino acids. The inhibition by β-DL-phenylserine and its reversal would imply a role for phenylalanine in hemolysin elaboration.

The experimental data available do not provide sufficient evidence to evaluate adequately the role of the active fraction (AF) in the production of streptolysin S′. It is possible that a polynucleotide is either a major moiety or a cofactor for the hemolysin. The concept that polynucleotides may be endowed with enzymatic or coenzymatic activity is a challenging one. Binkley (1952) isolated cysteinylglycinase from pig kidney. The enzyme appeared to be largely a polynucleotide.

However, the data are also consistent with an enzyme stabilization mechanism. The extreme lability of streptolysin S and S′ has been reported (Herbert and Todd, 1944; Bernheimer, 1950). The AF might be acting as a protective agent for the labile hemolysin. The phenomenon of protein stabilization by nucleic acids is supported by the findings of Greenstein and Hoyer (1950).

Reference has been made to the theory of active cosynthesis of enzymes and nucleic acids. As indicated, the production of streptolysin S′ differed from the induced enzyme synthesis in several respects. However, this difference can not be taken as a direct evidence against such a mechanism for the hemolysin formation.

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SUMMARY

The production of streptolysin S′ by resting cells of *Streptococcus pyogenes* strain C203S was studied. Below 0.05 mg/ml of active fraction (AF) the maximum yield of hemolysin was dependent upon the concentration of AF. Among 10 amino acid analogues tested, β-DL-phenylserine was the most potent inhibitor. Fifty per cent inhibition was obtained by 1×10^{-3} M of the antagonist which could be completely reversed by DL-phenylalanine. Benzimidazole caused 84 per cent inhibition at 1×10^{-2} M.

Freshly harvested cells were found to contain about 1.5 per cent (dry weight) of endogenous amino acid pool which was partially depleted by incubation in a nitrogen free medium. The AF did not appreciably enhance the over-all uptake of the intracellular amino acids.

Under optimum conditions of the production of streptolysin S′, the ratio of the yields of extracellular to intracellular hemolysin was 70:1.

The effect of streptomycin, chloramphenicol, aureomycin, terramycin and tetracycline was tested. The last three antibiotics were extremely potent inhibitors. Penicillin G and azaserine were inactive.

These data support Bernheimer's view that the appearance of hemolytic activity in the suspension medium of resting cocci involves a synthetic process.

Two mechanisms were advanced to explain the role of AF in the production of streptolysin S′.

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