

THE CELLULAR FORMS OF STREPTOLYSIN S AND
THEIR RELATIONSHIP TO EXTRACELLULAR STREPTOLYSIN S

by

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INTRODUCTION

Extracellular SLS-like Hemolysins

Streptolysin S (SLS) is the most potent hemolysin known (14). It is the oxygen-stable hemolysin (104) made by Group A, C, E, G, H, and L streptococci (39). It differs from streptolysin O (SLO) in that 1) the activity once lost cannot be regenerated by reducing agents (48) (15), and 2) it is not antigenic (39). No special substances are required for SLO production (10), but SLS formation depends upon the addition of any of several different substances to the growth media or to harvested cells. The role of these additives on SLS formation or production is uncertain. A brief history of SLS will be presented to illustrate the dependence of SLS upon these additives and to present a nomenclature of SLS.

Although it was known in 1895 (71) that streptococci produced at least one hemolysin, it was not until 1938 that Todd (104) showed that streptococci produced two hemolysins, SLS and SLO. The two hemolysins differed in several ways including formation under different growth conditions, antigenicity, and in ability to be reactivated. SLS was produced only in the presence of serum, while serum neither promoted nor inhibited SLO production. Optimal production of SLS occurred in the absence of glucose; SLO production was not inhibited by glucose. SLO was antigenic while SLS was not. SLO was inactivated by oxygen, but could be activated by reducing agents. SLS was not inactivated by oxygen and not reactivatable by reducing agents once

the activity was lost by storage, by brief heating, or by treatment with dilute acid. Todd also noted that the hemolysis by SLS took longer than that by SLO and, therefore, he termed the SLS a "slow" hemolysin. Whether the serum needed to obtain SLS was necessary to stimulate production of SLS, to extract SLS as suggested by Weld (109), or to stabilize SLS was not determined. Herbert and Todd (48) concluded from physicochemical studies that the lipoproteins from the serum were associated with, or part of, the hemolysin because of the inactivation of partially purified hemolysin by treatment with hydrophilic organic solvents. The SLS formed in the presence of serum will be referred to as serum-SLS.

It was later found that yeast nucleic acid could replace serum (77) and that the hemolysin formed had many of the characteristics of serum-SLS (15). The hemolysin exhibited a delay in the onset of hemolysis, was irreversibly inactivated by dilute acid or brief heating, was non-antigenic, and its production was inhibited by glucose. Interestingly, Bernheimer and Rodbart (15) found that, if the medium contained 3×10^{-3} M glucose, 5×10^{-5} M maltose stimulated SLS production. Higher concentrations of maltose were not effective and the yield of SLS was often decreased. The necessary component in the nucleic acid mixture was found to be ribonucleic acid, and its SLS-promoting effect was markedly enhanced by treatment with pancreatic RNase (15). SLS was formed either with RNA or the RNase-resistant RNA-core by both growing cells or cells harvested and suspended in a defined resting cell medium. The medium, referred to as Bernheimer's basal medium (BBM) contained a fermentable carbohydrate such as

maltose, Mg^{2+} , K^+ , phosphate ions, cysteine as a reducing agent, and RNA-core (9). Bernheimer (9) concluded that "the development of streptolysin S in a resting cell system depends neither upon autolysis nor upon physical extraction of preformed toxin but upon toxin synthesis". His conclusion was based upon the effect of various enzyme inhibitors and temperatures upon the "development" of SLS. The fact that the hemolysin was inactivated by several proteolytic enzymes led to the conclusion that at least part of the hemolytic activity was due to a protein. However, other than showing some of the RNA-core was hydrolyzed during incubation with streptococci, the role of RNA-core in promoting hemolysin production or stabilizing hemolysin was undetermined. The hemolysin formed in the presence of RNase-resistant RNA-core will be termed RNA-core SLS and that formed in the presence of RNA will be termed RNA-SLS.

Another hemolysin with characteristics of SLS was demonstrated by incubating washed streptococci with red blood cells (109) (92) (40) (44) (100) and observing the resultant hemolysis. Since no extra-cellular hemolysin could be demonstrated, this hemolysin was referred to as Cell Bound Hemolysin (CBH). Demonstration of maximal hemolytic activity was dependent on the presence of maltose, Mg^{2+} , and cysteine. In the absence of maltose, 80% of the maximal hemolytic titer was still obtained. Separation of streptococci from erythrocytes by Millipore filters or a thin layer of agar prevents lysis by this hemolysin (45) (67). Ginsburg et al. (40) (41) have suggested that this may be the hemolysin released from streptococci with detergents, such as Tween-80 or Triton, serum albumin, and lipoproteins. The effective portion of albumin was found to be lipid contaminating the albumin

so that the protein was not responsible for the effect described (40). The formation or extraction of this hemolysin, which will be referred to as Tween-80-SLS or albumin-SLS, was maximal in the presence of glucose (40) or maltose (44), cysteine, Mg^{2+} , and albumin or Tween-80. Although RNA-core SLS formation was very sensitive to UV irradiation (40) and to the action of β -DL phenylserine (115), Tween-80-SLS or albumin-SLS formation was not (40). These results, plus the fact that in the resting cell system albumin-SLS and serum-SLS were formed earlier than RNA-core SLS (41), led Ginsburg to state that albumin, Tween-80 and serum may remove a preformed hemolysin (CB) from streptococci (40). However, the dependence of hemolysin formation on glucose or maltose and the fact that he found no cellular hemolysin after sonication suggested to him that the hemolysin was being synthesized and then removed (40) (43) (39).

All of these hemolysins are similar in their inhibition by two inhibitors. They are all inhibited by serum lipids (39), especially lipoproteins (50), which might explain some of the earlier unconfirmed reports of inhibition of SLS by antibody. The factors important in the inhibitory effect of lipoproteins are their concentration and physical state, since lipoproteins under optimum conditions promote SLS formation (48). All the SLS-like hemolysins, with the possible exception of serum-SLS of Todd, are inhibited by the dye, trypan blue. Trypan blue, however, can also promote SLS formation (44) under appropriate conditions. RNA-core is also an inhibitor when used in excess amounts (47).

These extracellular hemolysins differ in several ways (Table 1). Because of these differences, the question has arisen whether different

Table 1

Properties of Extracellular SLS-like Hemolysins

<u>SLS Preparation</u>	<u>Alkali Stability</u>	<u>Acid Stability</u>	<u>Heat Stability</u>	<u>Precipitation by Ammonium Sulfate</u>	<u>Chloroform Extraction</u>
Serum SLS of Todd	ND	Destroyed by dilute acid	50% lost in 10 hr at room temperature; 50% lost in 3 days at OC	Precipitated; % not stated	40% inactivated by one extraction
RNA-SLS from growing cells	ND	Destroyed by dilute acid	Inactivated by "brief heating"	ND	ND
RNA-SLS or RNA-core SLS from resting cell system	Inactivated at pH 11	Stable at pH 3	Stable at 3C for one week; most activity lost after a few hr at 25C	ND	Stable
Albumin-SLS	Stable at pH 11	Inactivated at pH 3	Most activity lost after a few hr at 25C	ND	ND
Tween-80 SLS	Stable at pH 11	Stable at pH 3	Most activity lost after a few hr at 25C	ND	ND

ND = not done or reported

Table 1 (Cont.)

<u>SLS Preparation</u>	<u>Ether Extraction</u>	<u>Papain Digestion</u>	<u>Trypsin Digestion</u>	<u>Ribonuclease Digestion</u>	<u>References</u>
Serum SLS of Todd	100% inactivated by one extraction	ND	ND	ND	48,104
RNA-SLS from growing cells	ND	ND	ND	ND	15
RNA-SLS or RNA-core SLS from resting cell system	Alcohol-ether precipitates; no loss in activity	Destroys	No effect	Destroys	9, 42, 97
Albumin-SLS	Alcohol-ether destroys	Destroys	No effect	No effect	42
Tween-80 SLS	ND	Destroys	No effect	No effect	42

ND = not done or reported

SLS molecules are produced by streptococci. Another possibility is that only one SLS hemolytic moiety is produced, but it associates with any of several protecting agents or carriers (42).

Little is known about the regulation of synthesis of streptolysin S except that its formation was decreased in the presence of glucose (38) (15). The repressive effect of glucose on enzyme or toxin production is often referred to as the "glucose effect" (32) (68). Okamoto et al. (70) studied the effect of glucose on RNA-core SLS formation in some detail. They found that a 0.3% or greater concentration of glucose in the growth medium totally inhibited RNA-core SLS production, as measured by the amount of RNA-core SLS accumulated after 20 hr of growth. At 0.1% glucose concentration, the amount of RNA-core SLS produced was half that produced in the absence of added glucose. When cocci were grown in glucose medium and tested for hemolysin production in the resting cell system, no RNA-core SLS production occurred. When they were transferred to a non-glucose containing growth medium the subsequent generations did produce RNA-core SLS. When cells were grown in non-glucose-containing broth and then transferred to a resting cell system which contained glucose, they continued to produce RNA-core SLS. Thus glucose inhibited production of RNA-core SLS prior to, but not after, the onset of production. He also noted that the glucose greatly enhanced the growth of streptococci. Reference was made in Okamoto's paper to an observation by Shoin that maltose did not have an inhibitory effect on RNA-core SLS production; no published reference was given for this observation.

Practically all of the work to determine the composition of any SLS hemolysin has been done on RNA-core SLS. Unfortunately, the majority of research has been done on the RNA-core and not the bacterial factor or hemolytic moiety involved. The RNA-core has been highly purified and is still active in promoting RNA-core SLS formation (61). Therefore, no contaminating substances, such as found with unpurified serum albumin, are responsible for the RNA-core SLS-promoting effect. The guanylic acid residues of RNA-core were shown to be most important in promoting SLS formation (103). Treatment of RNA-core by RNase T (from Taka Diastase) which releases 3'-guanylic acid from RNA-core markedly decreased the effectiveness of RNA-core in RNA-core SLS production (39). Synthetic polyguanylic acid also had excellent SLS-promoting activity (78). Koyama (58) determined that part of the RNA-core SLS is composed of oligoribonucleotides. He showed that this oligoribonucleotide portion can be dissociated into heterogeneous components with 8 M urea (62). Addition of 8 M urea to RNA-core SLS destroyed its hemolytic activity.

The other portion of RNA-core SLS has been presumed to be a peptide, because the activity of RNA-core SLS is destroyed by papain and chymotrypsin (15) and because highly purified RNA-core SLS contains amino acids (30) (57) (60). Koyama (57) published an amino acid composition of RNA-core SLS purified by zone electrophoresis and DEAE-cellulose column chromatography. There were twelve amino acids present, but no aromatic or sulfur-containing amino acids. Since he did not determine how many polypeptides were associated with the RNA-core, it was uncertain if all of the twelve amino acids are part of the peptide

portion of RNA-core SLS. The apparent assumption that the hemolytic moiety of RNA-core SLS was the only peptide binding to RNA-core was never tested. The degree of purification was reported only as the change in the HU/optical density at 260 nm, and not on the basis of protein. The reason given was that there was not enough protein to be measured by the Folin colorimetric reaction. Based on Koyama's data that the molar ratio of polypeptide to oligoribonucleotide was 0.3, Bernheimer (12) has calculated the molecular weight of the polypeptide moiety to be 2800.

It has been suggested that RNA-core SLS composed of an aggregated oligoribonucleotide (58) plus a peptide component (57). The fact that streptolysin S has a slower electrophoretic mobility than the purified oligoribonucleotide portion supports the idea that RNA-core SLS is a complex of peptide and oligoribonucleotide (58). The fact that once dissociated from the RNA-core, the polypeptide has no activity suggests that the RNA-core is necessary for the stability of peptide portion (58).

Although the role of the necessary additives, such as RNA-core, in inducing or stimulating SLS production is uncertain, they have been shown to function as carriers of the hemolytic moiety. Ginsburg (41) showed that the chromatographic behavior of an SLS hemolysin on DEAE-cellulose was exactly the same as the material added to promote its formation. For example, RNA-SLS eluted at the same salt concentration as RNA, Tween-80 SLS eluted where Tween-80 eluted, and albumin-SLS eluted where albumin alone did. He then showed that the hemolysin could be transferred from one carrier to another, and

that after transfer the hemolysin-carrier complex had the chromatographic characteristics of the second carrier (42). For example, 2000 hemolytic units (HU) of albumin-SLS were incubated with RNA; 50 HU eluted at the elution volume of albumin and 100 HU eluted at the elution volume of RNA. Similarly, the incubation of albumin-SLS with Tween-80 resulted in a mixture of Tween-80 SLS and albumin-SLS. This phenomenon of transfer was also confirmed by electrophoresis of mixtures of hemolysins and carriers on agar plates. Taketo and Taketo (98) have confirmed this by studies using methylated-albumin chromatography. When the hemolysin was transferred from albumin to RNA, it became stable at pH 3 and labile at pH 11. Although the hemolysin before transfer was not inactivated by RNase, after transfer it was. The transferred hemolysin then exhibited the characteristics of hemolysin originally obtained with the second carrier (Table 1). Ginsburg (39) proposed that the hemolytic moiety had a greater affinity for the extracellular carriers than for a cellular component when in the cell-bound form (40). He suggested that the hemolysin was synthesized by the cell and remained associated with the cell until removed by a substance such as a red blood cell or carrier to which it has greater affinity. The only common basis for binding of the proposed peptide hemolytic moiety by all the carriers was suggested to be hydrogen bonding (39).

Several facts led Ginsburg (39) to conclude that "The appearance of hemolysin in the suspension medium of resting streptococci depends upon an active metabolic process rather than on a physical extraction of a preformed intracellular hemolysin". First, streptococci possess

a large intracellular pool of amino acids (37) so that SLS could be synthesized. Streptococci producing SLS utilize about half of this pool during two hours (115). The absence of RNA-core did not change the rate of utilization of the amino acids. If streptococci were incubated for two hours without RNA-core, they produced only 25% as much SLS as when RNA-core was added, and the addition of a mixture of 19 amino acids did not restore SLS formation to its previous level. Although the Youthan and Barkulis data do not negate an active metabolic process, they did not prove that SLS is synthesized during the resting cell incubation. Koyama et al. (60) stated that de novo synthesis of the lysin did occur, because they could demonstrate the incorporation of C¹⁴-amino acids into RNA-core SLS. However, since it was uncertain just how many peptides were associated with RNA-core, one cannot be sure that the label was incorporated into the hemolytic moiety of SLS. The second indication that SLS formation depended upon metabolic processes was that the appearance of SLS in the extracellular medium was inhibited by enzyme poisons such as mercuric chloride, arsenite, cyanide, and azide (9). This also did not prove that SLS is synthesized during the resting period. Finally, Ginsburg doubted that the appearance of SLS in the resting cell media depended only on extraction of preformed hemolysin by the various carriers since neither Bernheimer (9), Youthan and Barkulis (115), nor Ginsburg and Grossowicz (40) could find appreciable amounts of preformed hemolysin after sonicating streptococci.

Cellular SLS-like Hemolysins

Schwab (87) did, however, report the presence of an intracellular hemolysin released on sonication of a Group A streptococci. Although he called the hemolysin "intracellular", he gave no proof that it was. Since true intracellular materials are located within the cell membrane (84), proof that the hemolysin was intracellular depended upon the demonstration that hemolysin was present within the cell membrane. Since sonication solubilizes cell membrane proteins (86) and degrades the cell wall (90), the sonicate of Schwab contained more than intracellular material. The cells to be sonicated were grown in a rich medium containing beef heart, proteose peptide, 0.3% glucose and other constituents for 15 hours and then harvested. It was not stated to what stage of growth the 15 hours corresponded. He did state that the length of treatment of cells by sonication markedly affected the hemolytic titer. Prolonged sonication by a Raytheon 9KC sonicator for periods up to 8 hours continued to increase the titer of hemolysin, even though essentially all cells had been broken by 60 minutes. He explored this phenomenon by sonicating a cell suspension for 30 minutes, centrifuging the sonicate at 20,000 RPM for 20 minutes in a Spinco 40 rotor, and resonicating the supernatant and sediment. The sediment contained 2 HU prior to resonation and 28 HU afterwards; the supernatant contained 20 HU prior to resonation and 41 HU afterwards. He concluded from this "that the increase in activity was due to elution of lysin from the cell debris and further dissociation of soluble complexes, yielding more

active material". No data were given, however, on whether whole cells were present in the debris, so that whether the increase in titer of the sediment was due to further disruption of cocci is uncertain. Although he suggested that the hemolysin may have been a structural component of the cell, he did not further explore this possibility. The hemolysin lost 35% activity at 4C in 24 hours and 78% in 120 hours, but could be partially reactivated by lowering the pH to 2.0 and then elevating it back to neutrality.

Schwab showed that the hemolysin was similar to RNA-core SLS in several ways, but also differed in other ways (88). Neither was inhibited by rabbit-anti-streptolysin O or cholesterol. Neutralized glutathione (4×10^{-2} M) which activated SLO had no effect on either. Both were inhibited by Ca^{2+} , leithitin, and normal rabbit serum. The rate of hemolysis at various temperatures was the same for both. They differed, however, in the ratio of hemolytic activity with sheep and rabbit red blood cells. The sheep cells were more sensitive to lysis by RNA-core SLS than by Schwab's hemolysin. He compared the amount of "intracellular" hemolysin obtained from strain C203U which makes very little RNA-core SLS to that of strain D-58 which does make RNA-core SLS. Strain C203U contained 11 HU of "intracellular" hemolysin, and formed 3.5 HU of RNA-SLS per mg cell N after 3 hours incubation at 37 C. Strain D-58 contained 38 HU of "intracellular" hemolysin, and formed 520 HU of RNA-SLS per mg cell N after 3 hours incubation at 37 C. Since the ratios of RNA-SLS to intracellular hemolysin differed (C203U ratio 0.33; D-58 ratio 13.5), he concluded that the significant difference in amount of intracellular hemolysin was evidence for distinction of RNA-SLS from intracellular hemolysin.

These two hemolysins also differed in their buffer and pH optima for hemolysis, and in the relative effect of Na^+ and K^+ on their hemolytic activity. They also differed in stability, since intracellular hemolysin was more labile at 4C. All of these facts led Schwab to conclude that intracellular hemolysin was distinct from streptolysin S. This conclusion was strengthened by his later finding that intracellular hemolysin was weakly antigenic (89), although RNA-core SLS is not.

Taketo and Taketo (97) confirmed the presence of a cellular hemolysin released by sonication. They also could release the hemolysin by grinding frozen cells with quartz sand. They centrifuged the broken cells at 30,000 X g for 20 minutes and 50 minutes and then at 105,000 X g for 60 minutes. The clear supernatant was then treated with 2.5% streptomycin sulfate for 15 minutes and then centrifuged at 15,000 X g for 20 minutes. The supernatant was then treated with 60% saturated ammonium sulfate for 15 minutes and centrifuged at 15,000 X G for 20 minutes. The precipitate was then dialyzed and the retentate referred to as AS fraction. This fraction contained hemolytic activity which could be increased 3.5 times by incubation with RNA-core at 37C with or without Bernheimer's basal medium. Chloramphenicol, 5-methyltryptophan, ribonuclease, KCN, dinitrophenol, sodium arsenate, and sodium arsenite did not inhibit the increase. The increase was dependent on the temperature and time of incubation. No increase occurred unless phosphate was present. Ethylenediaminetetraacetic acid reduced the increase. Preincubation of AS at 37C for 60 minutes and shaking with chloroform

at OC both decreased the hemolytic titer of AS and prevented the increase in titer upon incubation with RNA-core. Irradiation with UV had no effect on either the titer or increase in titer upon incubation. The increase in titer occurred only with RNA-core and not with untreated yeast RNA. The fact that the increase in titer was dependent upon the initial hemolytic titer and not on the protein content suggested to them that the increase in titer was due to activation or release of preformed hemolysin.

The incubation of AS with RNA-core resulted in the presence of a new hemolysin which had a different migration on zone electrophoresis and a different elution volume on a methylated albumin column, than AS hemolysin. The migration pattern and elution volumes did, however, correspond to those of RNA-core SLS. The new hemolysin and RNA-core SLS were said to behave identically on DEAE-Sephadex, DEAE-cellulose, and CM-cellulose columns. All the activity of AS hemolysin and the ability to obtain an increase in hemolysin using AS fraction was lost on these latter columns. However, they later showed (98) that the new hemolysin eluted after AS hemolysin on G-200 Sephadex. The new hemolysin was like RNA-core SLS in several characteristics (97) (summarized in Table 2): both were precipitated by 0.1% protamine sulfate (as was AS hemolysin), both were stable in dilute acetic acid or dilute NH_4OH , both were inhibited by trypan blue, both exhibited a long lag period prior to hemolysis, both were inactivated by pronase (as was AS hemolysin), neither was substantially inactivated by trypsin (50% of AS hemolysin was), and neither was inhibited by

Table 2

Comparison of AS Hemolysin, the Resultant Hemolysin after Incubation of AS Hemolysin with RNA-core, and RNA-core SLS

<u>Preparation</u>	<u>Chloroform Extraction</u>	<u>Protamine Sulfate(1%)</u>	<u>Dilute Acetic Acid or NH₄OH</u>	<u>Trypan Blue</u>	<u>Character Hemolysis</u>	<u>Pronase</u>	<u>Trypsin</u>	<u>Ribonuclease</u>
AS Hemolysin	I	P	NE	Part I	F	I	50% I	NE
Resultant Hemolysin	NE	P	NE	I	D	I	I	NE
RNA-core SLS	NE	P	NE	I	D	I	I	NE

I = inactivated or inhibited

NE = no effect

P = precipitated

D = delayed hemolysis

F = fast hemolysis

ribonuclease. The AS hemolysin preparation also contained an SLO-like hemolysin which was inhibited by antistreptolysin O and which caused hemolysis without an observable lag (97). Although the addition of RNA stimulated the formation of RNA-core SLS found in the growth medium, it had no effect on the level of AS hemolysin. After the addition to the growth medium of 1.0% glucose, which is known to inhibit RNA-core SLS production, very little AS hemolysin was present in the cell sonicate and no increase in hemolytic activity occurred on incubation of AS with RNA-core.

Taketo and Taketo (97) (99) concluded that the new hemolysin formed was identical to RNA-core SLS. They believed that the new hemolysin was formed by transfer of the hemolytic moiety of AS hemolysin from a cellular-carrier to RNA-core. They proposed that the transfer may involve the release of the hemolytic moiety from "masking substances associated with the cellular carrier, which resulted in the simultaneous increase of hemolytic activity". Since the addition of RNA-core did not induce an increase in synthesis of AS hemolysin, they further postulated that RNA-core may "induce the successive synthesis of the hemolytic moiety of AS hemolysin by removing out the produced hemolytic moiety through complex formation, thus resulting in an accumulation of RNA-core SLS in the media." They referred to AS hemolysin as intracellular streptolysin S, but, since it was obtained by sonication, the intracellular nature has not been proved. Contrary to Schwab, they reported that strain C203U which did not produce RNA-core SLS did not contain "intracellular" SLS. Finally, they stated that "it appears from the results of the

chromatography that intracellular-SLS is larger in molecular weight than RNA-core SLS, or liable to aggregate" (99).

Several Japanese investigators have used an enzymatic procedure to lyse Group A streptococci to obtain protoplasts and subcellular fractions effective in production or release of SLS (60) (73) (93) (94) (95). Maruyama, Sugai, and Egami (73) reported that protoplasts in the presence of RNA-core, BBM, and succinate formed RNA-core SLS. However, they gave no proof that the hemolysin formed was RNA-core SLS. They did not show that the hemolysin was inhibited by trypan blue and was not inhibited by antistreptolysin O. Although they showed production of hemolysin in the presence of purified and unpurified RNA-core, they did not present data from any control experiments to determine if hemolysin was produced in the absence of RNA-core. The protoplasts did form hemolysin more rapidly than did whole cells, but whether there were the same number of whole cells as there were protoplasts in the incubation mixtures was not stated. If the protoplast was incubated in the absence of succinate, no hemolysin was formed. They used this fact as proof that the hemolysin formation was not due to remaining intact cells (74). Two of the authors (95) then went on to show that the sedimentable fraction (10,000 RPM for 10 min) of protoplasts lysed in hypotonic buffer form RNA-core SLS in the presence of a mixture of RNA-core, succinate, thioglycollate, Mg^{2+} , phosphate, and maltose. Three-hundred hemolytic units were formed by 2 mg (dry weight) of ghosts in 60 minutes. No hemolysin was formed in the absence of RNA-core, which

was the only evidence presented that the hemolysin formed was RNA-core SLS. They did not show that the hemolysin formed was not inhibited by antistreptolysin O antiserum, even though the reducing agent would activate SLO. The presence of any active hemolysin in the protoplast cytoplasm was not reported. It is important to note that after lysis of the protoplasts the sediment was a highly viscous clump which was dispersed with DNase. It was later shown (94) such sediment, referred to as the ghost fraction, even after washing, contained 36% of the RNA of intact cells. The ghost fraction, which contained electron dense material attached to transparent membranes, was a heterogeneous fraction. No data were given to prove the absence of whole cells or protoplasts in the ghost preparation.

Sugai (94) further studied the ghost fraction to determine optimum conditions for hemolysin production. It was noted that if the fraction was washed with 0.06 M phosphate buffer that all streptolysin S-forming activity was lost. Apparently the wash supernatant was not tested either for hemolytic activity or hemolysin-forming potential. If the wash contained Mg^{2+} and sodium succinate instead of phosphate, optimal hemolysin production was obtained from the ghost fraction. The formation of hemolysin was inhibited both by 1×10^{-3} M chloramphenicol and 1×10^{-3} M- β -DL phenylserine, a potent inhibitor of RNA-core SLS production by whole cells. The addition of a mixture of 17 different amino acids enhanced production of hemolysin almost 7.5 fold. Sugai concluded from his studies that

the RNA-core SLS production appeared to be carried out by a process resembling protein synthesis. Since succinate enhanced the formation of hemolysin in his system, but inhibited production of RNA-core SLS by whole cells, he concluded that the ghosts were the structures responsible for hemolysin production. However, he offered no proof that neither whole cells nor intact protoplasts were present. It is possible that the very small production of hemolysin could have been due to either of these structures. It was later reported that ATP could replace maltose in the incubation medium of the ghost fraction to obtain hemolysin (60). However, the data show that ATP is only one-third as effective as maltose and that the hemolysin yield from the incubation mixture was very small.

Sokawa and Egami (93) later reported that the hemolytic component of streptolysin S was released by ATP and Mg^{2+} from the protoplast membrane fraction in the presence of RNA-core. However, this fraction was prepared by the same procedure as that used by Sugai (94) and, therefore, was more properly designated as the ghost fraction, which is heterogeneous. A more serious objection to the data in their paper was that whole cells probably remained in the ghost fraction. They did not present data to show that whole cells did not remain in their preparation. In their Materials and Methods section, however, they reported that not all the cells were lysed when treated with muralysin, but described no procedure to remove whole cells from their preparation. Forty HU of an hemolysin with SLS-like characteristics was released from 2 mg of their preparation in the presence of succinate, $MgSO_4$, KH_2PO_4 , thioglycollic acid, RNA-core,

and ATP. The release was both time and temperature dependent. No hemolysin was produced if ATP and Mg^{2+} were omitted from the mixture. GTP, UTP, and CTP could replace ATP, but the monophosphates, glucose, and maltose could not. Mn^{2+} could replace Mg^{2+} and Tween-40 could replace RNA-core. Trypsin abolished the appearance of the hemolysin but RNases, chloramphenicol, actinomycin D, and chemicals such as sodium fluoride had no effect. Preincubation of their preparation for 10 min at 37C in the absence of ATP or of ATP and RNA-core caused a 50% loss in potential hemolysin. Preincubation of their preparation in the presence of the complete incubation mixture or with only RNA-core deleted caused a 90-100% loss in potential hemolysin. Their preparation in the presence of ATP and Mg^{2+} did lyse rabbit erythrocytes. These results suggested that the hemolytic component was released by ATP and Mg^{2+} and was stabilized in the presence of RNA-core or Tween-40. If cells were preincubated at 37C in 6.7×10^{-2} M phosphate buffer for 15 minutes, the ghost fraction no longer contained potential or releasable hemolysin. If maltose or maltose and RNA-core was present during the preincubation, the ghost fraction subsequently isolated did contain potential or releasable hemolysin. However, when cells in the resting cell system containing maltose were preincubated with chloramphenicol, puromycin, or streptomycin, the ghost fraction contained little potential or releasable hemolysin. Trypsin under the same conditions had little effect. Sokawa and Egami believed that the data indicated "that the hemolytic component may be synthesized by resting cells by using maltose as an energy

source and bound to the membrane fraction in some form." They thought that the destructive effect of trypsin on the isolated ghost fraction was due either to destruction of enzymes necessary for the release of hemolysin or due to destruction of the hemolysin.

It must be emphasized that the ghost preparations of Sugai (94) and Sokawa and Egami (93) were not pure. The only evidence that the fractions contained membrane was the electronmicroscopy data of Sugai; Sokawa and Egami (93) presented no such data. Therefore, there is no definitive proof that streptolysin S is either formed at or releasable from the membrane of streptococcal cells.

None of the work by any of the Japanese researchers proved that streptolysin S is an intracellular substance. The data of Sugai (94) and Sokawa and Egami (93) do not rule out the possibility that potential SLS may be associated with contaminating cell wall material. Even if SLS is produced at the cell membrane, that does not provide evidence that SLS is necessarily intracellular. It could be made at the membrane and immediately released into the periplasmic space between the cell wall and cell membrane, be incorporated into cell wall, be excreted into the growth medium, or accumulate on or in the membrane. None of these locations are intracellular.

It is difficult to compare the amount of hemolysin obtained from subcellular fractions by Sugai and Egami (95), Sugai (94), and Sokawa and Egami (93) to the amount of extracellular RNA-core SLS hemolysins obtained by Bernheimer (9) and Koyama and Egami (61).

Sugai (94) noted that the amount of hemolysin he obtained from protoplast ghosts was 10-20% of that obtainable from whole cells under similar conditions. However, he stated later in the same paper that the conditions used to obtain hemolysin from ghosts inhibited production of RNA-core SLS by whole cells. Sugai and Egami (95) obtained a total of 300 HU of hemolysin in 60 minutes from 2 mg (dry weight) of ghosts. Sokowa and Egami (93) reported that at the optimum time of 20 minutes they obtained 40 HU of hemolysin from 2 mg (dry weight) membranes (more correctly called ghosts). Both groups (94) (93) used purified RNA-core. Bernheimer (9), using unpurified RNA-core, obtained 620,000 HU of RNA-core SLS in 3 hours from 350 mg (dry weight) cocci in BBM, while Koyama and Egami (63) obtained 2,800,000 HU RNA-core SLS from cells suspended in BBM for 60 minutes. They used the same medium and growth conditions and harvested their cells at the same time as Bernheimer, but harvested 4 l cells instead of 5 l as used by Bernheimer and used purified RNA-core in the resting cell medium. On the assumption that they harvested 280 mg dry weight of cocci, they obtained 10,000 HU/mg dry weight cocci while Sugai and Egami (95) obtained 150 HU/mg dry weight ghosts. This assumption may, of course, not be correct.

None of the Japanese workers who used muralysin to prepare protoplasts or lyse cells have reported the presence of an active cellular or intracellular hemolysin. Yet both Schwab (87) and Taketo and Taketo (97) reported that active cellular ("intracellular") SLS-like hemolysins are present. Whether this is a true contradiction is uncertain because of the lack of data on the presence of any

hemolysin in the lysate of cells broken by muralysin treatment. That the sonication treatment to release cellular SLS may be doing more than simply releasing hemolysin on cell breakage is suggested by Schwab's finding continued enhancement of the titer of his "intracellular" hemolysin by sonication after cell destruction (87). Taketo and Taketo (99) reported that prolonged sonication of cells decreased the titer of the "intracellular" SLO and resulted, to some extent, in the increase in titer of "intracellular" SLS. Whether or not this confirmed the phenomenon reported by Schwab is uncertain, but if both reports are true, the observations suggest activation of a latent hemolysin. It seemed possible, therefore, that no active intracellular SLS is present in Group A streptococcal cells, but that the hemolysin obtained from sonicated streptococci is active because of some process occurring during sonication.

STATEMENT OF PROBLEM

The primary questions asked in the research presented in this thesis are as follows:

1. Is there an active cellular SLS-like hemolysin present in Group A streptococci and is it the same hemolysin as that released by sonication?
2. Does sonication have a potentiating effect on any cellular SLS-like hemolysin of Group A streptococci?
3. In what ways are the extracellular SLS hemolysins related to any cellular SLS-like hemolysins?

MATERIALS AND METHODS

I. General Growth Conditions

Media or other solutions as required were sterilized by autoclaving for 15 min at 15 PSI and 250F. All reagents and media were prepared with deionized and distilled water.

A. Media

1. Brain Heart Infusion medium (BHI): The broth was prepared by dissolving 37 g dry powder (Difco) per liter of distilled water. The final percentage of glucose was 0.2%. BHI - 0.7% glucose was prepared by adding 900 mg glucose dissolved and autoclaved in 2 ml distilled water to 180 ml of separately autoclaved BHI prepared as above.

2. Heart Infusion medium (HI): The broth was prepared by dissolving 25 g dry powder (Difco) per liter of distilled water. HI - 0.3% maltose was prepared by adding 1 ml of an autoclaved 300 mg/ml maltose solution in distilled water to 100 ml of separately autoclaved HI prepared as above.

3. Todd Hewitt medium (TH broth or agar): The broth was prepared by dissolving 30 g dry powder (Difco) per liter of distilled water. TH agar was prepared by adding 15 g agar (Difco) per liter of the broth. TH ice was prepared by dissolving 60 g of dry powder per liter of distilled water and freezing the solution.

B. Cultures

Streptococcus pyogenes C203S, obtained from Dr. Alan

Bernheimer, New York University Medical Center, was used for all the studies on SLS or related hemolysins. A Group C streptococcus and its phage, obtained from Dr. H. L. Torney of Dow Chemical Co., was used to prepare muralysin.

C. Growth Conditions and Harvest

1. Stock cultures: Both the Group A and Group C streptococcal strains were maintained on blood agar slants. One slant was used as a master culture for monthly transfers of the Group A streptococci, or for transfers every other month for the Group C streptococci. All slants were grown for 24 hr at 37C. The Group C slants were stored in the refrigerator, and the Group A slants at room temperature.

Master lysates of the Group C phage were stored frozen at -70C. Concentrated phage of high titer in Todd Hewitt broth was stored frozen at -20C.

2. Experimental cultures: A three-step growth procedure was used to prepare Group A streptococci for either extracellular or cellular SLS production. All incubations were at 37C under stationary conditions.

Step 1: Streptococci from a slant were inoculated into 5 ml BHI broth and incubated 17-21 hr.

Step 2: 1 ml of the BHI culture was inoculated into 180 ml BHI containing 0.7% glucose and incubated 13-15 hr. These cells were then centrifuged at 2000 X g for 15 min at room temperature, washed once with 37C broth, and suspended in 8 ml HI.

Step 3: An appropriate number of replicate final cultures was made by inoculating 1 ml of the suspension into 100 ml HI supplemented with 0.3% maltose per 250 ml flask, and incubating for the appropriate time.

After incubation in Step 3, the flasks were cooled in an ice bath for 5 min and the cultures centrifuged at 5000 X g for 10 min. When large batches of streptococci (greater than one liter) were harvested at one time, the uncooled streptococci were centrifuged in 200 ml containers in the GSA head of the model RC-2 Sorvall centrifuge at 5000 X g for 10 min at 4C. The cells were washed 1 to 3 times with 100 to 200 ml sterile saline solution, depending upon the requirements of the experiment. The washed bacteria were then suspended in the appropriate volume of either 0.03 M phosphate buffer pH 6.5 containing 0.85% NaCl, or in Bernheimer's basal medium (BBM) (9).

II. Preparation of Muralysin

A. Correlation of optical density with viable Group C streptococcal counts

A two-step growth procedure was used to obtain Group C streptococci for the preparation of muralysin.

Step 1: Streptococci from a slant were inoculated into four 5 ml replicates of TH broth and grown 17 hr at 37C.

Step 2: The streptococci (20 ml) were inoculated into 980 ml TH broth and grown at 37C to the appropriate optical density before the Group C phage were added.

A graph relating the number of bacteria per ml to the corresponding optical density in Step 2 was first constructed. The optical density at 520 nm was determined in 15 X 100 mm colorimetric tubes in a Bausch and Lomb Spectronic 20 spectrophotometer and the number of bacteria per ml was determined by colony counts on the TH agar plates.

B. Bacteriophage Stock Suspension and Preparation of Bacteriophage of High Titer

A bacteriophage stock suspension was made by adding 1 ml of Group C streptococci from Step 1 growth to 50 ml of warmed TH broth, incubating the mixture at 37C until just beyond the beginning of the log phase of growth, and then adding 0.1 ml original stock bacteriophage. After lysis in 40 to 60 min, the mixture was centrifuged to remove bacterial debris at 10,000 X g for 30 min at 4C. The supernatant was divided into 5 ml replicates and stored at -70C.

Bacteriophage for centrifugation to high titer was prepared similarly except that 8×10^{10} plaque-forming units (PFU) of bacteriophage were added to 1000 ml of Group C streptococci (4×10^{11}) grown to mid log phase in Step 2. After removal of bacterial debris as above, the bacteriophage were centrifuged in the type 30 rotor for 2 hr at 20,000 RPM and 4C in the Beckman Model 2 ultracentrifuge, and the pellet suspended in 5 ml TH broth. This procedure for the concentration of bacteriophage was developed after unsuccessfully attempting to use the procedure of Fox (34).

C. Titering Bacteriophage

Because of a report (54) that the plaques of Group C phage were somewhat difficult to see using a pour-plate method such as used by Krause (63), a new procedure for titering bacteriophage was developed. The bacteriophage suspension was diluted in half-strength TH broth so as to include the proper dilutions (10^{-6} to 10^{-9} dilutions). TH agar plates (previously dried overnight at 37C were lawned with 0.15 ml mid-log phase Group C streptococci and then incubated 1 hr at 37C. Phage dilutions (0.05 ml) were spread over the lawn on triplicate plates, and the PFU counted after 8 hr incubation at 37C.

D. Preparation of Muralysin

Muralysin was prepared from a phage lysate of Group C streptococci by the method of Fox (34), with minor modifications in the procedure, and use of a new method for the preparation and titering the phage of high titer (described above).

To 1000 ml Group C streptococci grown to late log phase in Step 2 above was added high titer bacteriophage at a 3:1 multiplicity (PFU: bacterium). Usually 1.2×10^{12} PFU were added to 4×10^{11} bacteria/liter at the corresponding OD_{520nm} of 0.90. After incubation at 37C for 25 min (prior to lysis) and chilling by pouring over crushed TH ice, the infected bacteria were concentrated in either of two ways. Some batches of cells were harvested in the coldroom with the Szent-Györgyi and Blum continuous flow apparatus for the Sorvall SS-3. The cells were sedimented at 8000 X g at a flow rate of 40-60 ml/min. Other batches were harvested by centrifugation as for large batches

of Group A streptococci described above. After centrifugation, the cells were immediately suspended in 10 to 20 ml buffer consisting of 0.85% NaCl, 0.01 M sodium phosphate (pH 7.0), 1×10^{-3} M MgCl_2 , 1×10^{-3} M 2-mercaptoethanol, and 5 $\mu\text{g/ml}$ DNase. The mixture was incubated at 37C until lysis occurred, or for 15 min. The debris was removed by centrifugation at 10,000 X g for 30 min at 4C. Some preparations were further purified by additional centrifugation at 100,000 X g for 2 hr at 4C.

E. Titering of Muralysin

The muralysin was titered by the method of Markovitz and Dorfman (69) with slight modification. Stationary phase Group A streptococci grown in BHI were washed once with, and suspended in, 0.03 M sodium phosphate buffer pH 7.0 to an $\text{OD}_{520 \text{ nm}}$ between 0.30 to 0.40. To 3.0 ml of streptococcal suspension were added 0.1 to 0.01 ml dilutions of muralysin. The mixtures were incubated at room temperature for 1 hr. The optical density of the bacterial suspension was read at 625 nm and compared to control tubes without added muralysin. To obtain the titer the following formula was used:

$$\frac{1}{\text{dilution}} \times \frac{1}{\text{OD change}} \times 3 = \text{muralysin units/ml}$$

For example, a change in OD of 0.20 for a 1/1000 dilution of muralysin corresponds to a value of 600 muralysin units (MU) per ml.

III. Buffer

Unless otherwise stated, the buffer referred to as "buffer" or "standard buffer" is 0.03 M sodium phosphate, pH 6.5, containing 0.125 M NaCl.

IV. Disruption of the Cells

A. Sonication: Each of the three different Bronwill Biosonic Sonicators used - model BP-1 with a 3/5" diameter probe, the Biosonic 2 with a 1/2" probe, and the Biosonic 4 with a 3/4" probe - required different conditions for operation. The usual volume for models BP-1 and Biosonic 2 was 3-4 ml, unless very large lots of bacteria were sonicated with the Biosonic 2 at a volume of 5-10 ml. No less than 5 ml was sonicated with the Biosonic 4. The bacterial suspensions were surrounded by an ice bath during sonication. Only the model BP-1 probe could be water cooled during operation. All probes were cooled in an ice slurry before use. The model BP-1 and Biosonic 2 probes were cooled in ice every 20 min during use, and the model Biosonic 4 probe was cooled every 1.5 to 2 min.

The BP-1 was operated at a power intensity of 75 and the usual period of sonication was 20 to 25 minutes. The Biosonic 2 was operated at a power intensity of 60 for periods of 20 to 25 minutes. Higher intensities resulted in foaming and longer periods of sonication resulted in warming of the sonicate. The Biosonic 4 was operated at an intensity of 50 in the high power range for periods of 1.5 to 2 min. When periods of prolonged sonication were required, the sonication was intermittently interrupted and both the probe and the preparations cooled in ice for a few minutes. Failure to cool the probe and the sonicate after 2 min resulted in sonicate temperatures greater than 37C. The sonicates were centrifuged at 20,000 X g and 4C for 30 min in most experiments, and the sediment discarded unless otherwise stated.

B. Muralysin Treatment: Heavy bacterial suspensions were incubated for either 20 min at 23C or for 30 min at 37C, as required, with the muralysin preparation described above. The number of muralysin units to be added to each suspension was calculated by the following formula: 32 units X ml suspension X OD_{520nm} of the suspension. The debris was removed by centrifugation at 20,000 X g and 4C for 30 min.

C. Homogenization: The washed streptococci were suspended in 10 ml buffer and placed in the precooled 75 ml stainless steel flask of the Braun model MSK homogenizer with 50 grams of Superbrite glass beads (3M Co.), Type 100-110 regular, which had been washed once in distilled water and precooled. The flask was then placed in the chamber of the Braun homogenizer and the bacteria were homogenized 3 min while the chamber was cooled with liquid CO₂. Buffer (20 ml) was then added to the mixture; the beads were removed by centrifugation for 3 min at 3,000 X g at 4C. The supernatant was then centrifuged at 20,000 X g and 4C for 30 min to remove debris.

V. Preparation and Purification of Nucleic Acid and RNA-core

A. Nucleic Acid: Nucelic acid from S. pyogenes was prepared from cells grown for 4.5 or 8.5 hr in Step 3 above, and then disrupted with muralysin. All procedures except shaking were done in an ice bath or under refrigeration. To the streptococcal lysate was added an equal volume of phenol, washed and equilibrated with the buffered saline solution used for bacterial suspension. The mixture was vigorously shaken 5 min, and centrifuged at 10,000 X g for 15 min. Phenol extraction of the aqueous layer was repeated 4 times. The preparation was washed 3

times with ether to remove phenol and then gassed with N_2 for 30 min to remove ether. Two volumes of 100% ethanol were added and the preparation stored at $-20^{\circ}C$. The RNA precipitate was suspended in appropriate buffer as required.

Foreign nucleic acid preparations were used in a few experiments. Escherichia coli RNA prepared by the method of Erikson (33) was donated by Miss V. Spangler. Rat rRNA prepared after Bloemendal (19) and rat tRNA prepared after Kay (55) were donated by Dr. R. Nelson.

B. RNA-core

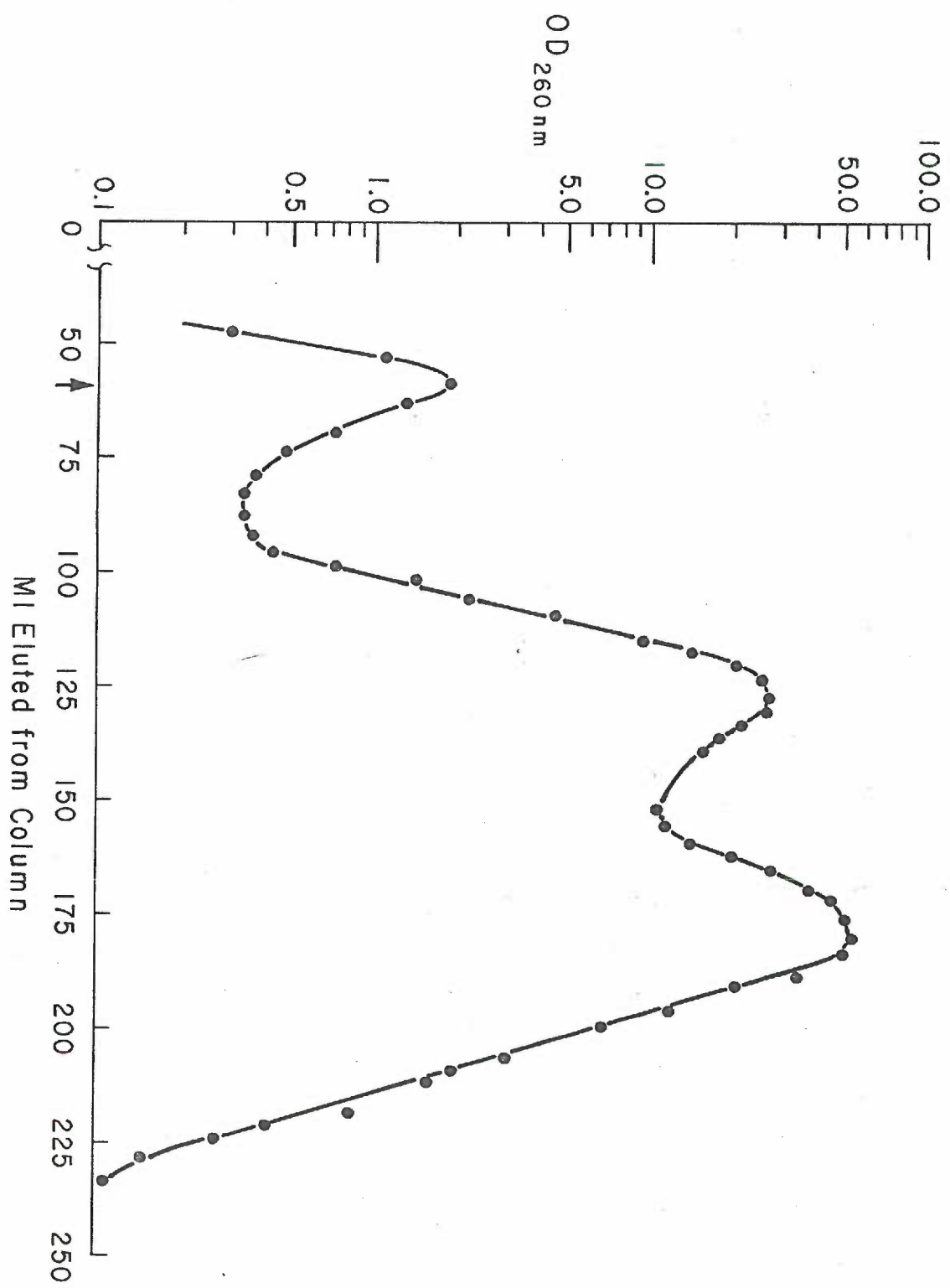
Yeast RNA (Schwartz) was digested with pancreatic Ribonuclease-A (Sigma) by the method of Ginsburg (41). Yeast RNA was dissolved in distilled water (100 mg/ml) and the pH adjusted to 7.0 with NaOH. RNase (0.1 mg/ml) was added and the mixture incubated 5 hr at $37^{\circ}C$. The mixture was then dialyzed 24 hr against cold running tap water and then 24 hr against distilled water. The dialysis retentate was lyophilized and stored at $-20^{\circ}C$. This will be referred to as unpurified RNA-core.

Further purification was achieved by phenol extraction as described above. The number of extractions necessary varied from 4 to 12 depending upon the original purity of the yeast RNA. The unpurified or phenol-purified core was also purified by chromatography on G-100 Sephadex. There were two large peaks after the void volume which were measurable at 250 nm (Fig. 1). The peak at the void volume was probably due to the undigested RNA and the amount present in different batches of RNA-core varied depending upon the extent of RNA digestion. The fractions between the first and second troughs were pooled and that RNA-core referred to as peak 1 RNA-core. In some experiments the

Figure 1.

Elution Profile of RNA-core from G-100 Sephadex

RNA-core (140 mg) dissolved in 1.5 ml buffer was mixed with 1 ml of sucrose solution (120 mg/ml), chromatographed on G-100 Sephadex, and eluted with standard buffer. Void volume indicated by arrow.



void volume RNA was included in this peak 1 pool when a void volume marker was needed. The fractions between the second trough and the end of the OD₂₅₀ nm-adsorbing eluate material were pooled and referred to as peak 2 RNA-core.

Some RNA-core which was phenol extracted and chromatographed on G-100 Sephadex was further purified by addition of 80% acetic acid to the peak 1 RNA-core. The RNA-core was soluble in the 80% acetic acid - 0.03 M sodium phosphate buffer containing 0.125 M NaCl. Any precipitate was removed by centrifugation and the RNA-core then precipitated from the acetic acid mixture with 2 volumes absolute ethanol.

VI. Preparation of SLS

A. RNA-core-SLS:

RNA-core SLS was prepared from resting cells by the procedure of Bernheimer (9) as modified by Ginsburg (41). Harvested late log-phase Group A streptococcal C203S were washed twice with 0.9% NaCl. The cells from 1 liter growth media were suspended in 10 ml of Bernheimer's Basal Medium consisting of 1×10^{-3} M maltose, 1×10^{-3} M Mg^{2+} , cystine-HCl, the appropriate amount of RNA-core and 0.03 M sodium phosphate, pH 6.5 containing 0.125 M NaCl. The cells were incubated at 37C for 1 hr, cooled, and centrifuged at 10,000 X g for 10 min. The supernatant usually contained 15,000 to 25,000 HU/ml.

B. Tween-40 SLS:

Tween-40 SLS was prepared by the procedure used to prepare RNA-core SLS except that Tween-40 (25 mg/ml) was used instead of RNA-core. The supernatant usually contained 2000-3000 HU/ml.

VII. Assay of Hemolysin

The assay procedure was a modification of that used by Schwab (87). Sheep red blood cells (RBC), freshly supplied commercially in Alsever's solution, were centrifuged at 3,500 X g for 10 min and the supernatant and buffy coat discarded. The RBC were suspended in 0.8% NaCl in 0.03 M sodium phosphate, pH 7.0, or in 0.9% NaCl. One-half ml of a 4% RBC suspension was added to hemolysin diluted to 5.0 ml. One unit of hemolysin (1 HU) produced 50% lysis of 5.5 ml of the 0.36% RBC suspension in 75 min at 37C as determined by the amount of released hemoglobin measured at 540 nm.

VIII. Preparation of Sheep RBC Membranes

Sheep RBC membranes were prepared either by the method of Dodge (29) or as follows: 8 ml of packed RBC were lysed in 40 ml distilled water. The lysate was centrifuged at 20,000 X g for 20 min and the supernatant discarded. The pellet was washed alternately with distilled water and normal saline until the supernatant was colorless, which required 4 to 5 washes. The final pellet of membranes was suspended in 5 ml saline solution and the occasional small amount of non-dispersible material discarded. The membrane preparations were stored at -20C.

IX. Use of Inhibitors

A. Trypan Blue Inhibition

The concentration of trypan blue initially used was 25 µg/ml (97), but it was found that this concentration partially inhibited streptolysin O (SLO). Since 13 µg/ml was sufficient to inhibit RNA-core SLS and caused only slight inhibition of SLO, the lower concentration was used in later experiments. Trypan blue was mixed with hemolysin

preparation immediately before RBC were added. After incubation the RBC were collected by centrifugation, lysed in a volume of distilled water equal to that of the incubation mixture, and the degree of inhibition correlated with the amount of hemoglobin released.

B. Inhibition with Antistreptolysin O

The amount of antistreptolysin O serum (aSLO, BBL) to be added to the hemolysin preparations was determined with SLO reagent (BBL). However, when the SLO reagent was preincubated, as recommended by BBL, for 15 min at 37C most of it was inactivated. At 0C the SLO reagent was stable and could be inactivated by 15 min incubation with aSLO at this temperature. Under these conditions aSLO equivalent to 0.1 Todd unit under standard conditions inhibited 30 HU of SLO.

X. Measurement of Protein, Rhamnose, and Bacterial Nitrogen

A. Protein

Proteins were quantitated by the Folin-Ciocalteu procedure as modified by Reiner and Cheung (85). Crystalline bovine serum albumin (A grade, Calbiochem) was used as the protein standard, and a standard curve using 25 to 150 μ g BSA was prepared during each set of protein determinations.

B. Rhamnose

Rhamnose was determined by the method of Dische and Shettles (28). Duplicate dilutions of unknown, standard, and blank were prepared. To 1 ml sample was added 4.5 ml H_2SO_4 (1 part water to 6 parts concentrated H_2SO_4) while stirring in ice. The mixture was cooled to room temperature, then placed in a boiling water bath for 10 min, then cooled to room temperature. To one of the paired tubes was added 0.1 ml of 3% cysteine-HCl. The mixtures were left at room temperature

according to the following formula $(OD_{396nm} \text{ of tube with cysteine} - OD_{396nm} \text{ of tube without cysteine}) - (OD_{430nm} \text{ of tube with cysteine} - OD_{430nm} \text{ of tube without cysteine})$. The mg rhamnose/ml was then determined from a standard curve.

C. Bacterial Nitrogen Determination

The mg of bacterial N content of each cell suspension was calculated from the optical density at 520 nm of 0.1 ml whole cell suspension diluted in 9.9 ml water. Previous analysis in this laboratory using micro-Kjeldahl determinations of suspensions with varied optical density indicated that for Streptococcus faecium, the optical density multiplied by a factor of 265 results in mg bacterial N/0.1 ml. This formula was used to report bacterial N for S. pyogenes C203S.

XI. Purification Procedures and Physical Properties

A. Removal of Nucleic Acids

Streptomycin sulfate was used in some experiments to remove nucleic acid. The procedure used was that of Taketo and Taketo (97), but the final percentage of streptomycin sulfate used in later experiments was lowered from 2.5% when it was determined that streptomycin sulfate removed or inactivated a substantial amount of hemolysin precursor. Chilled 5% streptomycin sulfate solution was added to the desired concentration to the appropriate preparations in an ice bath. The mixture was stirred 15 min, centrifuged at 15,000 X g for 20 min, and the precipitate discarded.

B. $(NH_4)_2SO_4$ Precipitation of Proteins

Ammonium sulfate precipitation of protein was used to increase the specific activity of the hemolysin precursor or hemolysins. Usually,

a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was added dropwise to the appropriate preparation while stirring the mixture surrounded by an ice bath. The mixture was stirred for 20 min then centrifuged at 20,000 X g for 20 min. In other experiments, solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly to the appropriate preparations at a rate allowing all crystals to dissolve before more was added. After addition of the solid $(\text{NH}_4)_2\text{SO}_4$, the mixture was treated as above.

C. Gel Filtration on G-75, G-100 and G-200 Sephadex

Gel filtration was used in the purification of several hemolysins, to determine molecular weights of hemolysins, and to demonstrate transfer of hemolysin from one carrier to another. Sephadex G-75 beads (Pharmacia) were hydrated by heating in boiling distilled water for 20 min and allowing them to swell in water overnight. Sephadex G-100 and G-200 beads were hydrated by heating in boiling distilled water for 5 hr. The fines were decanted from the slurries after the beads had settled. The appropriate cold buffer was then added to the beads. After mixing and allowing the slurry to settle, the fines were again decanted. The fining procedure was repeated 3 times. The slurries were then brought to the desired concentration and degassed for 1 hr with a vacuum pump. To pack the G-75 and G-100 Sephadex columns, slurry was poured into a 2.5 X 45 cm column in the cold room, filled to one-third with the appropriate buffer. The pressure head was adjusted to 12-14 cm. As the slurry settled, the clear buffer was removed from the top, and more slurry was added until the column was packed to within 5 cm from the top. A sample applicator was then placed on top of the gel

bed. Buffer (100-300 ml) was run through the column at 12 cm pressure to allow for any further settling and to completely equilibrate. The G-200 Sephadex column was prepared by a similar procedure using a 2.5 X 100 cm column and an extension tube on top of the column to hold excess slurry. Samples of 1 to 5 ml in 10% sucrose were placed on the column by means of a curved-tip Pasteur pipet so that the sample was layered over the gel and under 5 to 10 ml buffer. Samples of 2 to 4 ml were collected on a Gilson Model TL linear fraction collector or on an Instrumentation Specialties Model 270 circular fraction collector. The volume of fraction collected in each tube was determined with calibrated pipets. The elution volume of marker proteins or hemolysins was determined by graphic estimation of the midpoint. The void volume of each column was determined with 0.2% Blue Dextran (Pharmacia) at the beginning of each experiment or group of experiments. The hemolysins were assayed as stated previously and the non-enzymatic marker proteins were assayed by adsorption at their specific wave lengths: myoglobin at 410 nm; chymotrypsinogen A, ovalbumin, and bovine serum albumin at 280 nm (all markers from Mann Research Laboratories). Blue Dextran was measured at either 220 nm or 520 nm. During the standardization of the column, proteins were applied in groups or sequentially so that no overlapping occurred. Molecular weight determination of SLS was done by applying SLS both with and after Blue Dextran application.

D. DEAE Cellulose Chromatography

DEAE cellulose (DE-52, Whatman) was precycled with one treatment with 0.5 N HCl and two treatments with 0.5 N NaOH according to

the directions of the manufacturer (N. & R. Balston, Ltd.). The DEAE cellulose was suspended in 0.5 M NaH_2PO_4 and degassed for 10 min with a vacuum pump. The pH was then adjusted to 7.0 with 0.05 M Na_2HPO_4 . The gel was then equilibrated by 0.03 M sodium phosphate buffer, pH 7.0, changes until the pH of the decanted buffer was 7.0. The fines were removed after allowing the slurry to settle in a graduated cylinder for a length of time calculated by the following formula: $\text{time} = 1.4 \times \text{height of slurry in cm}$. Slurry was poured into a 1.8 X 30 cm column containing 20 ml buffer. The column was packed with a Buchler polystatic pump using a flow speed of 25.3 ml/hr. Buffer was removed and gel added and packed until the column was filled to 2 cm from the top. Starting buffer (100 ml) was pumped through the column prior to use. The starting buffer was 0.01 M sodium phosphate, pH 7.0, with 0.3 M NaCl . RNA-core SLS was added to the top of the gel and allowed to adsorb. Two volumes of starting buffer were run through the column. The RNA-core SLS was eluted with 0.01 M sodium phosphate, pH 7.0 containing 0.5 M NaCl . Two to four ml fractions were collected with a Gilson linear fraction collector. The fractions were assayed for hemolysin and 260 nm-adsorbing material.

E. Sucrose Density Sedimentation

A sucrose gradient former was used to prepare a 5 to 20% sucrose gradient. No more than 0.25 ml sample was applied to the top of the gradient. The sample was overlayed with mineral oil, as required, prior to centrifugation at 200,000 X g for 90 min at 4C. Samples were removed in 0.2 ml amounts starting at the bottom of the gradient by

puncturing the bottom of the tube with a needle and forcing the sample out under pressure. Hemolysin precursor was assayed by sonication with RNA-core and measurement of activated hemolysin. SS hemolysin was assayed directly as stated under Assay of Hemolysin.

F. CsCl Density Sedimentation

CsCl (1.875 g) was weighed for each centrifuge tube. The sample to be centrifuged was adjusted to 3.0 ml and then added to the solid CsCl. The sample was gently mixed by pipetting until the CsCl was in solution. The remainder of the tube volume was filled with mineral oil. The tubes were centrifuged at 30,000 RPM in the SW-39 head of the Beckman Model L ultracentrifuge for 48 hr. The gradient was removed in 0.2 ml samples from the bottom of the tube. Any sediment in the tube was removed as a separate sample in 3 ml buffer. Hemolysin precursor was assayed by sonicating 0.02 ml sample plus 0.3 mg RNA-core plus buffer to 3 ml for 25 min at 60 intensity with the model 2 sonicator and assaying the sonicate for activated hemolysin. The insoluble sediment was sonicated similarly, but with 1.0 mg RNA-core, 0.3 ml sample and buffer to 3 ml. SS hemolysin was assayed directly.

G. Cs₂SO₄ Density Sedimentation

To 2.35 g Cs₂SO₄ was added 2.5 ml sample. The mixture was mixed gently until the Cs₂SO₄ was in solution. The mixtures were centrifuged at 30,000 RPM on the Beckman Model L-250 ultracentrifuge in the SW-50 head for 64 hr. The gradient was removed in 0.2 ml samples. Hemolysin precursor was assayed as described above for hemolysin precursor in the CsCl gradient.

H. Polyacrylamide Electrophoresis

Polyacrylamide electrophoresis in urea was done by the procedure of Martinez et al (72). Acrylamide gels (7.5%) were prepared according to the method of Hjertter et al (49). After polymerization, the gels were equilibrated with pH 7.0 buffer by electrophoresis at 3 ma/tube for 30 to 60 min at 4C. No spacer or sample gels were used. The pH 7.0 buffer for electrophoresis was made as follows: 1.21 g tris(hydroxymethyl)aminomethane and 240 g urea were dissolved in 900 ml water and the pH adjusted to 7 with 2 N HCl. Samples were applied in 10 to 100 lambda amounts. Electrophoresis was carried out at 8 ma/tube for 30 to 90 min at 4C. At the conclusion of the run, the gels were stained with amido black, as described by Davis (27).

Polyacrylamide electrophoresis in sodium dodecyl sulfate (SDS) was done by the method of Weber and Osborn (108).

XII. Chromatography Using Aminex A-5 Resin and Measurement of the Eluate for Peptides

RNA-core SLS was analyzed using a 23 cm X 0.9 cm diameter column of Aminex A-5 resin (Bio-Rad) at 50C. The column was developed using a linear gradient between 0.2 M pyridine acetate, pH 3.1, and 2 M pyridine acetate, pH 5.0, and washed with 2 M pyridine acetate, pH 6.5. Prior to sample application, columns were equilibrated with the pH 3.1 buffer. Buffers were pumped through the column at 30 ml/hr. Part of the effluent stream was analyzed using a peptide analyzer (Technicon, Model P-1) (23) which monitored the reaction of the effluent with ninhydrin

both before and after hydrolysis with NaOH. The analyzer was modified to utilize 12 ml/hr for analysis and the remainder 18 ml/hr collected in a fraction collector. The appropriate fractions corresponding to the Aminex A-5 peaks were pooled and lyophilized.

XIII. Measurement of Ninhydrin-Reacting Material Before and After Alkaline Hydrolysis

The procedures used were based on those used in the automated peptide analyzer of Catravas (23).

A. Ninhydrin Assay

One ml sample was mixed with 2 ml ninhydrin solution (50% ninhydrin and 0.35% hydrindantin in 50% methyl cellosolve) and 2 ml diluent (50% methyl cellosolve and 2 N sodium acetate made to pH 5.5 with HCl). The mixture was heated in boiling water for 20 min and then cooled. The OD of the cooled solution was read at 570 nm using the appropriate blank. Norleucine was used as the standard.

B. Alkaline Hydrolysis

One volume of 50% NaOH was mixed with 4 volumes of sample and the mixture heated at 95-100C for 20 min. The pH was then lowered to 5.5 with acetic acid.

RESULTS

Development of a Growth System for Production of High Titer Extracellular RNA-core SLS and Cellular SLS

Requirements for a growth system for the production of extracellular and cellular SLS were several in number. First, it was desirable to obtain high yields of RNA-core SLS so that purification procedures could be undertaken on samples of adequate size. Second, production of SLS by cells in the non-stationary phases of growth was essential to minimize release of autolytic materials into the extracellular medium. Third, in order to determine possible changes in the cellular location, large numbers of cells at any stage of growth were required. Fourth, a growth step which repressed SLS formation so that such repressed cells on transfer to another medium would then make SLS was a requirement to determine the temporal relation between extracellular and cellular SLS. Fifth, since two different hemolysins -- SLS and SLO -- are made by Group A streptococci, a medium minimizing production of SLO was desirable.

Growth media used to obtain good yields of Group A streptococci have generally contained high concentrations of glucose (0.1 - 0.25%) and have been rich in essential growth constituents. In a comparison of five different growth media, very good growth of strain C203S was obtained in Brain Heart Infusion broth (BHI) (0.2% glucose) and Todd Hewitt broth (TH) (0.2% glucose); very little growth was obtained

with BBL Trypticase Soy broth (0.25% glucose) or Heart Infusion broth (HI) (0% glucose); no growth was obtained with Difco Penassay broth (0.1% glucose). Production of RNA-core SLS has been reported to be completely inhibited by glucose concentrations greater than 0.3% and to be partially inhibited by lesser concentrations (79). Early experiments with small batches of cells showed that high yields of extracellular RNA-core SLS were obtained from cells growing in both HI and BHI (Table 3). After 9 hr of growth there was much more RNA-core SLS in the HI supernatant than the BHI supernatant. However after 19 hr of growth, there were equal amounts of hemolysin. The increase in hemolysin from the BHI cells was associated with a decrease in turbidity of cell mass, suggesting that the increase in hemolysin might be related to cell autolysis. Therefore, the BHI medium was unsuitable for this research. The HI medium allowed little cell growth which was also undesirable.

The problem was, therefore, to find a growth system in which RNA-core SLS was produced during the log phase of growth by an adequate number of cells suitable for further experimental procedures. The alternatives were either to use no sugar or to use a sugar which did not repress RNA-core SLS production. Maltose was tested as a non-repressing sugar, since Bernheimer and Rodbart (15) had shown that 5×10^{-5} M maltose promoted RNA-core SLS production by growing cells ten times better than any other sugar; higher levels of maltose were inhibitory. Cells of strain C203S grown in HI - 0.3% maltose did grow very well and produced large amounts of RNA-core SLS. Those grown in HI grew very poorly, but produced large amounts of RNA-core SLS, as before.

Culture	HU/ml		OD _{660nm}	
	9 hr	19 hr	9 hr	19 hr
BHI	9	1	0.75	0.70
BHI + core	400	2000		
HI	2	2	0.36	0.36
HI + core	3000	2000		

Table 3. Cumulative SLS Levels in the Presence and Absence of Crude RNA-core in BHI and HI.

A loop of Group A streptococci was transferred from slant culture to 5 ml tryptose broth (Difco) and grown 24 hr. Three ml were then transferred to 30 ml BHI or HI with or without 30 mg crude RNA-core. The cultures were grown at 37 C. Initial OD_{660nm} = 0.1.

Cells grown in HI - 0.3% glucose grew very well, but produced very little, if any, RNA-core SLS.

The 3-step growth system, as described in Materials and Methods, was devised to utilize the effects of various growth media on both streptococcal growth and RNA-core SLS formation. Growth in Step 1 (BHI-0.2% glucose) provided an adequate inoculum for Step 2. The length of growth in Step 1 could be manipulated to decrease or increase the lag in growth in Step 2 (BHI-0.7% glucose). Unfortunately, variation in the rate of growth in Step 1 often caused undesirable variations in the time of onset of growth in Step 2. Variations in growth in Step 2 also influenced the lag prior to growth in Step 3. The third step, in HI-0.3% maltose, yielded large amounts of cells which formed large amounts of RNA-core SLS during the log phase of growth. These points are illustrated in Fig. 2 and Fig. 3. The hemolysin produced in the presence of core is equivalent to the sum of SLO and SLS (8), while the core-independent hemolysin can be ascribed to SLO (15). Even though the rate of growth is greater for the HI-glucose-grown cells, there is always a final higher yield of cells from the HI-maltose medium. The yield of BHI-grown cells usually exceeds that of the HI-maltose-grown cells. There is no production of RNA-core SLS by cells transferred from Step 2 to Step 3 for at least 1.5 hr. The greatest amount of RNA-core SLS is formed in either HI or HI-maltose, but the amount formed per cell is greater in HI. Almost all of the hemolysin in HI-glucose medium can be attributed to SLO in the early and mid-stages of growth. In the stationary phase of growth,

Figure 2.

Rate and Extent of Growth of S. pyogenes C203S in Various Media

Cells were grown in Steps 1 and 2 as in Materials and Methods, and inoculated into 100 ml medium with or without 7 mg peak 1 RNA-core. Data shown for flasks without RNA-core, but were the same for flasks with RNA-core. (□) HI, (▲) HI-0.3% maltose, (●) HI-0.3% glucose, (●) BHI.

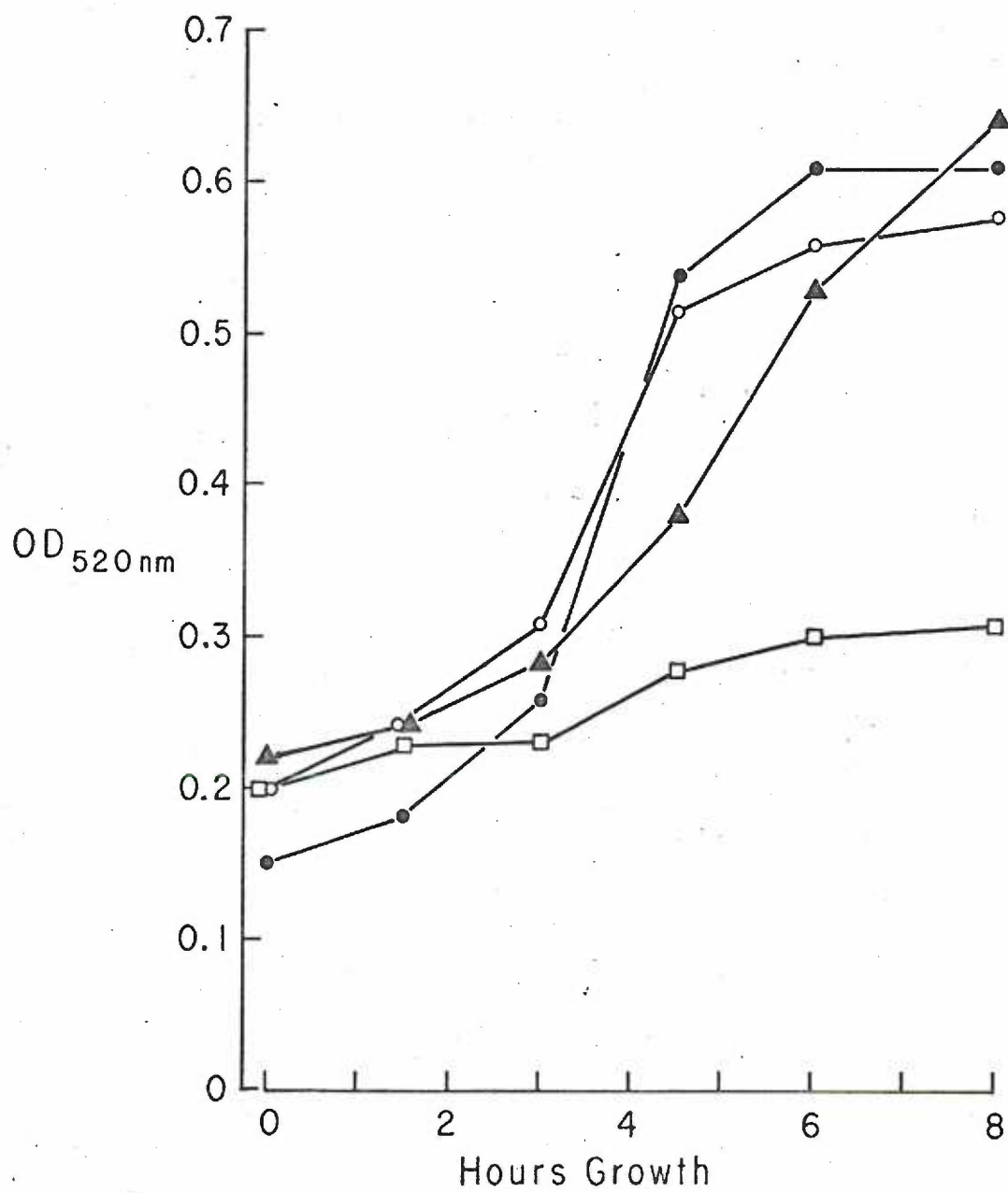
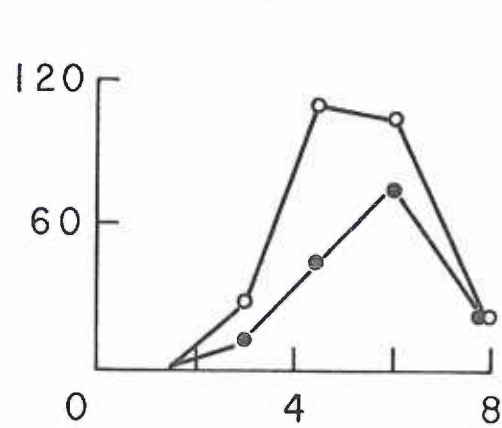
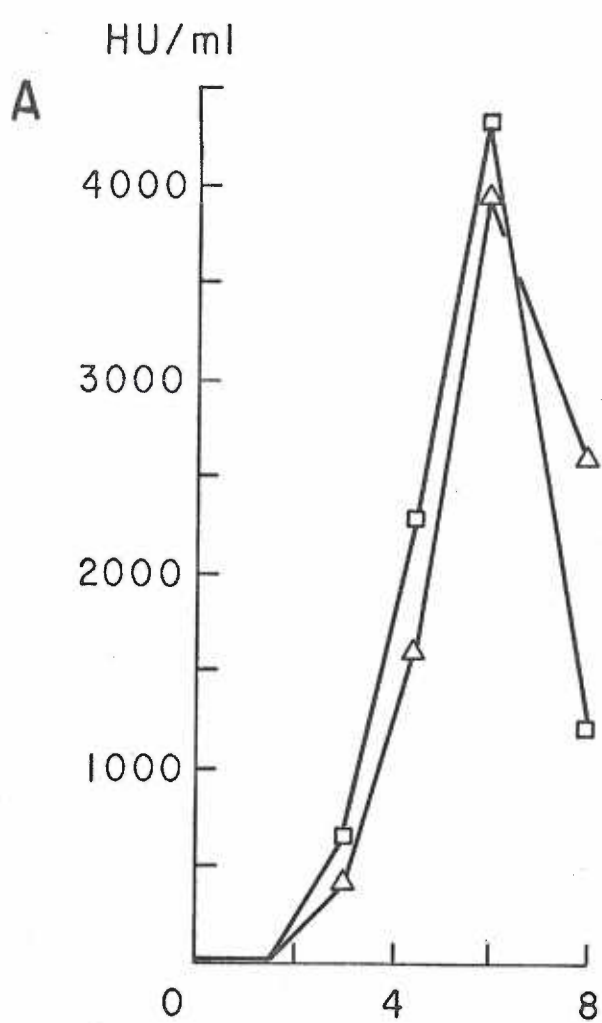
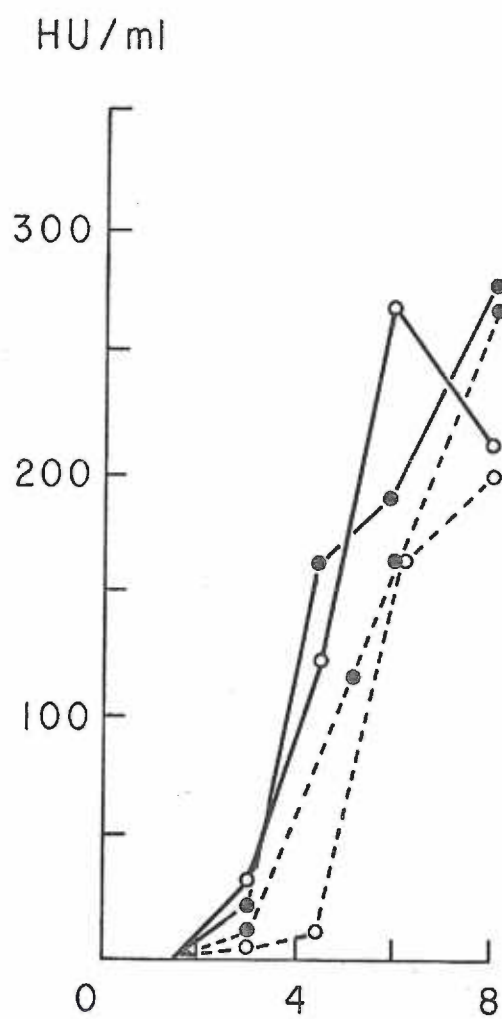


Figure 3.

Production of RNA-core-dependent Hemolysin (RNA-core
SLS) and Non-RNA-core-dependent Hemolysin (SLO)

- A. RNA-core-dependent Hemolysin. Seven mg peak 1 RNA-core added per 100 ml medium.
- B. Non-RNA-core-dependent Hemolysin. No RNA-core added.
(□) hemolysin in HI, (Δ) hemolysin in HI-0.3% maltose, (○) hemolysin in HI-0.3% glucose, (●) hemolysin in BHI. Dashed lines = hemolysin in BHI+RNA-core minus that in BHI or hemolysin in HI-0.3% glucose + RNA-core minus that in HI-0.3% glucose.



Hours Growth

however, there is a rise in the core-dependent hemolysin in both the BHI and HI-glucose media (Fig. 3A). Prior to the stationary phase of growth, RNA-core SLS production has peaked in both the HI and HI-maltose media. The addition of either maltose or glucose enhances SLO production in the HI medium.

Measurement of the Levels of Sonically-released Cellular Hemolysin During the Growth Cycle

Strain C203S cells, grown in different media, were sonicated to confirm the presence of a sonically-releaseable hemolysin and to determine its cellular level during the growth cycle. After harvesting and washing, cells were sonicated on the model I sonicator as described in Materials and Methods. As can be seen in Fig. 4, most of the cell destruction, as measured by change in optical density, has occurred by 20 min. Longer sonication times were avoided in this study to minimize any processes due to sonication alone.

The growth procedure initially used was the same as that described in Materials and Methods except that Step 3 growth media consisted of HI + $5 \times 10^{-5}\text{M}$ maltose. HI was used to minimize the amount of SLO which might be present. The maltose was added because of the report of Bernheimer and Rodbart (15) that it enhanced the SLS production; maltose might be expected to enhance the levels of cellular SLS. This very small amount of maltose had no effect on the growth rate or SLO production.

Sonically-released hemolysin from Step 3 HI + $5 \times 10^{-5}\text{M}$ maltose-grown cells (Fig. 5A) was first detected in the lag phase of growth.

Figure 4.

Rate and Extent of Cell Disruption by Sonication

Five ml of harvested cells grown to stationary phase in BHI (●) or HI (□) were sonicated using the model 1 sonicator at 60 intensity.

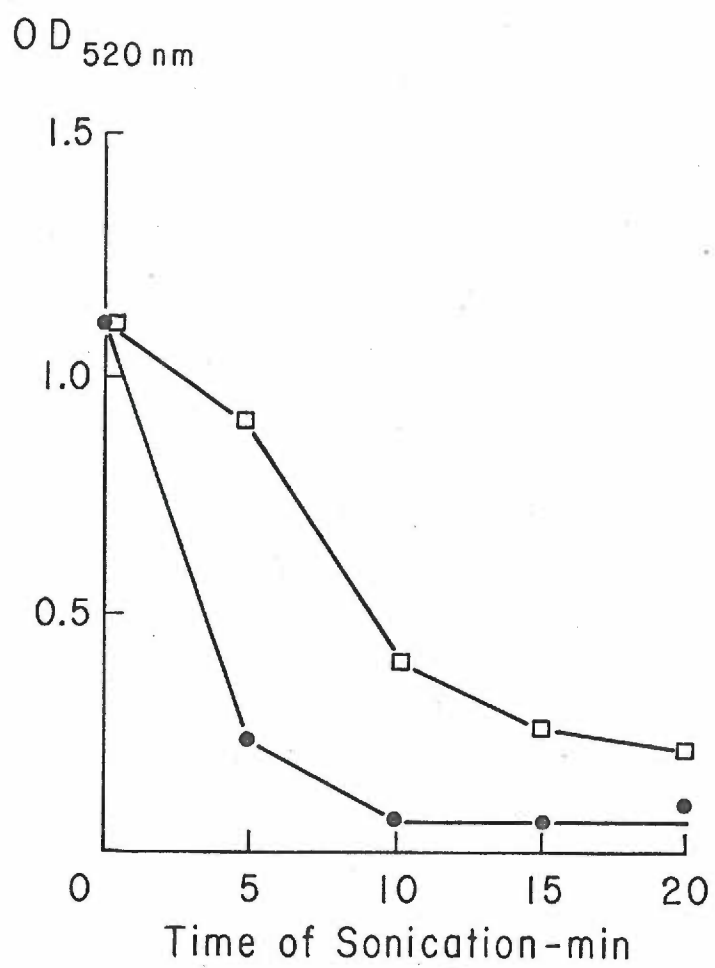


Figure 5.

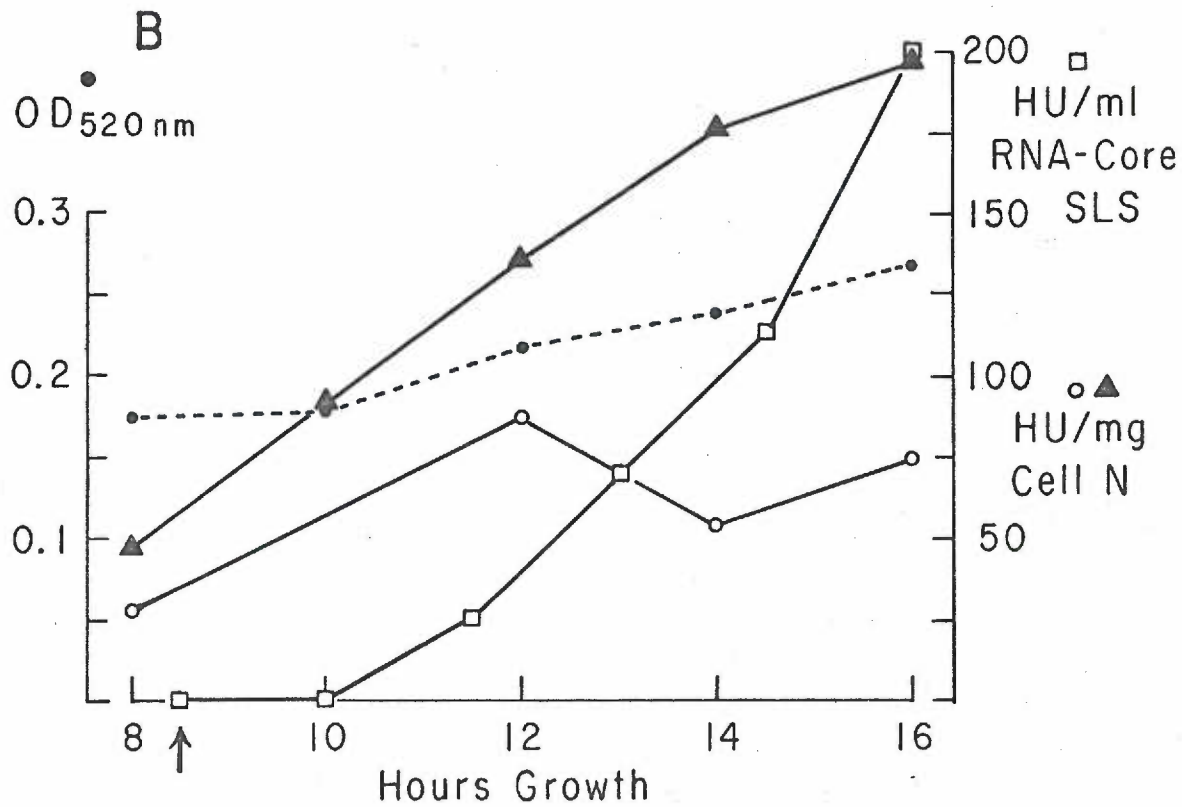
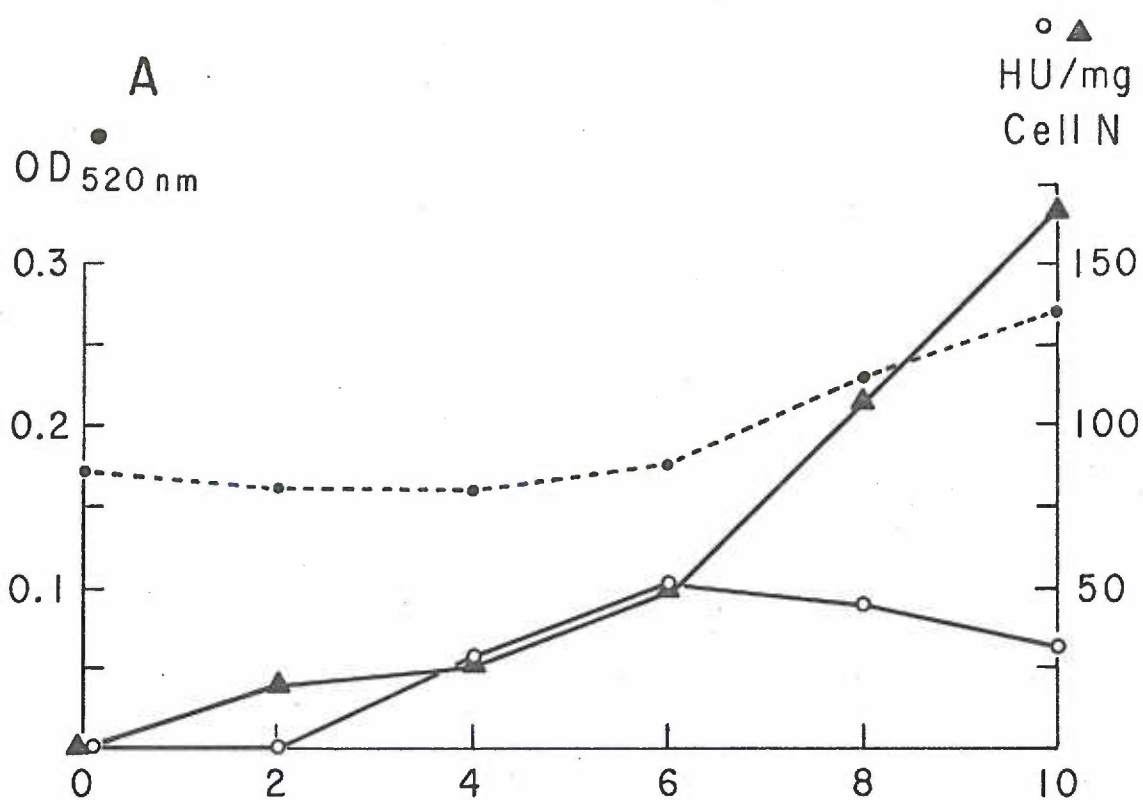
Sonically-released Hemolysin from $HI+5 \times 10^{-5}$ M Maltose-grown Cells

Cells harvested, sonicated with the model 1 sonicator and centrifuged as in Materials and Methods.

A. From cells in early stage incubation.

B. From cells in late stage incubation. Crude RNA-core (33 mg) added to each of 2 flasks with 50 ml cells at 8.5 hr (arrow) and 2.5 ml sampled from alternate flasks as indicated.

(□) RNA-core SLS, (▲) sonicate supernatant hemolysin, (○) sonicate sediment hemolysin, (●) OD_{520nm} .



No such hemolysin was detectable at the start of the growth cycle, i.e., in cells from Step 2. No attempt was made during these experiments to determine if the hemolysin present in the sediment of the 20,000 X g for 30 min centrifugation was due to viable or intact cells, to trapped hemolysin, or to bound hemolysin. The hemolysin in the sediment did not constitute the major portion of the hemolysin during the total and mid-phase of growth. No extracellular hemolysin was detected until 10 hr of growth and then represented only 0.3 HU/ml. The titers were lower than might be expected because the assay time was shorter (40 min) than described in Materials and Methods; therefore, the amount of hemolysin was a relative amount, not the absolute amount. The hemolysin released by sonication was characterized by a lag in hemolysis and, therefore, is SLS-like in character (8) (59).

Cells were assayed for sonically-released hemolysin during the late stage of incubation (Fig. 5b) during which little cell growth occurred. As stated earlier, the variation of growth in Step 1 and 2 markedly affected the lag phase in Step 3. Since growth was so poor in HI + 5×10^{-5} M maltose, it was almost impossible to compare separate experiments. However, the sonically-released hemolysin did increase as the cell mass increased. A small amount of extracellular hemolysin, ranging in titer from 0.2 HU/ml at 8 hr, to 3.4 HU/ml at 16 hr, was present in the absence of RNA-core and was presumably SLO. The hemolysin released by sonication again was characterized by a lag prior to the onset of hemolysis. The formation of extracellular SLS by cells to which crude RNA-core had been added indicated that these cells were competent to form RNA-core SLS. Also, the lag in

formation of RNA-core SLS while sonically-releasable hemolysin was present suggested that the formation of the latter may precede the RNA-core SLS found in the extracellular medium.

Although the HI + 5×10^5 M maltose supported minimal SLO production, the poor growth of cells made it almost impossible to relate the level of sonically-releasable hemolysin to events in the growth cycle. A different medium was needed for that purpose.

Since high yields of RNA-core SLS were obtained from cells grown in HI-0.3% maltose, the level of sonically-released hemolysin in such cells was examined as an alternative to HI + 5×10^5 M maltose-grown cells. The pattern of sonically-releasable hemolysin formation was the same for cells grown in either medium (Fig. 6A). First, there was no such hemolysin measurable at the start of growth in Step 3. Second, the onset of hemolysin production began during the long lag phase of growth and increased during the log phase of growth. There was a little extracellular non-core-dependent hemolysin (SLO) detectable late in the lag phase, a few hours after the sonically-releasable hemolysin was first measurable. The titer of sonically-releasable hemolysin declined during stationary phase (Fig. 6B) and a second peak of activity occurred late in stationary phase. Only a trace of extracellular non-core-dependent hemolysin was present prior to and after this second peak. The secondary peak was, therefore, probably not due to SLO.

Both RNA-core SLS and sonically-released hemolysin from cells grown in HI-0.3% maltose were initially detectable during the lag

Figure 6.

Sonically-released Hemolysin from HI-0.3% Maltose-grown Cells

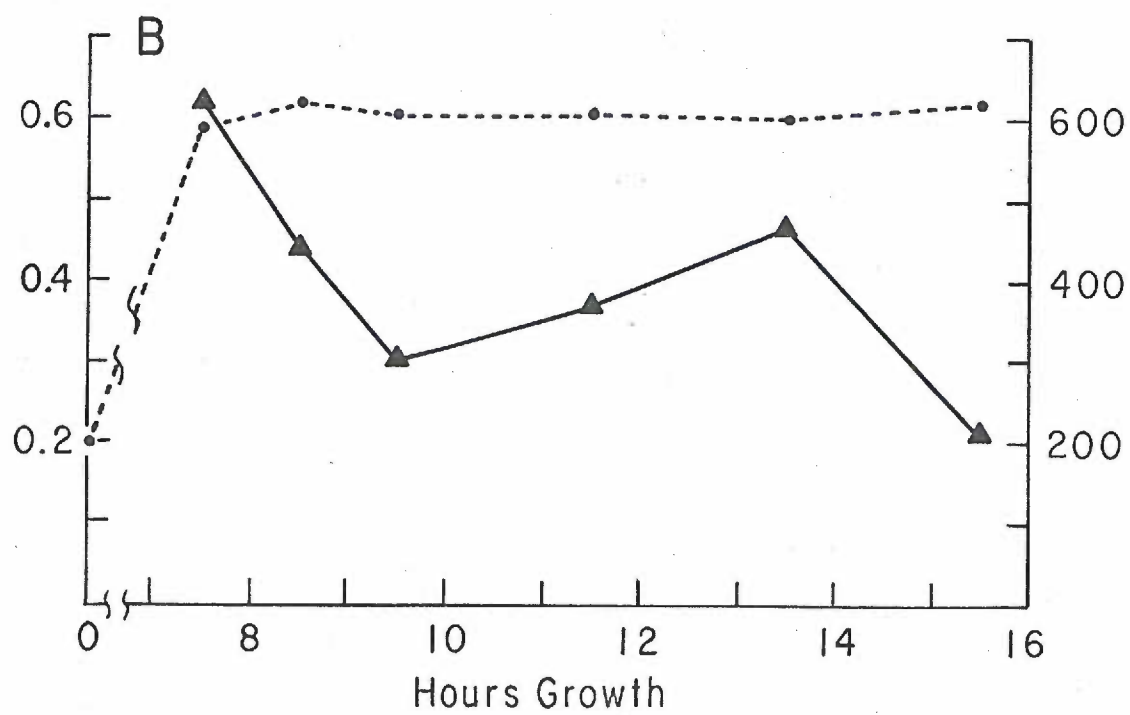
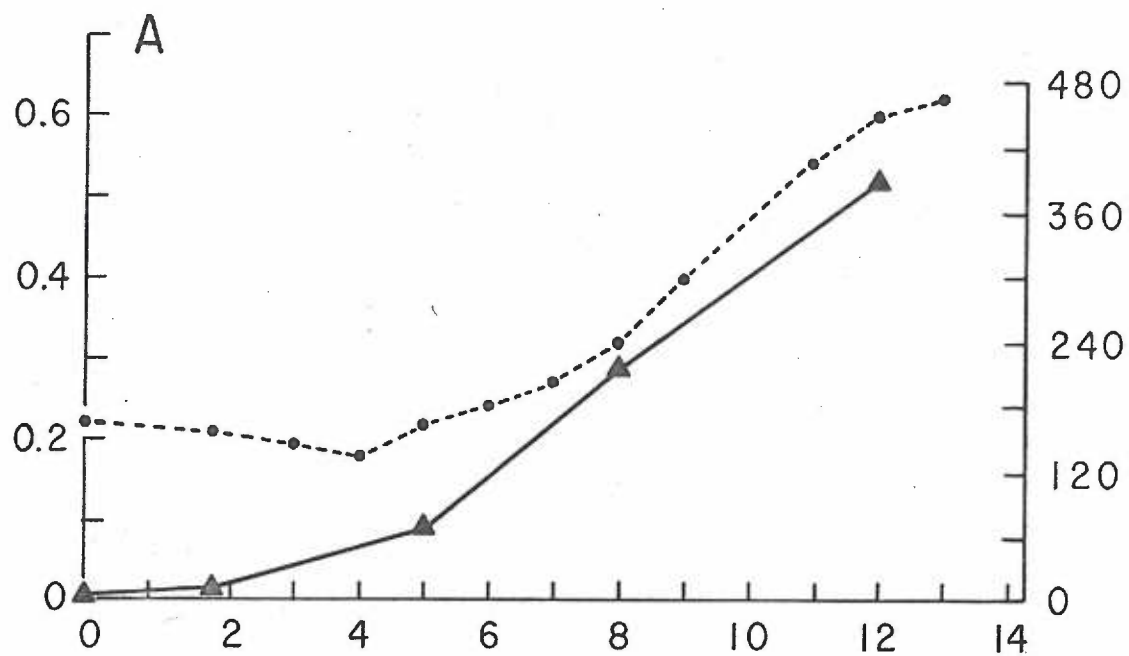
A. From cells in early log phase of growth.

B. From cells in stationary phase of growth.

(▲) sonicate hemolysin, (●) OD_{520nm} .

OD_{520 nm}

HU/mg
Cell N



phase, increased during the log phase, and declined during stationary phase (Figs. 2, 3, 7). In these experiments the extracellular RNA-core SLS and sonically-releasable hemolysin were not assayed from the same cells, because preliminary experiments had shown that there were differences in the amounts of sonically-releasable hemolysin depending upon the presence or absence of crude RNA-core. Further experiments, reported subsequently, were done in an attempt to confirm that RNA-core affected the amount of sonically-releasable hemolysin.

Muralysin-released Cellular Hemolysin

The previous results indicated that the highest levels of sonically-released cellular hemolysin occurred just prior to the onset of stationary growth in HI-0.3% maltose. (All further experiments, unless otherwise stated, were done with HI-0.3% maltose-grown cells.) Muralysin was used to disrupt strain C203S to determine if a cellular hemolysin could also be demonstrated after enzymatic lysis of the cells. The disruption of strain C203S cells by muralysin was done at 23C to minimize inactivation of any labile hemolysin. As shown in Fig. 8, the bulk of the cells lysed by 25 min, the length of incubation subsequently used.

When cells from either mid- or late-log phase of growth were disrupted by sonication or treatment with muralysin, the hemolysins were noted to have different initial rates of hemolysis (Fig. 9). The sonication supernatant (SS) showed delayed hemolysis, with onset of lysis at 15 to 20 min after the start of incubation. The muralysin-treatment supernatant (MS) did not have such a lag, and

Figure 7.

Production of RNA-core SLS and Sonically-released Hemolysin
During Growth of S. pyogenes C203S in HI-0.3% Maltose

- A. RNA-core production. Crude RNA-core (32 mg per 100 ml culture) was added at 0 hr, the initiation of Step 3 as described in Materials and Methods. Reported hemolysin titers in culture supernatants corrected for titers of SLO present in control flasks without RNA-core. ($\square$) extracellular RNA-core SLS, ($\bullet$) OD_{520nm} .
- B. Sonically-released hemolysin production. Cells were harvested from 100 ml medium without RNA-core; sonicated as in Materials and Methods. Cell N values calculated from optical density at 520nm. No SLO detectable in the sonicates. ($\blacktriangle$) sonically-released hemolysin, ($\bullet$) OD_{520nm} .

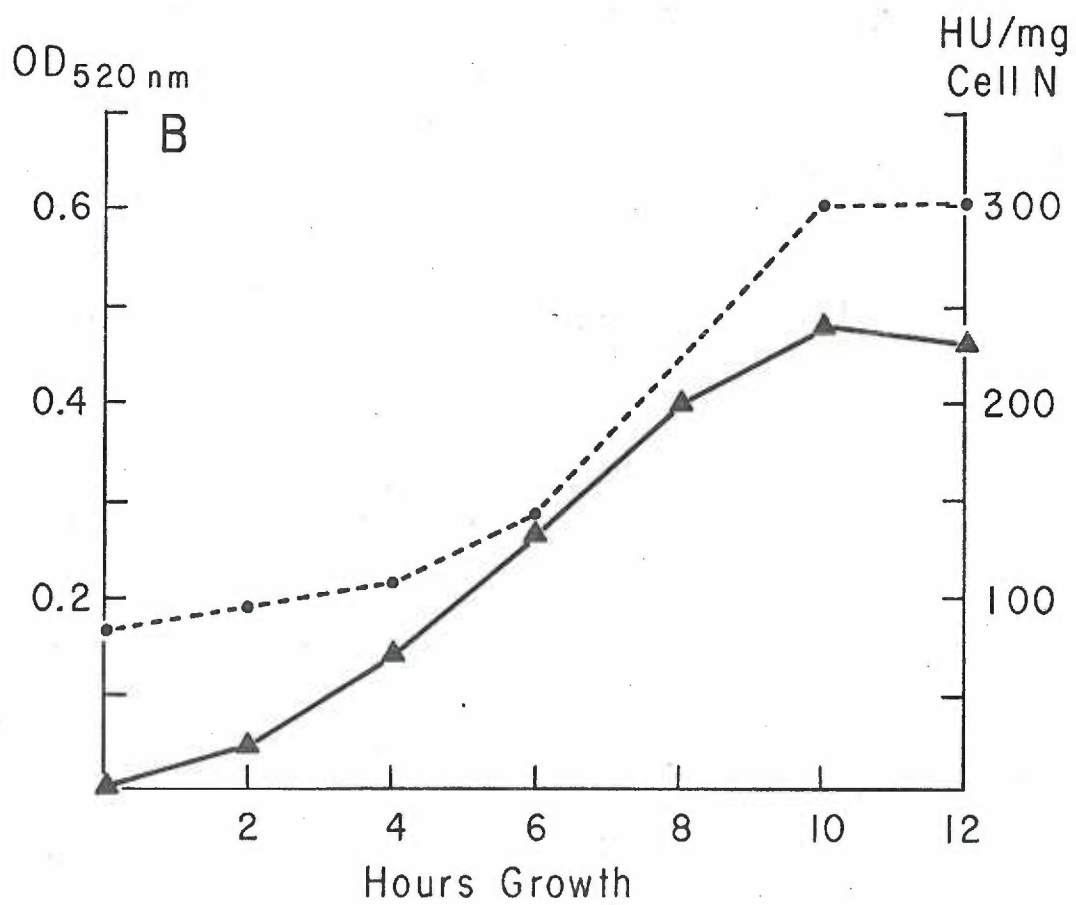
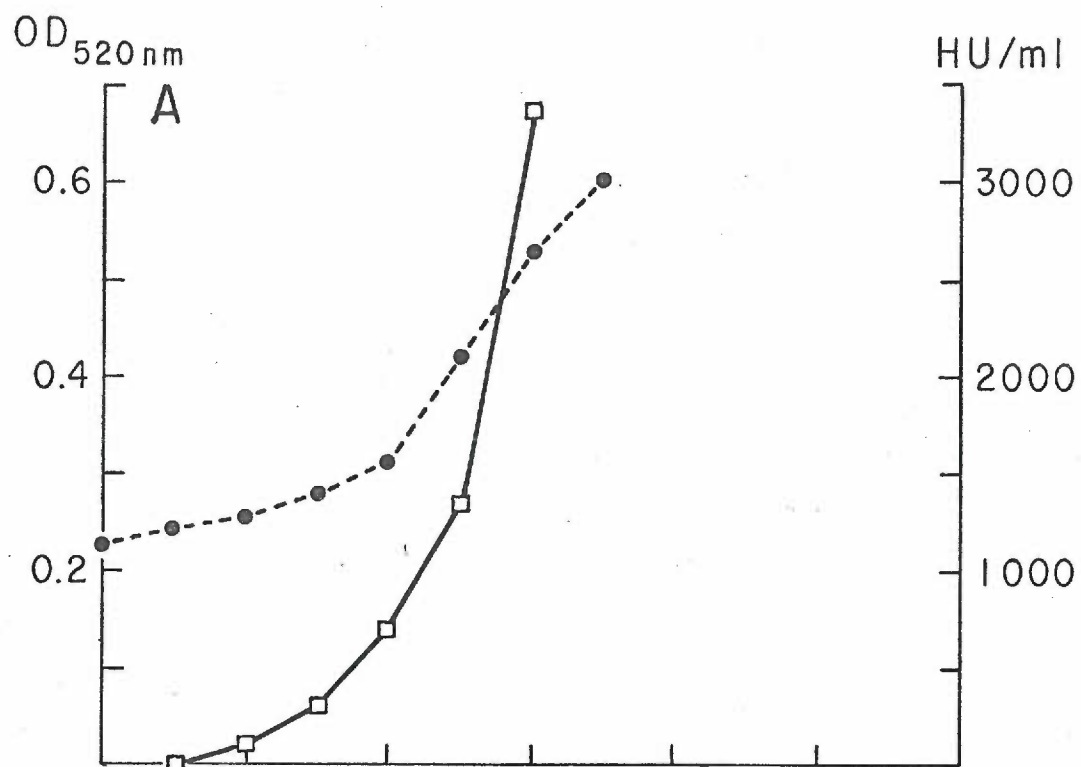


Figure 8.

Lysis of Stationary Phase Group A Streptococci with Varying Amounts of Muralysin

To 3.5 ml washed stationary-phase Group A streptococci C203S was added 90 (▲), 18 (△), 9 (○), 1.8 (□), or 0.9 (●) muralytic units of muralysin and the mixtures incubated at room temperature (23 to 25C).

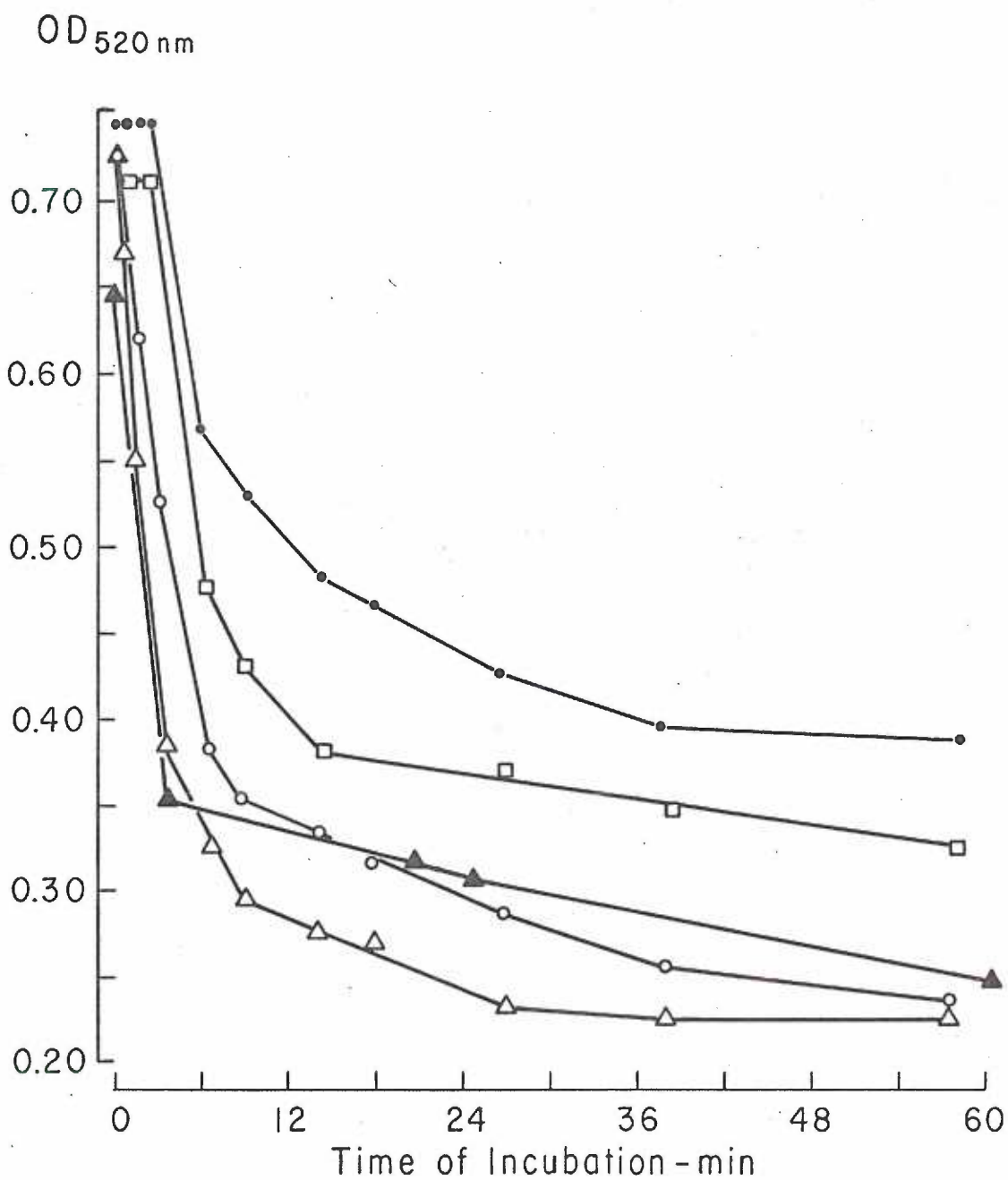
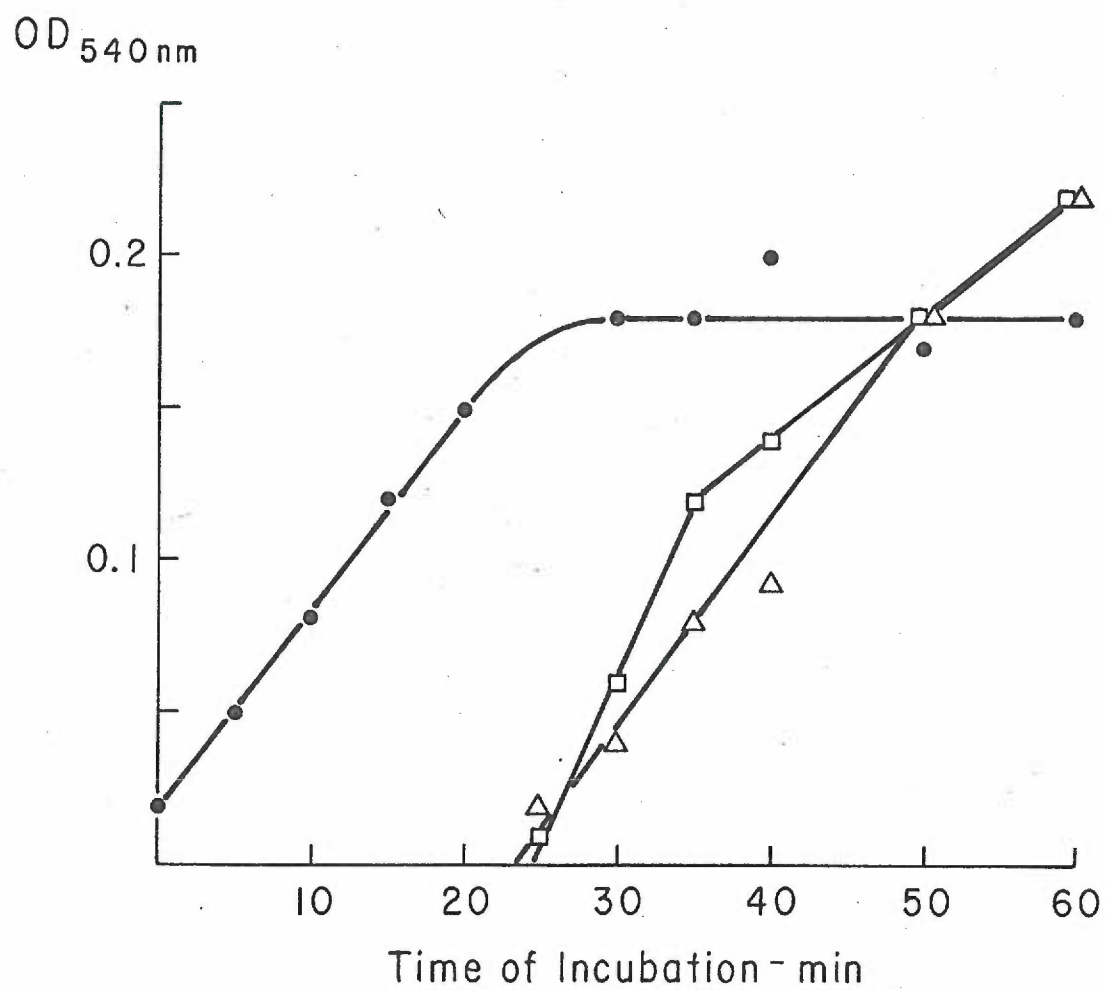


Figure 9.

Rates of Hemolysis by One Unit of Hemolysin

A series of replicate tubes containing RBC and 1 hemolytic unit of hemolysin were incubated at 37C for the appropriate time. The remaining unlysed cells were sedimented at 3000 x g for 5 min and the amount of hemoglobin released from the lysed cells measured at 540 nm. (•) muralysin-treatment supernatant (MS), (□) sonication supernatant (SS), (△) SS preincubated 20 min at 20C with 0.1 unit muralysin.



undiluted preparations containing >200-400 IU caused essentially immediate, total hemolysis. These findings could be explained by the presence of two different hemolysins. SLS is known to exhibit a delay in hemolysis, while SLO does not (8). A mixture of SLO and SLS would be expected to exhibit no delay in hemolysis; therefore, the presence of any SLO would mask the presence of SLS. The disruption of cells with muralysin would tend to result in higher titers of SLO, since 2-mercaptoethanol, present in the muralysin preparation, would provide the reducing conditions necessary for optimum SLO activity. Sonication does not inactivate RNA-core SLS (99). Intracellular SLO has been reported after cells have been broken by sonication by some (99), but not by other investigators (87); the difference could be due to differences in length and intensity of sonication. The sonication procedure I used may have inactivated SLO leaving delayed-type hemolysin.

The two disruption procedures on the same batch of cells resulted in preparations with different titers. This might be expected if the two procedures differed in their efficiency of cell breakage or in the degree of inactivation of a particular hemolysin. However, there was no consistency in the ratio of hemolysin titer from cells lysed by sonication to that from cells lysed with muralysin. In some preparations the hemolysin titers were approximately equal. In others, the hemolysin titer from muralysin-treated cells markedly exceeded that from sonicated cells. This was especially true for early or mid-log phase cells. However, the SS hemolysin titer exceeded the MS hemolysin titer from late-log or stationary phase cells. These results

suggested that differences in the efficiency of cell breakage were not the explanation.

Neither the difference in titers nor character of hemolysis was due to the muralysin preparation itself, which had neither hemolytic nor inhibitory activity. The addition of muralysin to SS either 20 min before or with the RBC did not affect the lag or rate of hemolysis (Fig. 9).

To determine if MS did contain a delayed-type hemolysin in addition to the fast hemolysin, an effective means of inhibiting each hemolysin was necessary. Cholesterol and anti-streptolysin O [aSLO] have been used as inhibitors of SLO, while trypan blue, Ca^{2+} and lecithin have been used as inhibitors of RNA-core SLS (88). The effect of cholesterol, aSLO, and trypan blue on SS hemolysin and MS hemolysin were used as an approach to determine whether the SLO-like and SLS-like hemolysins were present in these preparations.

Trypan blue (25 $\mu\text{g}/\text{ml}$) (97) did inhibit the majority of hemolysin in MS. The MS hemolysin preparation used in the experiment in Fig. 9 had a titer of 210 HU/ml. Only 25 HU/ml were not inhibited by trypan blue. There should, therefore, have been 185 HU/ml of SLS-like hemolysin and 25 HU/ml of SLO-like hemolysin in the MS preparation. The hemolysis by 1 HU of MS hemolysin in Fig. 9 should, therefore, exhibit a lag in hemolysin or only minimal initial hemolysis, since there would be only 0.13 HU/ml of SLO present. That the hemolysis by 1 HU of MS was characteristic of SLO, not SLS, suggested that 25 $\mu\text{g}/\text{ml}$ trypan blue inhibited intracellular SLO. An alternative explanation -- that small amounts of SLO in the diluted MS preparation were potentiated by delayed (SLS) hemolysin -- was shown to be incorrect.

The mixture of 0.5 HU of both MS and SS hemolysis exhibited the same initial rate of hemolysis as 0.5 HU of MS, but showed an increase in rate of hemolysis at 25-35 min which would be characteristic of a SLS-like hemolysin (Fig. 10). The two hemolysins were also partially additive and SS hemolysin did not prevent the fast hemolysis of MS.

Antistreptolysin O antiserum was tested as a specific inhibitor of SLO and the fast cellular hemolysin. The procedure, as described in Materials and Methods, required the incubation of the hemolysin and antiserum at 0-4 C to avoid or minimize inactivation of the hemolysin by incubation alone. Even though the extracellular SLO (BBL) was inhibited on incubation of 7 min or less with aSLO, a 20 min incubation was used to insure maximal inhibition of SLO. Since the antiserum (BBL) was unpurified, the possibility existed that it may inhibit SLO because of the presence of some other factor, such as serum lipid, rather than antibody.

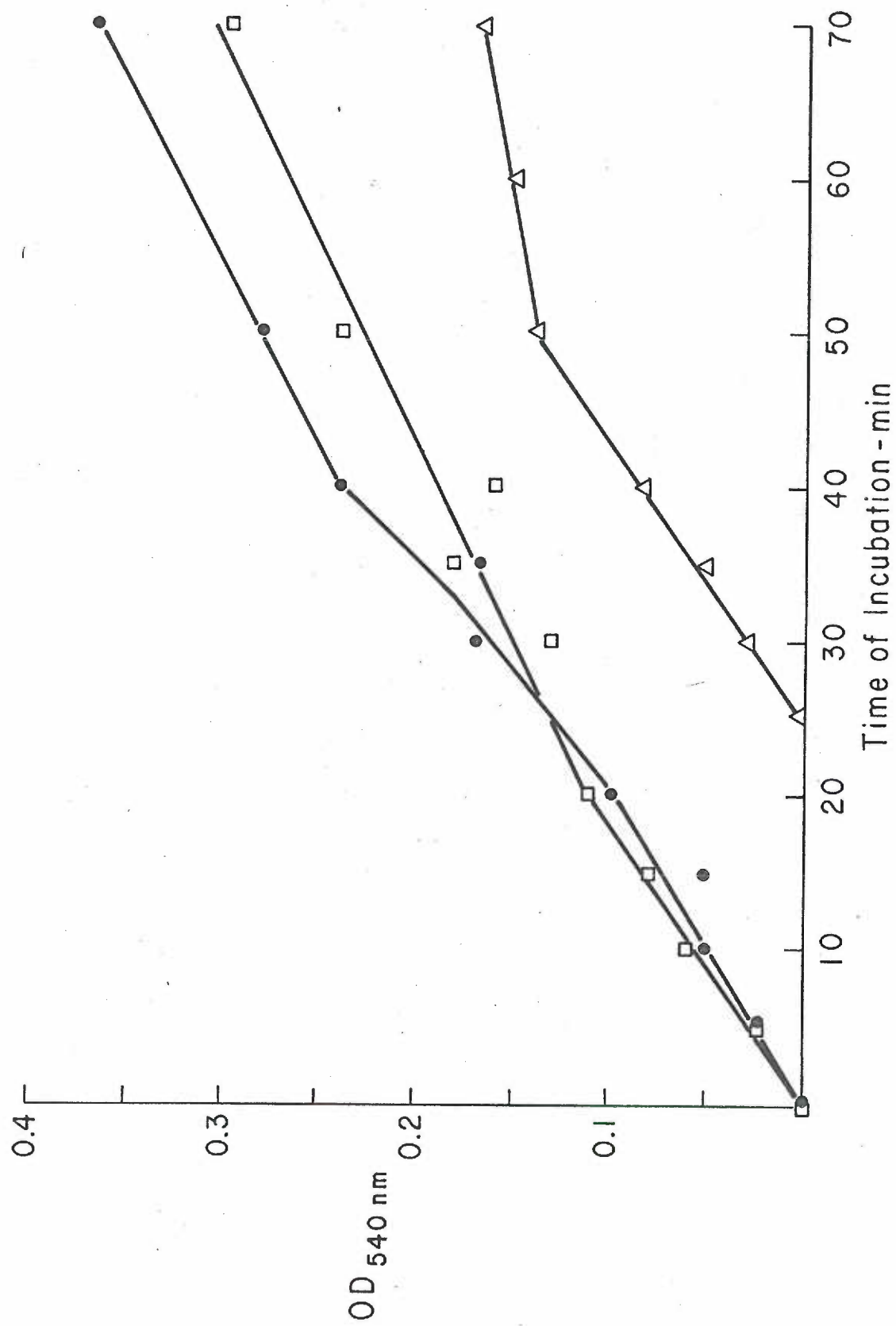
SS hemolysin from late log phase cells was not inhibited by aSLO, but some of the MS hemolytic activity was inhibited (Table 4, 8.5 hr cells). The sum of the hemolysis uninhibited by trypan blue and aSLO should be equal to the titer of MS hemolysin, but were not. In some experiments, there were wide differences between the total MS hemolysin and the sum of that uninhibited by trypan blue and aSLO. For example, in one MS preparation there were 1500 HU, of which 250 HU were uninhibited by trypan blue and 70 HU uninhibited by aSLO, leaving 1180 HU unaccountable.

Although delayed cellular hemolysin resembling RNA-core SLS apparently was present in the MS preparations, it was difficult using

Figure 10.

Rate of Hemolysis of MS Hemolysin, SS Hemolysin, and a Mixture of Both

A series of replicate tubes containing RBC and 0.5 HU of MS hemolysin ($\square$), 0.5 HU of SS hemolysin ($\triangle$), or 0.5 HU of both ($\bullet$) were incubated at 37C for the appropriate time. The remaining cells were sedimented at 3000 x g for 5 min and the amount of hemoglobin released from the lysed cells measured at 540 nm.



Treatment	HU/ml					
	4.0 hr cells			8.5 hr cells		
	MS	MS _S	SS	MS	MS _S	SS
None	194 _F	100 _D	250 _D	90 _F	140 _D	390 _D
aSLO (0.6 Todd Units)	5 (-)	54 _D	180 _D			
aSLO (0.4 " ")				30(-)	135 _D	400 _D
Cholesterol* (0.1 mg/ml)	0	31 _D	143 _D			
Trypan blue (25 μ g/ml)				35 _F	0	0

Table 4. Effect of Inhibitors on Hemolysins from 4.0 and 8.5 Hour Cells.

All incubations with inhibitors done at 0-4 C as described in Materials and Methods. *Cholesterol suspension prepared by adding 5 mg cholesterol to 5 ml distilled water and sonicating for 30 min on model 1 sonicator. The cholesterol was mixed with hemolysin at 0-4 C for 20 min prior to assay of hemolysin. MS_S = MS preparation sonicated 20 min on model 1 sonicator at 75 intensity. F = hemolysis without an observed lag; D = hemolysis with an observed lag; (-) = unsure of onset in hemolysis.

trypan blue (25 $\mu\text{g/ml}$) or aSLO to determine its titer with certainty or to attempt to characterize it. A lower concentration of trypan blue (13 $\mu\text{g/ml}$) inhibited RNA-core SLS, but still caused some inhibition of extracellular SLO. The lower concentration was used in the remainder of the experiments. Lower concentrations of aSLO did not totally inhibit SLO and could not be effectively used. In these experiments, even RNA-core SLS was slightly inhibited by the minimal concentrations of aSLO required to totally inhibit extracellular SLO (0.1 Todd unit/30 HU SLO). It should be noted that in Table 4 the inhibition of SS hemolysin from 4.0 hr cells by aSLO (0.6 TU) occurred only in this one experiment. Cholesterol did inhibit most of the fast hemolysin (Table 4, 4.0 hr cells), but in some experiments there were again wide differences between the total MS hemolysin and the sum of cholesterol-uninhibited and trypan blue-uninhibited hemolysins.

MS was then sonicated on the assumption that SLO would be inactivated. The rates of hemolysis did change from immediate to delayed, and the pattern of inhibition changed from that of SLO or a mixture of SLO and SLS to that of SLS (Table 4). The titer decreased as expected when MS from early to mid-log phase (4.0 hr) cells was sonicated, but increased when late log-phase (8.5 hr) cells were sonicated. This indicated that sonication of MS did more than inactivate SLO. One could not be sure then that delayed hemolysin (SLS) existed prior to sonication.

There was a pattern in the ratio of SS hemolysin to MS hemolysin at different stages of growth and in the titer changes of MS hemolysin on sonication. In early log-phase cells, the ratio was approximately 1

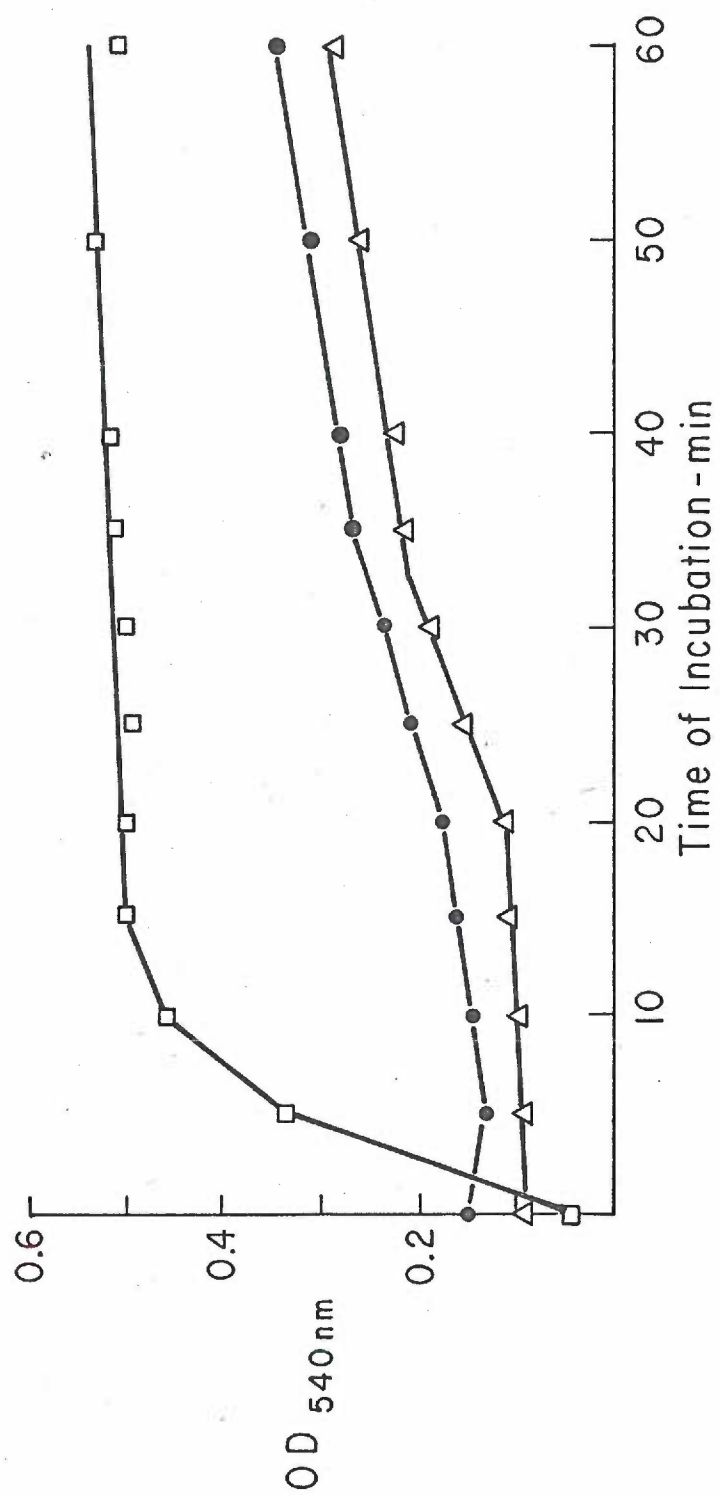
(Table 4). Almost all the MS hemolysin was inhibited by aSLO and the titer decreased on sonication. In late log-phase cells, the titer of SS hemolysin was two to four times that of MS hemolysin. Some of the MS hemolysin was not inhibitable by aSLO and the titer increased on sonication. The time at which an increase in titer of MS hemolysin on sonication could be demonstrated correlated with the time when sonically-released hemolysin was maximal, as reported before. The time at which a decrease in titer of MS hemolysin could be demonstrated correlated with the time when there were only small levels of sonically-releasable hemolysin.

Since none of the known procedures for selectively inhibiting one cellular hemolysin were useful, two procedures were devised to demonstrate the existence of an active cellular hemolysin with SLS-like characteristics prior to sonication. If the fast hemolysin were SLO, a sulfhydryl-group inhibitor such as N-ethylmaleimide (NEM) ought to inactivate it, leaving functional other cellular hemolysin. At 2.5 mM, NEM did inhibit 85% of MS hemolysin, but did not affect hemolysis by SS or sonicated MS. After 20 minutes of incubation, the rate of hemolysis by 1 HU of NEM-insensitive hemolysin in MS was similar to that of SS hemolysin and characteristic of RNA-core SLS hemolysin (Fig. 11). Inhibition of the fast hemolysin by NEM (or cholesterol) indicated the presence of a second delayed hemolysin. Since some SLO escaped inhibition, and because there was doubt as to whether the cellular hemolysin in question had been partially inhibited by NEM, another procedure to inhibit or remove SLO was desired.

Figure 11.

Rate of Hemolysis of One Unit of MS Hemolysin prior to and after
Addition of N-ethylmaleimide (NEM) or Cholesterol

A series of replicate tubes containing RBC and 1.0 HU of MS hemolysin ($\square$), 1.0 HU of (MS hemolysin + mM NEM) ($\bullet$), or 1.0 HU of (MS hemolysin + 0.05 mg cholesterol) ($\triangle$) were incubated at 37C for the appropriate time. The remaining cells were sedimented at 3000 x g for 5 min and the amount of hemoglobin released from the lysed cells measured at 540 nm.



The presence of a second hemolysin in MS was clearly demonstrated after the adsorption at 0-4°C of all the fast, SLO-like, hemolysin by sheep RBC membranes. The residual hemolysin in the membrane-adsorbed preparation (MS_m) was again of the delayed type, like that revealed by NEM or cholesterol treatment, and in some experiments was of considerable magnitude. For example, of an initial 187 HU/ml of fast hemolysin in one MS preparation from late log-phase cells, about 60 HU/ml of delayed hemolysin remained after adsorption with either sheep RBC membranes (RBC_m) or sheep RBC (Table 5). The lag in hemolysis by MS_m hemolysin (Fig. 12) was the same as that by SS hemolysin, and is characteristic of RNA-core SLS. N-ethylmaleimide (20 mM) did not diminish the activity of MS_m hemolysin and did not affect the lag in, or rate of, hemolysis (Fig. 12). The MS_m hemolysin activity, therefore, was not a result of the reducing conditions caused by the 2-mercaptoethanol in the muralysin preparation. This delayed-cellular hemolysin in MS_m was totally inhibited by 13 µg/ml trypan blue, and was very labile, losing about half the activity during 4 hr in an ice-water bath (Table 6). In contrast, the delayed cellular hemolysin in SS lost no activity under the same conditions.

The initial adsorptions of MS with RBC_m were done with a large excess of membranes to insure that all SLO would be adsorbed. For example, in one series of experiments, the minimum of cellular SLO adsorbed by membranes from 1 ml of packed sheep RBC was found to be 7000 HU. The membranes could be reused for several adsorptions. When such large amounts of membranes were used, a second adsorption of MS_m

<u>MS Preparation and Treatment</u>	<u>Titer (HU/ml)</u>	<u>Type</u>
Preadsorption	187	Fast
After Adsorption with RBC _m prep #1	60	Delayed
After Adsorption with RBC _m prep #2	60	Delayed
After Adsorption with RBC	55*	Delayed

Table 5. Effect of Adsorption of MS with RBC_m or RBC.

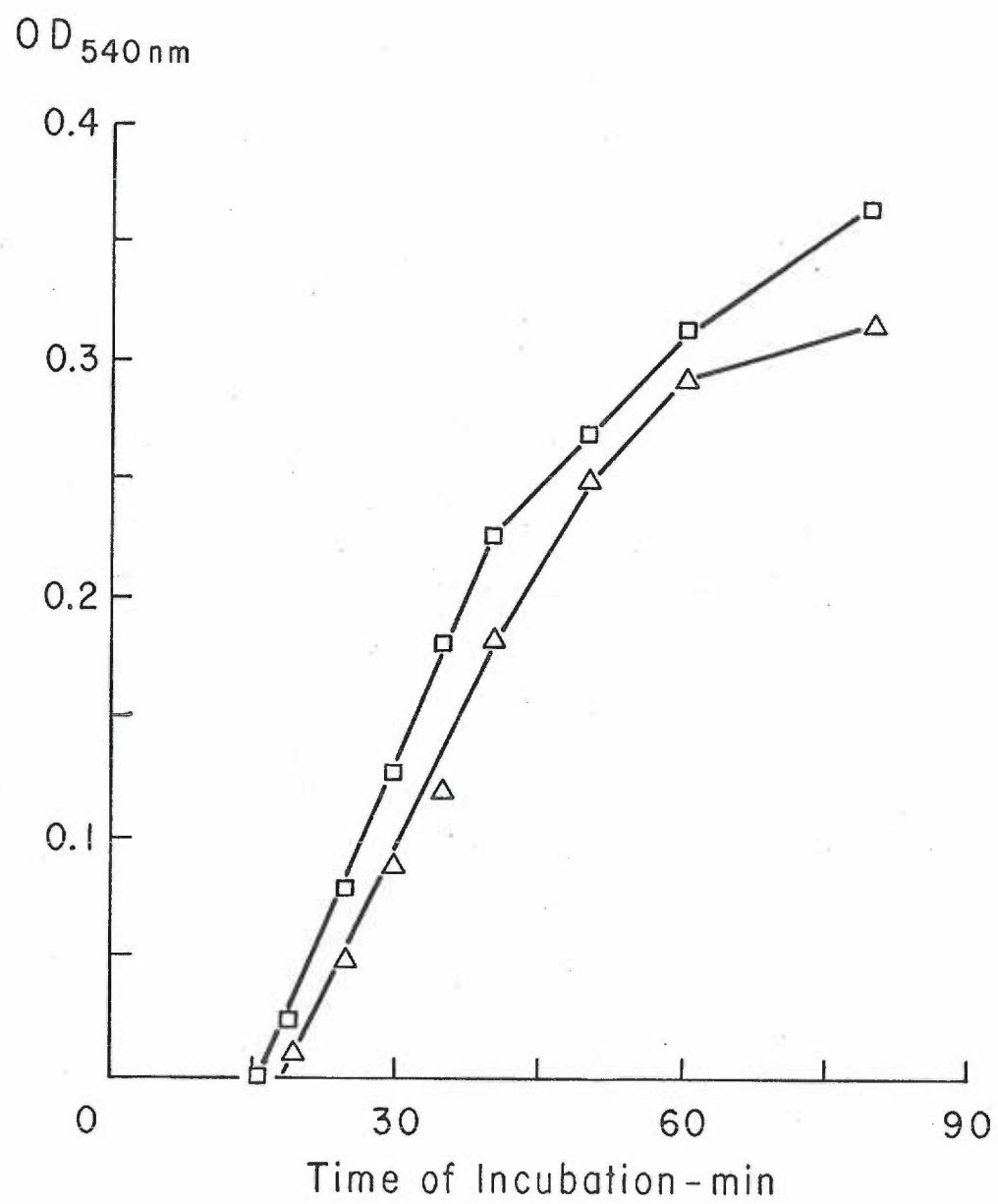
MS prepared from mid- log-phase cells. RBC_m preparation #1 prepared as in Materials and Methods. RBC_m preparation #2 prepared by the procedure of Dodge et al. (29). Whole RBC: 0.4 ml of packed cells. RBC_m preparations contained membranes from approximately 0.5 ml packed cells.

*Slight hemolysis was present after removal of RBC.

Figure 12.

Rate of Hemolysis of One HU of MS_m Hemolysin with or without NEM

A series of replicate tubes containing RBC and 1.0 HU of MS_m hemolysin with (Δ) or without ($\square$) 20 mM N-ethylmaleimide were incubated at 37 C for the appropriate time. The remaining cells were sedimented at 3,000 x g for 5 min and the amount of hemoglobin released from the lysed cells measured at 540nm.



<u>Time (hr)</u>	<u>MS_m Titer (HU/ml)</u>
0	193
1.5	153
3.0	119
4.0	94
20.0	13

Table 6. Decrease of MS_m Hemolysin Titer at 4 C.

MS_m from late log-phase cells held in ice-water bath and sampled at the indicated times.

did result in a small decrease in titer. For example, one re-adsorption resulted in a decrease from 46 to 40 HU/ml and in another from 55 to 42 HU/ml. These changes were minimal compared to the 100 to 400 HU/ml of SLO removed. Adsorption of purified RNA-core SLS with RBC_m , on the other hand, did not result in any decrease in titer.

Treatment of MS_m hemolysin with aSLO did result in a decrease in titer from 97 HU/ml to 43 HU/ml after treatment with 0.4 Todd units of aSLO. However, RNA-core SLS prepared from the resting cell system was also slightly inhibited by aSLO, as stated before. That the inhibition of MS_m hemolysin is due to specific antibody is doubtful since RNA-core SLS which is also slightly inhibited is not antigenic (39).

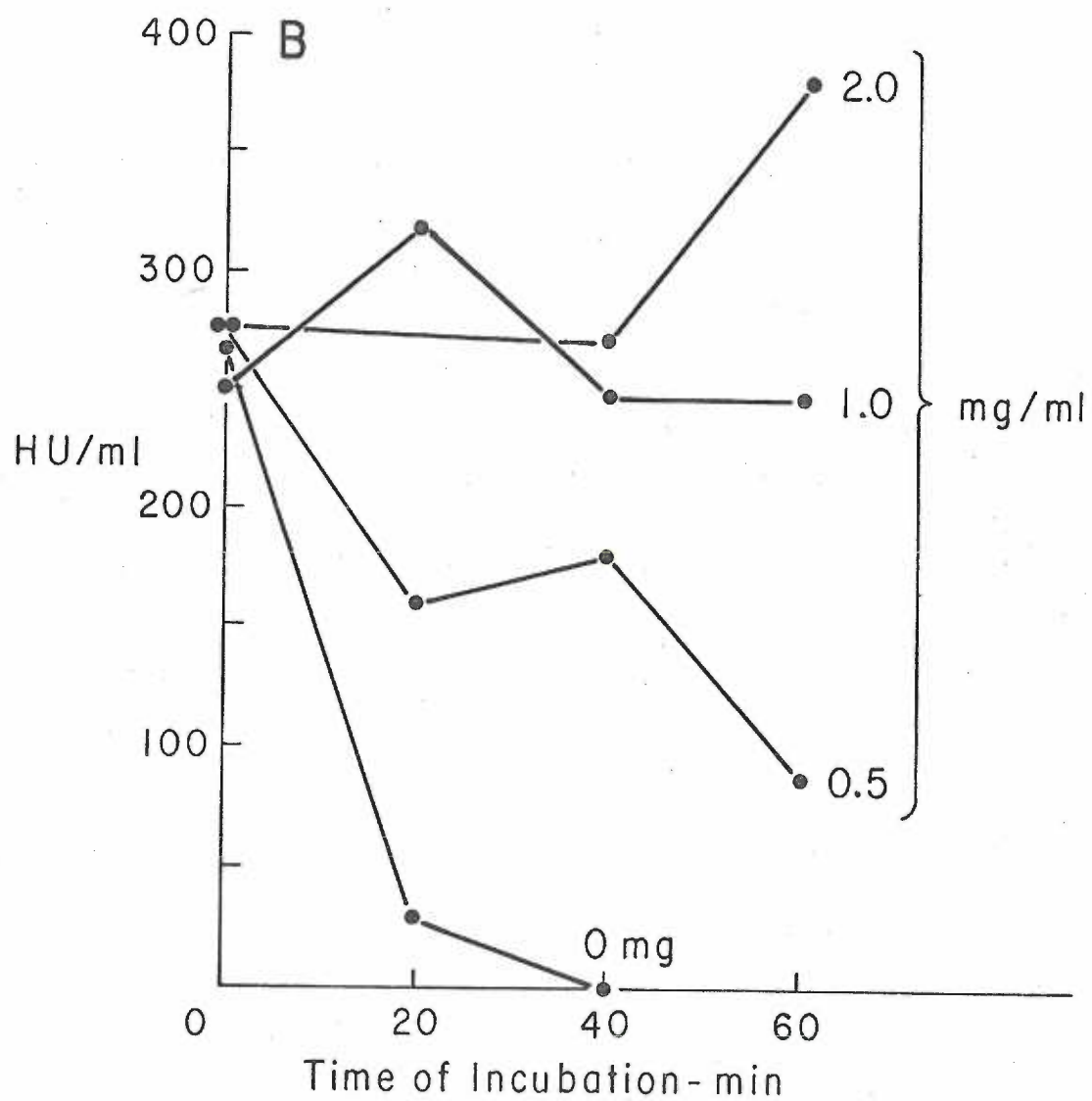
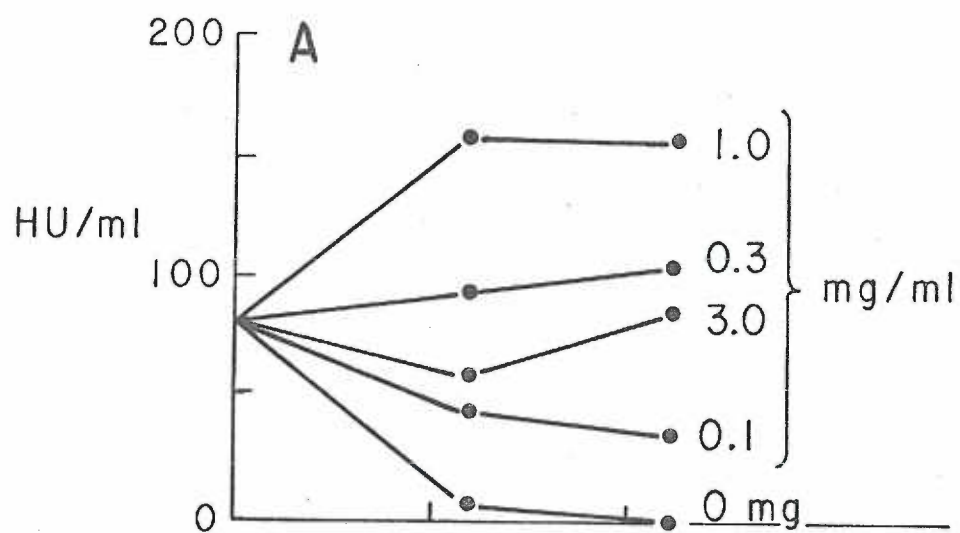
Characterization of MS Hemolysin (LACH)

One of the primary characteristics of SLS is its stabilization by RNA-core (42). Such stabilization of MS hemolysin (hereafter abbreviated as LACH for Labile Active Cellular Hemolysin) would be strong support that the hemolytic moiety is that of RNA-core SLS. When an MS_m preparation was incubated at 37C in Bernheimer's basal medium with varying concentrations of RNA-core, an RNA-core dependent stabilization of the delayed hemolysin occurred (Fig. 13A). Stabilization of SS hemolysin with RNA-core also occurred (Fig. 13B). Excess core has been shown to inhibit RNA-core SLS (47), and this may account for the inhibition of SS hemolysin by the highest concentration of RNA-core. Although the delayed hemolysins in MS and SS are both labile at 37C unless stabilized by RNA-core, the MS hemolysin is different in its lability at 0C.

Figure 13.

Effect on the Titer of Incubating MS_m Hemolysin or SS
Hemolysin from Late Log-phase Cells with RNA-core at 37C

- A. MS_m Hemolysin.
- B. SS Hemolysin.



The inhibitory effect of RNA-core may have been related to its effect on the RBC. When a mixture of RNA-core (0.02 mg/ml) and 0.5 ml RBC in 5.5 ml buffer was incubated at 37C for 30 min and the RBC sedimented at 3,000 X g for 10 min, the RBC remained as a very compact button of material. Even after vortexing the sedimented RBC for several seconds at high speed, some remained in clumps. When the sedimented RBC were resuspended in fresh buffer and reincubated with 1 to 2 HU of either SS hemolysin or MS hemolysin (containing fast hemolysin), no hemolysis occurred. Preincubation with RNA-core prevented not only hemolysis by SLS-like hemolysin, but also SLO-like hemolysin.

One of the interesting, unsolved problems concerning SLS is the role of Bernheimer's basal medium (BBM) in the production of RNA-core SLS. To determine if BBM is required for the stabilization or binding of LACH by RNA-core, various amounts of peak I RNA-core were added to a LACH preparation suspended in BBM or in phosphate-saline buffer. All the activity of LACH is lost after approximately 25 min incubation at 37C (Fig. 13A). However, the addition of 10.2 OD_{260 nm} units of peak I RNA-core stabilized 40% and 30.6 OD_{260 nm} units stabilized 57%, of a LACH preparation (40 HU/ml) from late-log phase cells during incubation at 37C for 30 min in the phosphate-saline buffer. Comparable stabilization occurred with the same amounts of peak I core in the BBM preparation. When peak I core was added to LACH and the mixture held in an ice-water bath, no stabilization of LACH could be demonstrated after 4 hr; both the LACH-core mixture and LACH alone had lost greater than 50% of their activity. After 24

hr only a small amount of activity remained in LACH alone or LACH plus core, but there was more activity in the preparation containing core. The stabilization of LACH by RNA-core then did not require Bernheimer's basal medium and was markedly enhanced by incubation at 37C.

The stabilization of LACH apparently involves binding to RNA-core. As will be described later, when LACH stabilized by RNA-core is chromatographed on a G-100 Sephadex column, it has the same elution volume as RNA-core SLS. Since the lability of LACH in the absence of RNA-core precludes determination of its elution volume, direct determination of whether the hemolytic moiety of LACH is transferred to RNA-core is presently impossible.

The lability of LACH also precluded a direct measure of any change in titer of hemolysin present in the supernatant after high speed centrifugation. When the normal loss due to lability was used as a correction factor to determine the amount of LACH unsedimented, 95% of the hemolysin could be accounted for in the supernatant after centrifugation at 200,000 X g for 60 min at 4C. For example, when 140 HU of LACH were centrifuged, 100 HU were recovered in the supernatant. The normal loss due to lability during the time of centrifugation was 33 HU. The corrected value for LACH in the supernatant was 133 HU. Therefore, the percentage remaining was $(133 \div 140) \times 100 = 95\%$. This result suggested that LACH was not attached to any subcellular component, but existed as an unbound molecule.

Although purification of LACH proved impossible because of its lability, certain interesting characteristics of this hemolysin were obtained during some attempts to do so. Removal of nucleic

acids with 2.5% streptomycin sulfate did not remove the majority of LACH (Table 7). That removed may have been trapped in the large precipitate that occurred, or a small fraction may have been bound to nucleic acid. The major effect of the removal of the nucleic acid was to decrease the potentiating effect of sonication on the MS_m titer. A small, but significant increase occurred prior to streptomycin sulfate treatment, but a marked decrease occurred after the nucleic acid removal. This suggested that the increase in titer with sonication treatment was dependent on nucleic acid and was possibly independent of LACH.

Large losses of LACH activity occurred during ammonium sulfate precipitation (Table 8). However, of that recovered, the majority of LACH was not precipitated by 50% saturated ammonium sulfate. After increasing the $(NH_4)_2SO_4$ concentration to 75% saturation, the only remaining active hemolysin was in the 75% SAS precipitate. The partial precipitation at the 50% SAS concentration suggested that LACH was larger in size than anticipated; RNA-core SLS is not precipitated by these concentrations of $(NH_4)_2SO_4$.

If LACH had a moderately high molecular weight (greater than 25,000 to 30,000), as suggested by its precipitation by 50% SAS, and is then bound to RNA-core, the resulting complex should elute from a G-100 Sephadex column at a different elution volume than does RNA-core. However, LACH stabilized by RNA-core eluted at the same volume as RNA-core. This suggested that the hemolytic moiety of LACH may be transferred to RNA-core as probably occurs with other SLS-like hemolysins (42).

<u>Preparation</u>	<u>HU/ml</u>
MS	334
MS _m	147
(MS _m) _s	198
Streptomycin Sulfate Precipitate	16
Streptomycin Sulfate Supernatant	121
(Streptomycin Sulfate Supernatant) _s	44

Table 7. Effect of Treating MS_m with 2.5% Streptomycin Sulfate.

The nucleic acids were removed with streptomycin sulfate as described in Materials and Methods. Precipitate removed by centrifugation at 15,000 X g for 20 min. The removal of nucleic acids was monitored by the change in optical density at 260 nm. The initial OD_{260 nm} reading for MS_m was 35.4 and after streptomycin sulfate treatment was 4.15. ()_s indicates that the mixture was sonicated for 20 min on model 1 sonicator at 75 intensity. HU/ml corrected to original volume.

<u>Treatment</u>	<u>HU</u>
MS _m	720
50% (NH ₄) ₂ SO ₄ precipitate	102
50% (NH ₄) ₂ SO ₄ supernatant	204
75% (NH ₄) ₂ SO ₄ precipitate	70
75% (NH ₄) ₂ SO ₄ supernatant	0

Table 8. (NH₄)₂SO₄ Precipitation of LACH

Saturated (NH₄)₂SO₄ solution (SAS) was added to MS_m as in Materials and Methods. To the 50% (NH₄)₂SO₄ supernatant was added SAS to a final concentration of 75%. No correction for loss of activity by inactivation made for LACH.

Activation of Cellular Hemolysin by Sonication

The increase in hemolysin titer on sonication of MS from late-log phase (8.5 hr) cells (Table 4) indicated the possibility of another cellular form of SLS. One possible explanation for the increase was that sonication of MS resulted concomitantly in inactivation of SLO and activation of a latent hemolysin, with the ratio of presonation and postsonication hemolysin titers dependent on proportion of each hemolysin in the preparation. The hypothesis could also explain why sonication decreased the titer of early log phase (4.5 hr) cells. The report of Schwab (87) of rising titer of an intracellular hemolysin on sonication of streptococci for extended periods of time similarly suggested activation of a latent hemolysin. Since the removal of nucleic acids from an MS_m preparation changed the effect of sonication on the hemolysin titer from potentiation to inactivation, it seemed likely that nucleic acids played a role in the titer increase. The effects of sonication with RNA preparations on the titers of MS_m preparations from late-log phase cells were therefore tested. Sonication of a mixture of MS_m and RNA-core resulted in a marked increase in the titer above that obtained by sonication of MS_m alone (Table 9). It was necessary that RNA-core be present during the initial sonication of MS_m ; once MS_m was sonicated, no further activation was obtained by either addition of RNA-core or sonication with RNA-core (Table 9, Exp. 1).

Since endogenous RNA might function with RNA-core in the increase in titer on sonication, the effect of RNA from 4.5 hr and 8.5 hr

Preparations	HU/ml		
	Experiment No.		
	1	2	3
Unsonicated MS _m	38	89	76
Unsonicated MS _m + core	40	1--	---
Sonicated MS _m	178	87	194
" (MS _m + core)	260	362	378
" (MS _m + 4.5 hr RNA)	---	168	---
" (MS _m + 8.5 hr RNA)	---	240	---
" (MS _m) + core	171	---	---
" (MS _{m+s} + core)	176	---	---
" (Core) + MS _m	---	---	75
" (4.5 hr RNA) + MS _m	---	---	38
" (8.5 hr RNA) + MS _m	---	---	46

Table 9. Effect of Sonicating MS from Late Log Phase Cells with Various RNA Preparations and the Effect of Presonation of either MS_m or the RNA Preparations.

Sonicated () or MS_{m+s} : materials presonicated 20 min at 75 intensity on Model 1 sonicator. () indicates that the mixtures were sonicated together. 4.5 hr RNA and 8.5 hr RNA is purified streptococcal RNA from early (4.5 hr) or late (8.5 hr) log phase cells and was prepared as in Materials and Methods. Quantity of streptococcal RNA used= 23.5 OD_{260 nm} Units. Crude RNA-core concentration used= 0.3 mg/ml. Presonicated RNA or RNA core preparations were stirred 20 min at OC with MS_m prior to assay. 1-- indicates not done.

S. pyogenes C203S culture was examined. RNA from 8.5 hr cells was more effective than an equal amount of RNA from 4.5 hr cells (Table 9, Exp. 2), although it should be noted that the equivalency was based on optical density readings, which may not be a valid criterion. The increase in titer on sonication was thus partially dependent on the bacterial nucleic acid present and could explain the increase in titer when RNA-core was not present. However, the activation could not result solely from an effect of sonic energy on the nucleic acid, since no increase in titer of MS_m was obtained when sonicated RNA-core or sonicated streptococcal RNA preparations were mixed with unsonicated MS_m preparation (Table 9, Exp. 3). If MS_m was sonicated without core, the increase in titer was not as great as that which occurred on sonication in the presence of core. Re-sonication of sonicated MS_m after the addition of RNA-core did not result in further increases in titer suggesting that presonation had inactivated potential active hemolysin. None of the RNA preparations, sonicated or not, had hemolytic activity. The optimum time for sonication with RNA-core was about 30 min (Table 10). The optimum time of sonication also suggested that sonication, if conducted long enough, would inactivate the activated hemolysin, at least in the presence of RNA-core. Thus activation of MS_m by sonication with RNA or RNA-core required the simultaneous presence of both types of preparations, and increased to a maximum with prolongation of sonication.

The degree of activation of this hemolysin depended upon the amount of sonic energy utilized and differed when different sonicators were used. For example, the model 4 sonicator, as described in Materials

<u>Time (min)</u>	<u>Titer (HU/ml)</u>
0	50
5	133
10	160
20	230
30	279
40	235

Table 10. Effect of the Length of Sonication on the Titer of MS_m.

Six ml of MS_m were sonicated with 2.5 mg crude RNA-core. Samples (0.5 ml) were removed for duplicate assay at the times indicated.

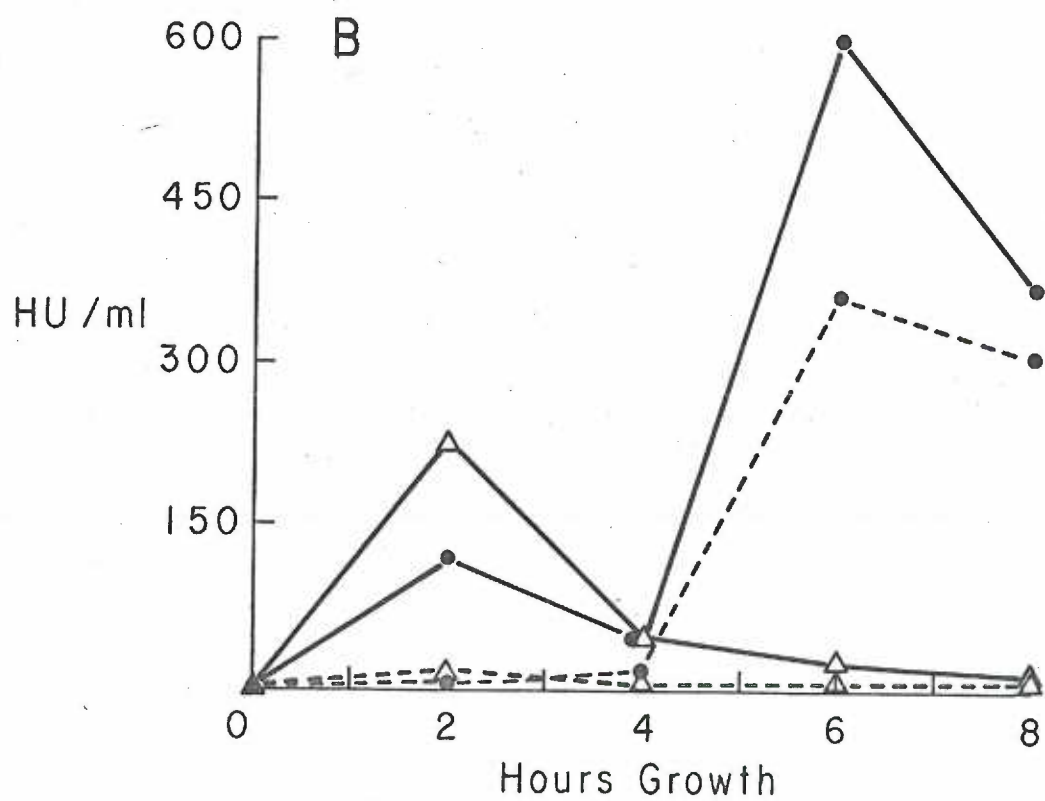
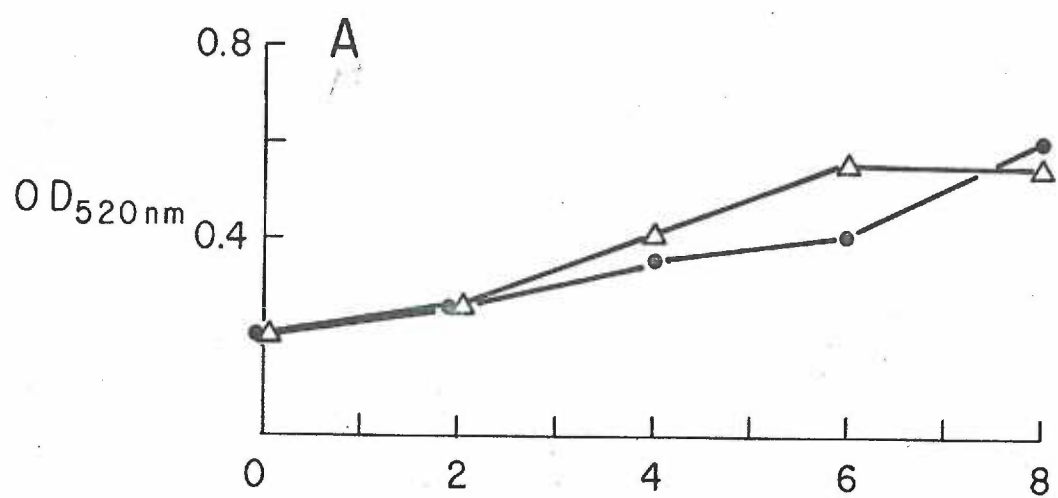
and Methods, activated the hemolysin five times faster than the model 1 sonicator. Although the model 1 sonicator inactivated all cellular SLO present, the model 2 sonicator did not. Direct comparison of the amount of delayed cellular hemolysin liberated by these two sonicators could not be made unless dilute bacterial suspensions were sonicated. Under such conditions, most of the cellular SLO was inactivated by the model 2 sonicator and the amount of delayed cellular hemolysin present was approximately the same as that activated by the model 1 sonicator.

A previous hypothesis to explain the effect of sonication on the hemolysin titer shown in Table 2 proposed that the final sonication titer was dependent upon the amounts of SLO and sonically-activatable hemolysin initially present. Data from experiments in which cells were sonicated with the model 2 sonicator supported this hypothesis (Fig. 14). In the early stages of growth, there was a peak of SLO activity, as measured by that amount of hemolysin adsorbed by RBC_m but little sonically-activatable hemolysin. The supernatant from cells lysed by muralysin at this time (Table 4, 4.0 hr cells) would exhibit fast hemolysis because of the SLO and decrease in titer upon sonication due to destruction of SLO. The small amount of sonically-activatable hemolysin was responsible for the SLS-like character of hemolysin remaining after sonication. In the late log phase of growth (Fig. 14), the majority of hemolysin was sonically-activatable hemolysin. The apparent large differences in pre- and post-adsorption hemolysin at 6 hr may be due to experimental error since the titer differences are due to less than one tube dilution difference in the assay. The supernatant from cells lysed by muralysin at this time

Figure 14.

Amount of Sonically-releasable Hemolysins from
HI-0.3% Glucose-grown and HI-0.3% Maltose-grown
Cells during the Growth Cycle

- A. Growth in HI-0.3% glucose (Δ) and in HI-0.3% maltose ($\bullet$).
- B. Amount of cellular hemolysin prior to adsorption of SS with RBC_m (solid lines) and after adsorption (dashed lines) from cells grown in HI with glucose (Δ) and maltose ($\bullet$). Cells harvested from 100 ml medium, suspended in 5 ml buffer, and sonicated 20 min at 60 intensity with the model 2 sonicator. RBC_m adsorption done with membranes from 0.25 ml packed RBC.



(Table 4, 8.5 hr cells) would exhibit fast hemolysis due to SLO, and increase in titer upon sonication due to inactivation of SLO and activation of a much greater amount of sonically-activatable hemolysin.

Purification of Hemolysin Precursor in MS_m

It was not possible using unpurified MS_m to determine whether sonication potentiated the activity of LACH or activated a hemolysin precursor. Since endogenous RNA was present in the MS preparation, it also could not be determined if part of the increase in titer was independent of nucleic acid. Although LACH was very unstable at -20C, most of the potential for activation ("hemolysin precursor") was retained on storage at this temperature (Fig. 15). The stability of this precursor therefore made purification procedures feasible. Initial purification experiments using streptomycin sulfate to remove nucleic acid, including any added in the muralysin preparation, followed by (NH₄)₂SO₄ (SAS) precipitation resulted in loss of about half of the hemolysin precursor at the streptomycin sulfate step, and recovery of most of the remainder in the 50% SAS precipitate. Calculation of the degree of purification of the precursor on the basis of changes in specific activity was dependent on the titer of hemolytic activity of its sonication product. This titer was found to depend on the amount of RNA-core present during the sonication of the hemolysin precursor (Table 11). The use of relatively large amounts of RNA-core resulted in high titers of hemolysin which approached the titers of RNA-core SLS obtained with the resting cell system of Bernheimer. Excess RNA-core was inhibitory as it was during the stabilization of MS_m (Fig. 13A). The measurement of the total amount of activatable hemolysin

Figure 15.

Stability at -20C of Active Hemolysin (LACH),

Activated Hemolysin, and Hemolysin Precursor in MS_m

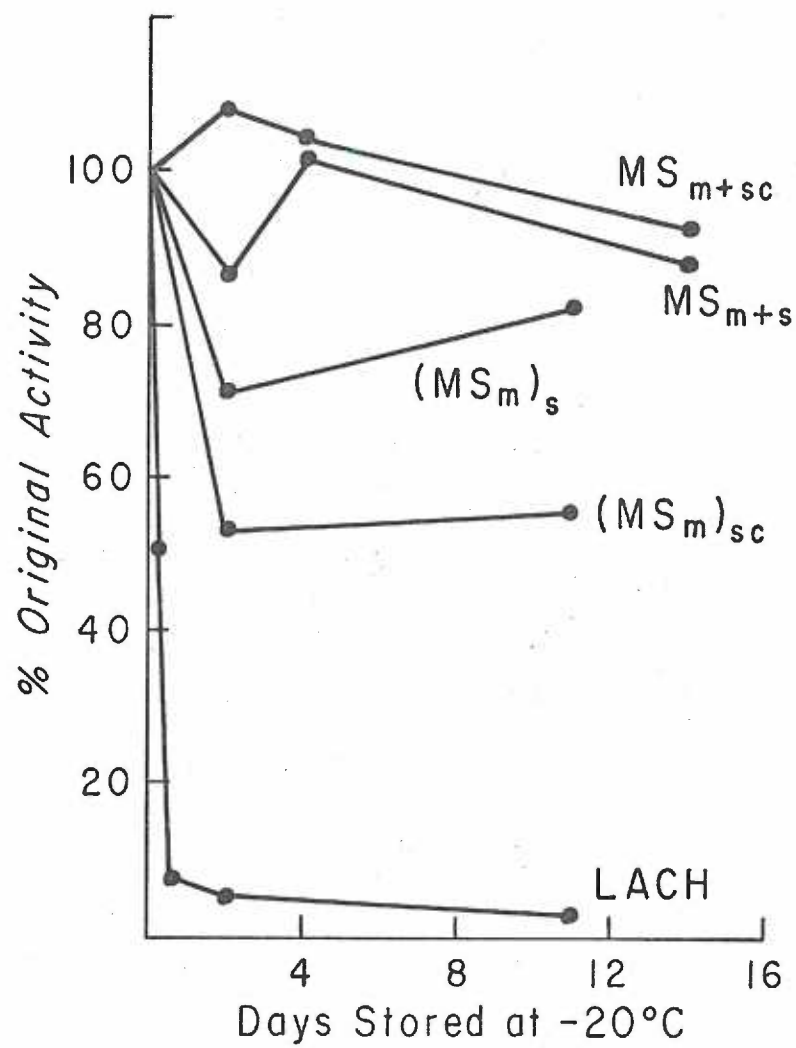
LACH = active MS_m hemolysin.

MS_{m+sc} = MS_m sonicated with crude RNA-core and stored.

MS_{m+s} = MS_m sonicated and stored.

$(MS_m)_s$ = MS_m stored and then sonicated.

$(MS_m)_{sc}$ = MS_m stored and then sonicated with crude RNA-core.



<u>Preparation</u>	<u>HU/ml</u>
Hemolysin Precursor	28
Hemolysin Precursor sonicated	444
(Hemolysin Precursor + 6 mg core) sonicated	1530
(Hemolysin Precursor + 10 mg core) sonicated	1824
(Hemolysin Precursor + 20 mg core) sonicated	1182
(Hemolysin Precursor + 30 mg core) sonicated	trace

Table 11. Effect of Crude RNA-core on the Activation of Hemolysin Precursor by Sonication

Sonication with the model 1 sonicator at 75 intensity for 25 min of MS_m precursor (from late log phase cells) precipitated in the 50% SAS fraction. Mg²⁺ (4 x 10⁻³M) present in all materials sonicated.

in a preparation thus requires several determinations to establish the appropriate amount of RNA-core. However, in order to simplify the experimental procedure, only one concentration of RNA-core was ordinarily used in subsequent experiments unless more exact titers were required.

Mg^{2+} was added to the 50% SAS-precipitated precursor because in the initial experiments with this preparation, Mg^{2+} enhanced the titer. However, it was later found with other preparations that Mg^{2+} had no effect and that chelation of divalent cations with ethylene diamine-tetraacetic acid (EDTA) potentiated the activation of precursor. Since Mg^{2+} was beneficial in some preparations and did not appear inhibitory, it was used in many of the subsequent activation procedures.

Subsequent purification experiments showed that the majority of the hemolysin precursor precipitated at 40% SAS (Table 12). Because of the problem of the optimal amount of RNA-core to be used, the hemolysin titers were based on the higher of two values using two concentrations of RNA-core. The streptomycin sulfate treatment resulted in the largest loss of material during purification, although less than 3% of the precursor was detectable in the streptomycin sulfate precipitate. Streptomycin sulfate (1.0%) added to activated hemolysin caused a 5-10% decrease in titer. Therefore, the lower value of hemolysin precursor in the streptomycin sulfate supernatant fraction than in the total of the SAS precipitates may be due in part to the inhibitory effect of streptomycin sulfate on the hemolysin after activation. Despite the loss of activity, the streptomycin sulfate treatment was retained in further purification experiments in order to reduce as much as possible the effects of endogenous

Preparation	Mixture sonicated		HU/ml	HU _t	% recovery	Specific activity HU/mg protein
	Mg protein	Mg RNA-core				
MS _m	3.1	10	3700	61000	100	600
Streptomycin sulfate supernatant	4.1	4	980	21500	35	120
40% SAS precipitate	1.2	6	6960	20880	34	1800
55% SAS precipitate	3.8	4	840	5060	8	230
70% SAS precipitate	11.0	6	720	2880	5	100

Table 12. Purification of MS_m Hemolysin Precursor from Late Log-phase Cells

The nucleic acids were removed with 1.3% streptomycin sulfate as in Materials and Methods. (NH₄)₂SO₄ precipitation done as in Materials and Methods. Protein determined as in Materials and Methods. Sonication done with the model 1 sonicator for 20 min at 75 intensity using 3 ml vol containing the mg protein of precursor and RNA-core in the table. Total HU (HU_t) calculated by multiplying the HU/ml activated hemolysin times the total vol of hemolysin precursor.

nucleic acid on hemolysin activation and to improve the $(\text{NH}_4)_2\text{SO}_4$ fractionation. Even with the streptomycin sulfate treatment, sonication of 40% SAS precipitate without RNA-core resulted in considerable activation, about half that obtained on sonication with RNA-core. Very little if any RNA was present in the 40% SAS precipitate. In such a precursor preparation containing 292 μg protein and 270 HU, there was less than 3 μg of orcinol-reacting material calculated as ribose (107) remaining in the supernatant after removal of protein by perchloric acid (21). The tailing of the precursor material into the 55% and 70% SAS precipitates may be due to the short time of exposure to $(\text{NH}_4)_2\text{SO}_4$ or to small amounts of hemolysin precursor material of different molecular weight and/or charge.

The hemolysin precursor precipitated by 50% SAS retained 100% of the potential for activation after incubation at 37C for 15 min, and 80% after incubation at 60C for 15 min. The addition of aSLO either before or after sonication had no effect on the hemolysin titer. However, trypan blue at 13 $\mu\text{g}/\text{ml}$ totally inhibited the activated hemolysin derived from the precursor precipitated by 40% SAS, as it did the hemolysins in SS and MS_m . The hemolysin precursor was also unaffected by pretreatment with pancreatic RNase (250 $\mu\text{g}/\text{ml}$) at 37C for 15 min. Considerable activation of the precursor could be obtained by sonication with a preparation of E. coli RNA in the presence of $3 \times 10^{-4} \text{ M}$ Mg^{2+} , but no activation could be demonstrated with rat liver r-RNA or t-RNA preparations.

The hemolysin precursor was precipitated on dropping the pH from 6.5 to 2.0 by the addition of 0.067 N HCl, and was stable for at least 10 min at pH 2.0. Ninety percent of the potential activity was recovered after solution of the precipitate in pH 6.5 buffer. The specific activity increased from 300 HU/mg protein prior to acid precipitation to 1300 HU/mg protein after solution of the precipitate in pH 6.5 buffer.

Attempts to Determine the Cellular Location of LACH and Hemolysin Precursor from Protoplasts and Cell Fractions

If the cellular locations of LACH and hemolysin precursor could be determined, their relationships to each other and to extracellular SLS could be explored. For example, if precursor was bound to the cell membrane and LACH was in the periplasmic space between membrane and wall, it would suggest that LACH may be the active intermediate between a cellular pool of hemolysin precursor and extracellular RNA-core SLS. The most reasonable approach to determine the location of each was to prepare protoplasts and compare the amounts and types of hemolysin present in the periplasmic space, the protoplast membrane, and the protoplast cytoplasm.

Protoplasts of Group A streptococci are prepared by removal of the cell wall with the enzyme muralysin. The removal of the cell wall is associated with a change in staining character of the cell from Gram positive to Gram negative. Since the cell wall is responsible for stabilizing the cell in various osmotic environments, it is necessary to stabilize protoplasts by suspending them in a

solution of ionic strength equal to the internal osmotic pressure of the protoplasts. When successfully stabilized in solutions such as 7% NaCl (36), 2 M sucrose (46), or 0.5 M sodium succinate (73), the protoplasts appear as Gram negative spherules larger in size than the original walled cocci. Demonstration that these organisms are protoplasts is shown by their lysis on subsequent transfer to a hypotonic medium such as 0.03 M sodium phosphate.

Since determination of the cellular location of LACH and hemolysin precursor depended upon successful stabilization of protoplasts, an intracellular marker was needed to determine whether there was leakage of intracellular material. Hexokinase was chosen as an intracellular marker, primarily because of ease of assay. However, only a trace of hexokinase activity was detected (116) in the 3 ml sonicate of 100 ml late-log phase cells grown in HI-0.3% maltose. The procedure used was either not sensitive enough, or these cells did not contain measurable amounts of hexokinase after sonication. An attempt to utilize acid inorganic pyrophosphatase as an intracellular marker was also unsuccessful, because none could be detected in sonicated cell supernatant. Since no useful intracellular enzyme marker was found, the liberation of nucleic acid, as measured at 260 nm, was utilized as an indication of release of intracellular material. Liberation of stringy, viscous material after treatment of cells with muralysin was also used as evidence of cell lysis and release of DNA (96).

The early attempts to stabilize protoplasts were done prior to discovery of hemolysin precursor and to discovery of RBC_m adsorption

of SLO. The cellular hemolysin measured after complete lysis of whole cells or rupture of protoplasts in a hypotonic medium was, therefore, a mixture of SLO and LACH. Using 0.5 M sodium succinate (73) as a stabilizing agent, hemolysin was detected in the supernatant after muralysin digestion of the cell wall. However, this supernatant contained large amounts of 260 nm adsorbing material which could be accounted for only partially by the addition of that present in the muralysin preparation. This indicated that the protoplasts were not stabilized in 0.5 M succinate, and that any or all of the hemolysin present in the supernatant could be due to that present within the protoplasts (intracellular). Increasing the concentration of sodium succinate to 1.0, 1.4, or 1.8 M did not prevent this partial lysis. Raffinose (0.8 M) and NaCl (2, 3, or 4 M) were also not effective as osmotic stabilizers. Later attempts to stabilize protoplasts when assaying specifically for LACH were also unsuccessful.

The location of hemolysin precursor was also explored. Initially the streptococci were suspended in 7% NaCl to stabilize the protoplasts (35). The cells were incubated with muralysin for 20 min at 37C, rather than 20C, to enhance the activity of muralysin and because hemolysin precursor is stable at this temperature. The 7% NaCl did not stabilize the protoplasts, however, and some of them lysed. When the treated cells were centrifuged at 800 X g for 10 min, there were two sedimented layers below the supernatant. The supernatant was slightly cloudy and contained only Gram negative amorphous material. Both portions of the sediment were stringy and sticky on pipetting.

They contained a few scattered Gram positive cocci per oil immersion field and much Gram negative amorphous material. The fractions were further centrifuged (Table 13) to separate all sedimentable material from soluble cell material released either on muralysin treatment (solubilized cell wall material) or on lysis of unstabilized protoplasts. It was hoped that sonication of these fractions and assay of activated hemolysin would give some idea of the distribution of hemolysin precursor in the sedimentable material. The original sediment after further centrifugation was washed in hypotonic buffer to complete the lysis of protoplasts and to remove any material adhering to the debris. Approximately 75% of the hemolysin precursor was in the sediment and 25% in the supernatant. The sediment containing 28,000 potential HU was resuspended in sodium phosphate buffer and centrifuged at 20,000 X g for 20 min. Twenty percent of the precursor was present in the supernatant. Thus, part of the precursor in the first sediment was either trapped or bound but releasable by washing with sodium phosphate buffer. There were less than 20 Gram positive bacteria per oil immersion field in this sediment; the majority of material was Gram negative amorphous debris including some Gram negative cocci or Gram negative ghosts (94). It seemed unlikely that the few Gram positive cocci and remaining protoplasts contained all the sedimentable precursor; therefore, some hemolysin precursor may be associated with the sedimentable debris of the cell.

NaCl (2 M) was used in other experiments to attempt to stabilize protoplasts. Even with this amount of salt, different amounts of muralysin, and older cells, DNA-like material was released. The

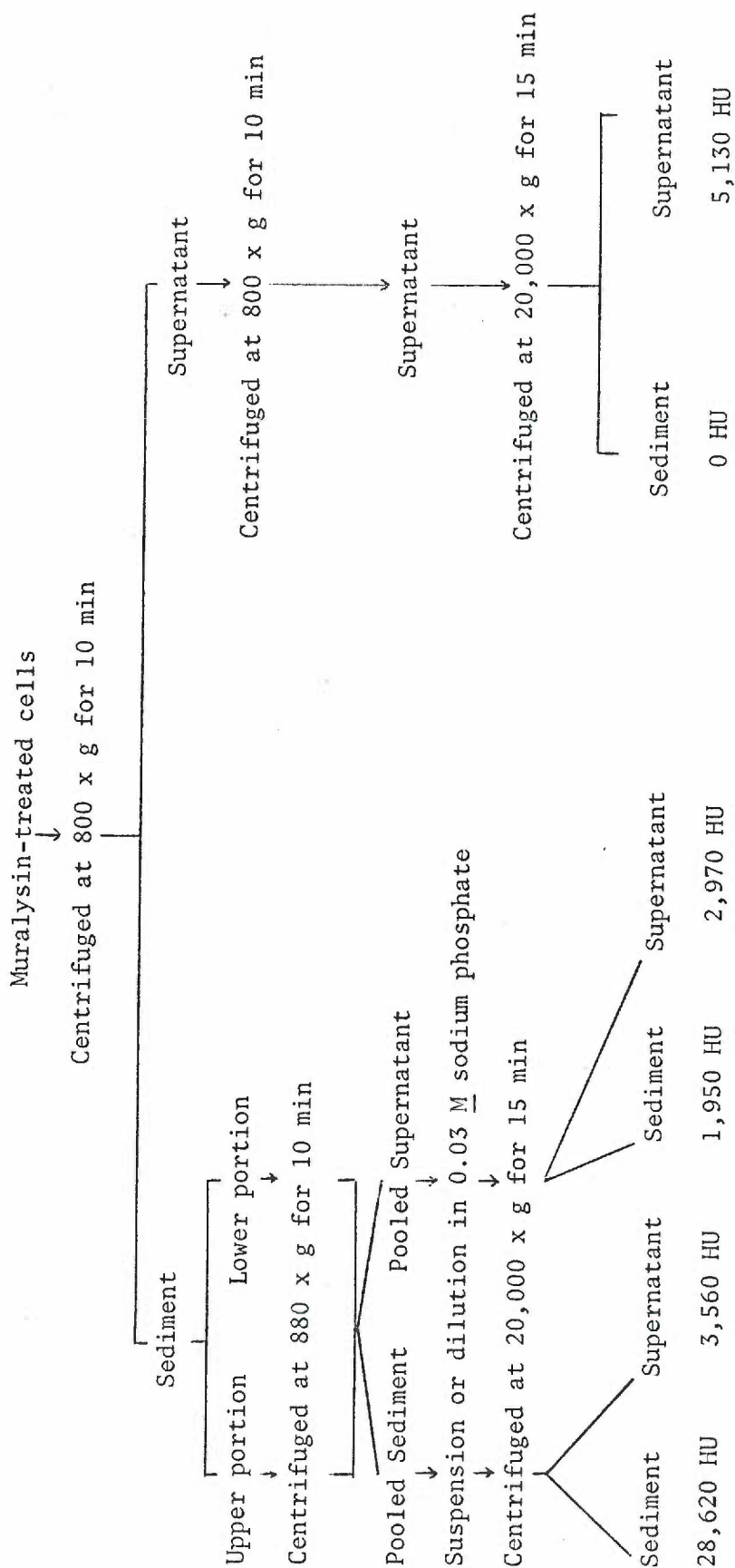


Table 13. Distribution of Hemolysin Precursor in Muralysin-lysed cells.

Cells harvested from late log-phase growth (1.6 liters) were incubated in 0.03 M sodium phosphate buffer plus 7% NaCl with 1100 muralytic units of muralysin for 20 min at 37 C. HU for each fraction was determined by sonication of samples with RNA-core for 25 min at 60 intensity on the model 2 sonicator.

Gram stain of formaldehyde-fixed protoplasts showed predominantly protoplasts in the supernatant of a 800 X g centrifugation for 15 min of muralysin-treated cells, but the sediment was usually gel-like and very difficult to disperse, even on treatment with pancreatic DNase. There were usually not enough protoplasts in the supernatant for further experiments. When amounts of muralysin which were insufficient to remove all the cell walls were used, the whole Gram positive cells which remained were very different to separate from the protoplasts, Gram negative debris, and gel-like material.

Since cells grew very little in HI broth but made large amounts of precursor, attempts were made to stabilize protoplasts from these relatively stationary cells, but these attempts also failed. Spermine (1×10^{-3} M), useful to stabilize Gram negative spheroplasts and some Gram positive bacteria (96), did not prevent lysis of these protoplasts. Since protoplasts could not be stabilized, it was impossible to determine if LACH or precursor was periplasmic or intracellular.

Because of several reports of production or release of RNA-core SLS from subcellular fractions of Group A streptococci (93) (94) (95), the lysates and sonicates of Group A streptococci were examined for RNA-core SLS production. BBM and RNA-core were added to the appropriate preparation prior to incubation of the mixtures at 37C.

When cells harvested from the late log phase of growth were lysed by muralysin, the lysate was not effective for continued and high titer production of RNA-core SLS, although unlysed cells were. Similarly, sonicated cell mixtures also were not effective. Both lysate and sonicate contained hemolysin, but their titer did not

increase by more than 100-200 HU while the titer of hemolysin in the resting cell medium of whole cells increased from initially 0 HU/ml to 4000 HU/ml after incubation. The supernatant from neither the lysate nor the sonicate centrifuged at 20,000 X g for 30 min was effective in high titer RNA-core SLS formation, while intact cells from the same batch were.

The results using uncentrifuged lysates of muralysin-treated cells indicated that no cell fraction would be effective in RNA-core SLS production. However, a fraction similar to the ghost fraction of Sugai (94), except centrifuged at 500 X g for 20 min to remove any whole cells, was examined for hemolysin production. No production could be demonstrated under the same conditions as those described by Sugai (94). The addition to a resting cell system of the same concentration of succinate as used in the buffer of Sugai did, however, partially inhibit RNA-core SLS production by whole cells. Production was also not demonstrable with the partially purified ghost fraction using phosphate buffer at either pH 6.5 or 7.0. Interestingly, this fraction contained neither precursor activatable by sonication nor active delayed hemolysin.

Comparison of the Chromatographic Behavior of Hemolysin Precursor, Sonically-activated Hemolysin, and Other SLS-like Hemolysins

Since both hemolysin precursor and sonically-activated hemolysin were reasonably stable substances, it was possible to determine some of the effects of sonication on precursor. Further, by comparing the chromatographic behavior of activated hemolysin to RNA-core SLS,

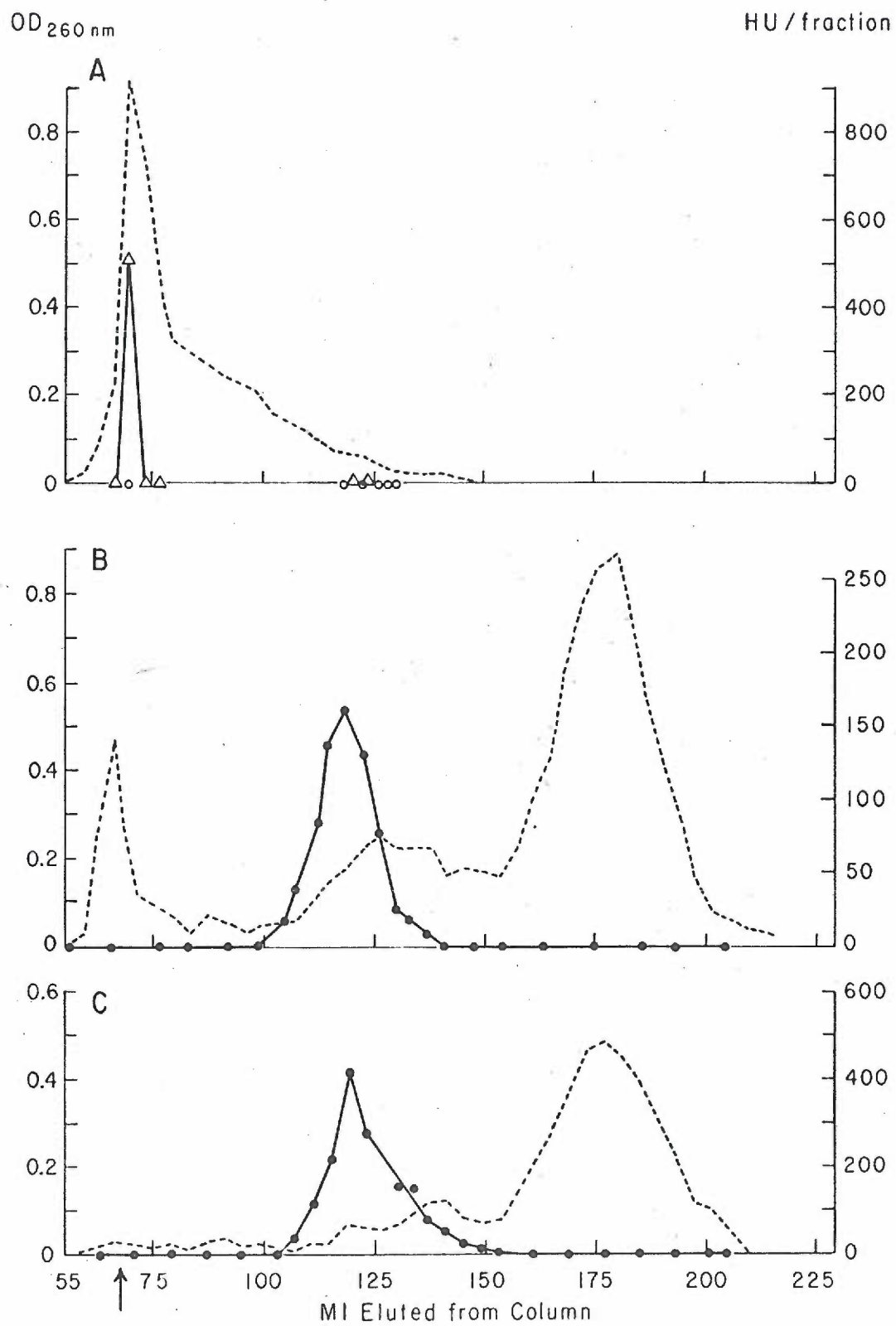
Tween-40 SLS, SS hemolysin stabilized by RNA-core, and LACH stabilized by RNA-core, some proof of identity or relationship between the hemolysins could be obtained.

Although no increase in specific activity of hemolysin precursor was obtained by chromatography on G-75, G-100, or G-200 Sephadex gels, it could be shown by chromatography on G-100 Sephadex gel that, after sonication, activated hemolysin was much smaller than precursor. Upon G-100 chromatography of a 40% SAS precipitate, precursor material was eluted at or just after the void volume (Fig. 16A), but after sonication with RNA-core, the activated hemolysin was eluted between 44 to 86 ml after the void volume (Fig. 16B). Prior to sonication there was no active hemolysin present in the fraction containing precursor, and neither precursor nor active hemolysin was detectable in the fraction corresponding to the elution profile of the activated hemolysin. The size of the majority of the protein in the 40% SAS precipitate did not change during sonication; the two additional peaks in Fig. 16B correspond to the elution profile of the RNA-core. The activated hemolysin eluted slightly before the first peak of RNA-core material. When the hemolysin precursor fraction eluting at 69 ml (Fig. 16A) was sonicated with RNA-core and then chromatographed, the activated hemolysin eluted in exactly the same position as the activated hemolysin derived by sonication of the 40% SAS precipitate. Sonication, therefore, activated a hemolysin precursor which eluted just after the void volume to an active hemolysin eluting 44 ml after the void volume. It did not activate any material eluting 44 ml after the void volume.

Figure 16.

Chromatographic Behavior on G-100 Sephadex of Hemolysin
Precursor, Activated Hemolysin, and RNA-core SLS

- A. Hemolysin precursor: 40% SAS precipitated precursor (2.6 mg) + 180 mg sucrose in 2 ml standard buffer applied to G-100 Sephadex column and eluted with the same buffer. The fractions were assayed for precursor (Δ) by sonicating 1.5 ml eluate + 2 mg crude RNA-core + 3×10^{-4} M Mg^{2+} + buffer to 3.0 ml for 20 min on the model 1 sonicator. Active hemolysin ($\circ$) also assayed.
- B. Activated hemolysin: 40% SAS precipitated precursor (1.04 mg) sonicated with 4 mg crude core. Then 2.2 ml of mixture (equal to 0.76 mg precursor) chromatographed and eluted with standard buffer. All fractions assayed for active hemolysin ($\bullet$).
- C. RNA-core SLS: 3200 HU plus 2.4 OD_{260nm} units of crude RNA-core chromatographed as in B. ($\bullet$) RNA-core SLS.
- Void volume indicated by arrow. OD_{260nm} = dashed line.

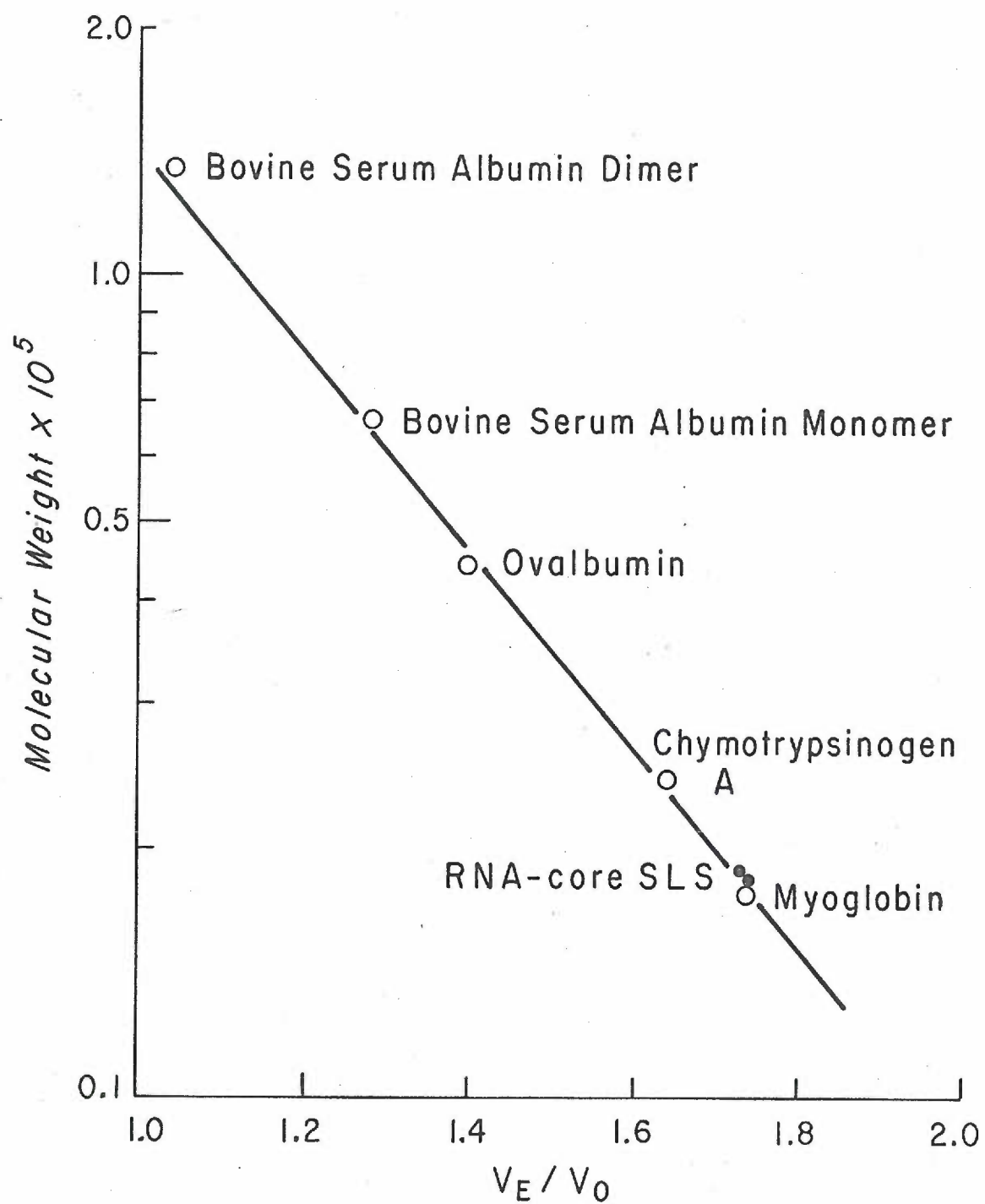


When RNA-core SLS was chromatographed, its elution profile (Fig. 16C) was exactly that of the activated hemolysin. The peak of the RNA-core SLS also elutes slightly before the first peak of RNA-core material. In other words, sonication of hemolysin precursor in the presence of RNA-core produced an activated hemolysin that had all characteristics of RNA-core SLS (inhibition by trypan blue, lack of inhibition by aSLO, delayed character of hemolysin, stabilization by RNA-core, and inhibition by excess RNA-core) including exactly the same chromatographic behavior (and thus presumably the same molecular weight) as RNA-core SLS on G-100 Sephadex. The properties of the sonically-activated hemolysin indicated that hemolysin precursor had been activated to RNA-core SLS.

The molecular weight of RNA-core SLS was determined by the method of Andrews (1) with bovine serum albumin, ovalbumin, chymotrypsinogen A, and myoglobin as markers. From a plot of elution volumes vs logarithm of molecular weight (Fig. 17), the elution volume of RNA-core SLS was equivalent to that of protein of 18,500 molecular weight. However, since RNA-core SLS is a complex of RNA and protein (39), the standards used to construct the curve should have included RNA-protein complexes of different size (2). This was not possible, so the molecular weight of activated hemolysin must be considered only a rough estimate, since its molecular nature is unknown. With these limitations in mind, a quantitative description of the shift in size of the hemolytic moiety from inactive to active form can be stated. During sonication the hemolytic moiety was shifted from the hemolysin precursor of molecular weight greater than 100,000 to active

Figure 17.

Standard Curve for Estimation of Molecular Weight of RNA-core SLS



hemolysin with molecular weight of about 18,500.

The cellular hemolysin which Taketo and Taketo (97) (99) obtained in the sonicate supernatant of Group A streptococci, and termed by them "intracellular" hemolysin, was similar to SS hemolysin because both were obtained by sonication, were relatively stable at 4 C, and were SLS-like in character. They did not report a hemolysin precursor, but did show that the size of the active hemolysin decreased after incubation of their "intracellular" hemolysin with RNA-core, as measured by chromatography. It was of interest, therefore, to determine the chromatographic behavior of SS hemolysin, the mixture of SS hemolysin and hemolysin precursor, and the active hemolysin after incubation of SS hemolysin with RNA-core. The purposes were to determine if 1) SS hemolysin and hemolysin precursor chromatographed similarly, 2) the hemolytic moiety of SS hemolysin was transferred to RNA-core, and 3) such transferred hemolysin had the same chromatographic elution profile as RNA-core SLS. Since Taketo and Taketo had also obtained intracellular SLO on sonication and showed that prolonged sonication decreased the titer of SLO, sonication of C203S was done at a lower intensity than before. Late log phase streptococcal cells were suspended in the phosphate buffer and sonicated for 30 min with a Bronwill Biosonic Sonicator, model 2, at an intensity of 60, instead of with the model 1 sonicator at an intensity of 75. The sonicate contained SLO, characterized by fast hemolysis (Table 14), and lack of inhibition of trypan blue. Chromatography on G-100 Sephadex gel showed two hemolytic components, the second of which corresponded to SLO (Fig. 18A). When the SLO was adsorbed with RBC membranes, only

<u>Hemolysin Preparation</u>	<u>HU/ml</u>	<u>Type of Hemolysis</u>
SS	320	fast
SS _m	4	delayed
SS _m incubated with core	0	----
Resonicated SS _m	460	delayed
Resonicated SS _m incubated with core	660	delayed

Table 14. Effect of Incubating RNA-core with SS_m before and after Resonication.

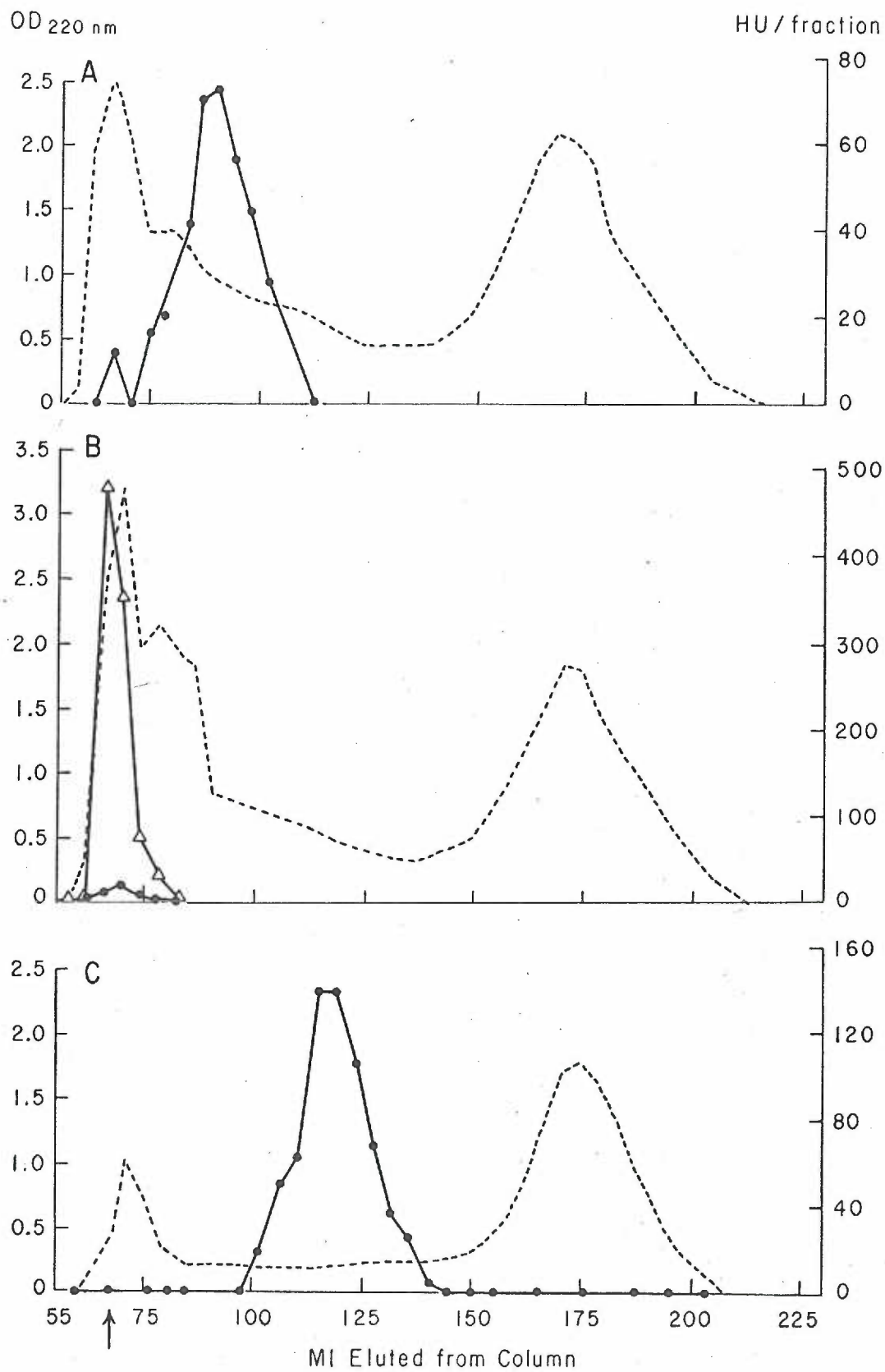
SS was prepared from 800 ml late log phase cells sonicated in 7 ml vol for 30 min. SS_m prepared from SS by adsorption with RBC_m (0.1 ml RBC_m per 1 ml SS). SS_m resonicated for 25 min at 60 intensity on the model 2 sonicator. SS_m or SS_m resonicated (1.5 ml of either) was incubated in Bernheimer's basal medium with 2 mg crude RNA-core in 3 ml total vol. for 60 min at 37 C. All titers corrected to original volume.

Figure 18.

Chromatographic Behavior of SS Hemolysin, SS_m Hemolysin (Resonicated) plus Hemolysin Precursor, and SS_m Hemolysin (Resonicated) after Incubation with RNA-core.

- A. SS hemolysin (prepared as described in Table 14): 2 ml plus 180 mg sucrose applied to G-100 Sephadex column and eluted with standard buffer. Active hemolysin (•) assayed.
- B. SS_m hemolysin (resonicated) + hemolysin precursor: 1.5 ml of SS_m preparation and 1.5 ml of resonicated SS_m preparation applied to column. [See Table 12 for details of preparation.] The eluate was assayed for active hemolysin (•) and also for hemolysin precursor (Δ) by sonicating 2 ml eluate + 1 ml buffer for 25 min on the model 2 sonicator.
- C. SS_m hemolysin (resonicated) after incubation with RNA-core: 2 ml of hemolysin core mixture prepared as described in Table 12 applied to column. The eluate was assayed for active hemolysin (•).

Void volume indicated by arrow. OD_{220 nm} = dashed line.



trace hemolytic activity remained. However, when this adsorbed supernatant (SS_m) was sonicated, delayed hemolysin which was relatively stable at 0C was obtained. Therefore, the adsorbed supernatant contained hemolysin precursor. The mixture of SS_m and resonicated SS_m would then be a mixture of hemolysin precursor and SS hemolysin. Chromatography of a mixture of resonicated material and adsorbed supernatant (Fig. 18B) revealed that both hemolysin precursor and SS hemolysin eluted just after the void volume. There was no significant difference in their elution profile. The first active peak in Fig. 18A also corresponded to the elution volume of resonicated SS_m or SS hemolysin. The majority of SS hemolysin activity was lost on the column. When the hemolysin from the resonicated SS_m (or SS hemolysin) was incubated in Bernheimer's medium with RNA-core, an increase in titer occurred (Table 14), as well as a shift in the size of the hemolysin to that of RNA-core SLS (Fig. 18C), and a stabilization of hemolytic activity during further incubation of moderate amounts of hemolysin at 37C (Fig. 19A). The stability of the SS hemolysin incubated with RNA-core was similar to the stability of a moderate amount of RNA-core SLS incubated at 37C. However, dilute amounts of RNA-core SLS incubated at 37C were much more labile than any of the other hemolysins (Fig. 19B). (At 0-4C, one hemolytic unit of RNA-core SLS from the resting cell system was 100% stable for at least 24 hr.) The majority of the RNA-core stabilized hemolysin was recovered from the column (Fig. 18C), as RNA-core SLS; the majority of SS hemolysin, was usually lost. It was of interest that there was no increase in titer when the original SS_m of low titer was incubated in BBM with

Figure 19.

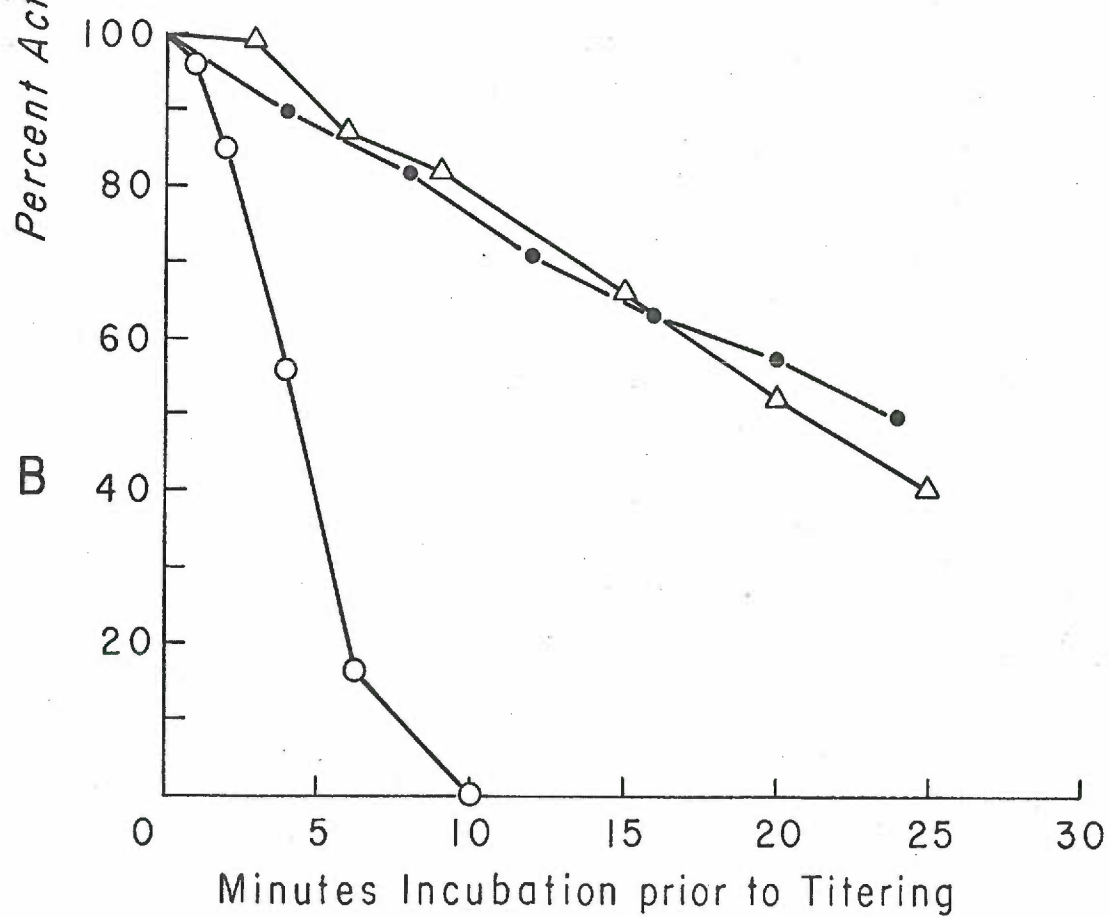
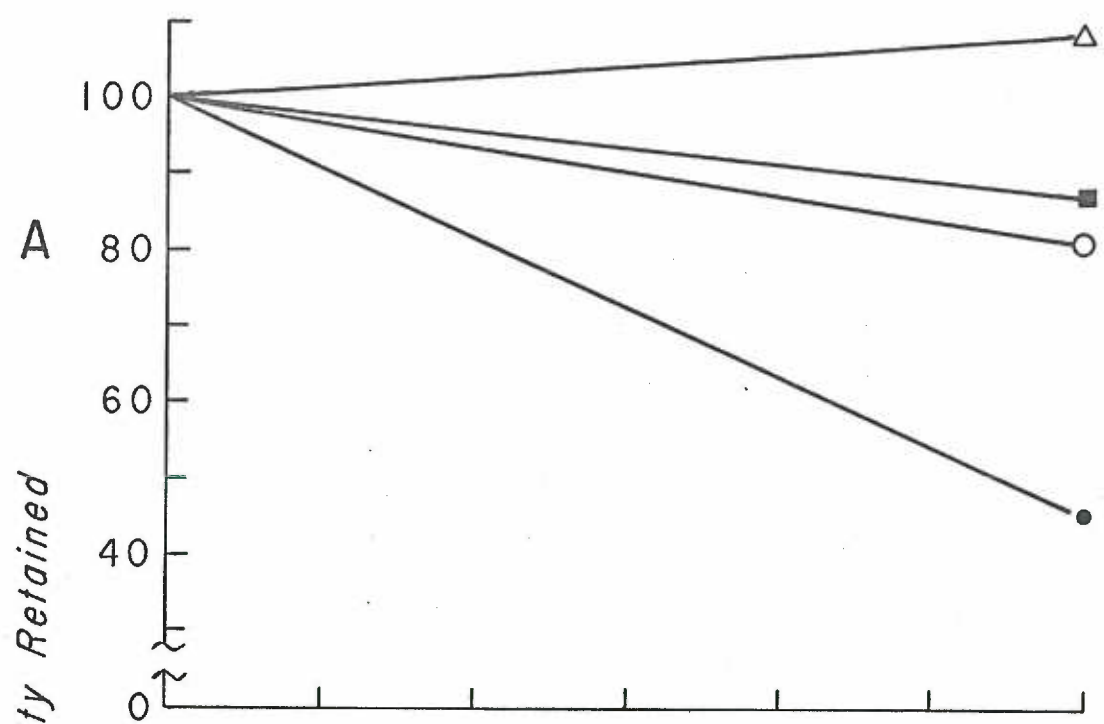
Effect of Incubation at 37C on the Hemolytic Activity of
Different Concentrations of SLS-like Hemolysins

- A. Effect on hemolytic activity of concentrated amounts of RNA-core SLS, SS hemolysin, SS hemolysin preincubated with RNA-core, and activated hemolysin. The hemolysins were titered, incubated in the amounts listed below, and retitered. One ml dilution containing 800 HU of RNA-core SLS (16,000 HU/ml) prepared in the resting cell system, 1 ml activated hemolysin (215 HU/ml), 0.5 ml of SS hemolysin (280 HU/ml), and 0.5 ml of the above SS hemolysin incubated with RNA-core (580 HU/ml) were used in the incubations. The SS hemolysin incubated with RNA-core was prepared by incubating 2 ml SS hemolysin (280 HU/ml) with 0.3 ml RNA-core (10 mg/ml) for 30 min at 37C.

Figure 19. (cont.)

B. Effect on hemolytic activity of 1 to 2 HU of RNA-core SLS, SS hemolysin, and SS hemolysin preincubated with RNA-core. Hemolysins were diluted to 1-2 HU in a final vol of 5 ml. They were incubated at 37C, and 0.5 ml RBC were added at the appropriate times. The tubes were incubated 75 min after the RBC were added and the per cent hemolytic activity remaining measured by the amount of hemoglobin released.

(O) RNA-core SLS, (●) SS hemolysin, (Δ) SS hemolysin preincubated with RNA-core, (■) activated hemolysin.



RNA-core (Table 14). Although hemolysin precursor was present in SS_m, it was not activated in the mixture of BBM and RNA-core. The SS hemolysin transferred to RNA-core, hemolysin sonically activated in the presence of RNA-core, and RNA-core SLS are alike in having the same molecular weight as determined by Sephadex chromatography, the same sensitivity to trypan blue, the same character of hemolysis, and the same stability when associated with RNA-core (Fig. 19A). Chromatography of a mixture of SS hemolysin plus precursor on a G-200 Sephadex column (Fig. 20) further showed that both were very large in size (greater than 200,000 molecular weight); no difference in size between the two could be shown by such chromatography. Therefore, the shift in size during transfer of hemolysin or sonic activation of hemolysin is from greater than 200,000 molecular weight to 18,500 molecular weight. The results of incubation of SS hemolysin or hemolysin precursor in BBM with RNA-core indicate that only active hemolysin is transferred to RNA-core. No activation of hemolysin precursor occurs; data from experiments where whole cell lysates or sonicates were incubated with RNA-core in BBM were presented to show that such incubations also do not result in the activation of hemolysin precursor.

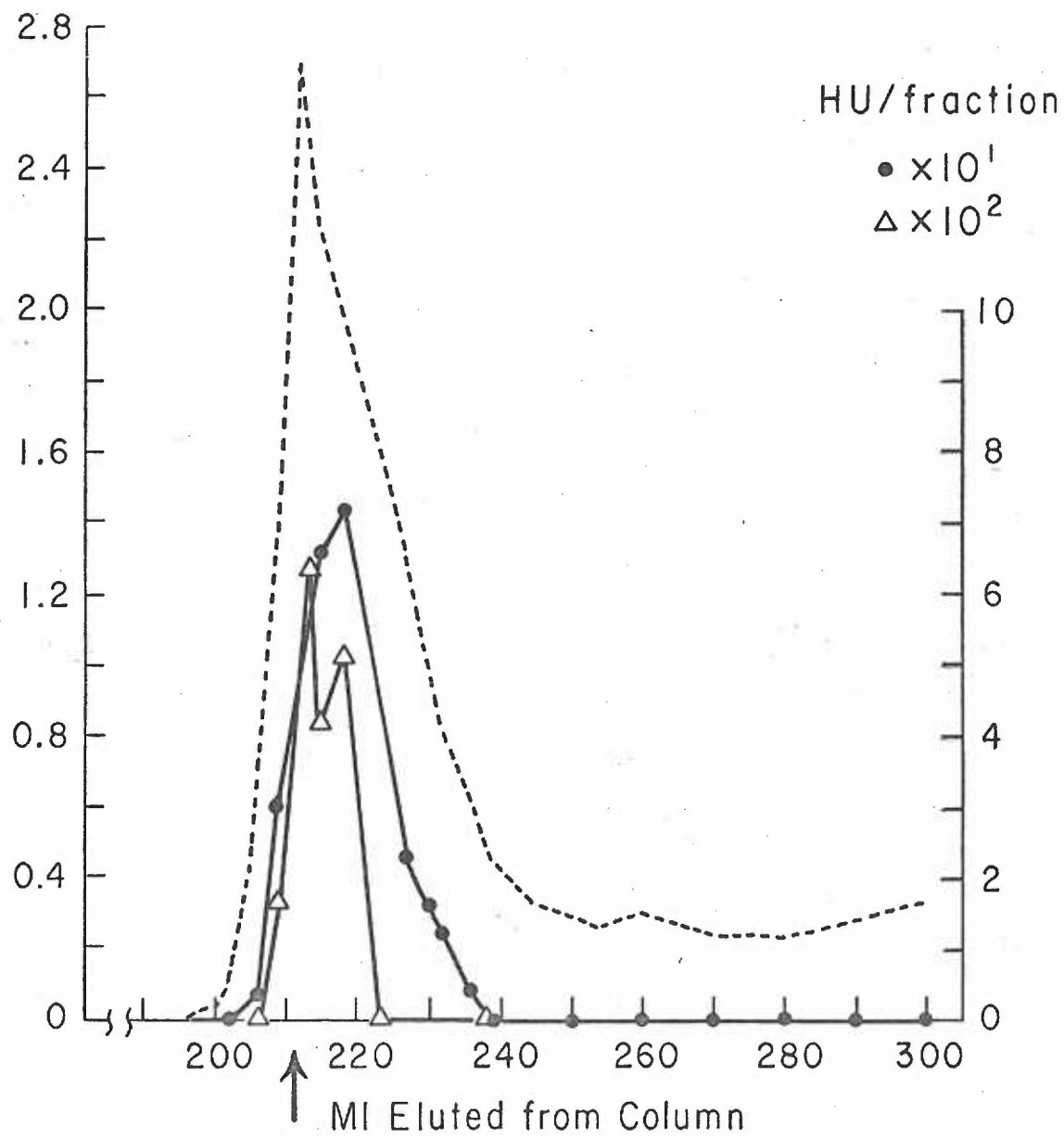
Since SS hemolysin is not present in vivo but hemolysin precursor is, the question arises as to how this hemolysin precursor could give rise to extracellular RNA-core SLS. The LACH could provide the intermediate between the latent hemolysin precursor and extracellular SLS. If the elution volumes of RNA-core SLS, and LACH stabilized by RNA-core, were the same, it would support the hypothesis that the two were related to each other and to the other SLS-like hemolysins.

Figure 20.

Chromatographic Behavior of SS Hemolysin
and Hemolysin Precursor on G-200 Sephadex

An SS preparation (2.5 ml) with 100 HU/ml of SS hemolysin activity was chromatographed on a G-200 Sephadex column and eluted with standard buffer. Active hemolysin (•) and hemolysin precursor (Δ) were assayed in the eluates. Hemolysin precursor was assayed by sonicating 1.0 ml eluate + 1 mg crude RNA-core + 2.0 ml buffer for 25 min with the model 2 sonicator. Void volume indicated by arrow. OD_{220nm} = dashed line.

OD_{220 nm}



Using a different G-100 Sephadex column than used in the experiments reported in Figures 16 and 18, LACH stabilized by RNA-core eluted at the same elution volume (Fig. 21A) as RNA-core SLS chromatographed prior to (Fig. 21B) and subsequent to (not shown) the stabilized LACH. The V_E/V_0 ratios of all three were identical. There was no difference in elution profile between LACH stabilized by RNA-core, and RNA-core SLS.

Tween-40 SLS is an example of another group of SLS-like hemolysins. The hemolytic moiety of Tween-40 SLS is also transferable to RNA-core (Fig. 22). Tween-40 SLS eluted at, or slightly after, the void volume of a G-100 Sephadex column (Fig. 22A). After incubation with RNA-core, almost all the activity was shifted to the elution volume of RNA-core SLS (Fig. 22B). In a separate study, the Tween-40 SLS which eluted just after the void volume was also found to be transferable to RNA-core, showing that RNA-core did not "activate" any hemolysin other than that eluting at or near the void volume. Incubation of RNA-core SLS with Tween-40 resulted in a small amount of active hemolysin eluting at the elution volume of Tween-40 SLS (Fig. 22C). Much of the activity of the RNA-core SLS plus Tween-40 mixture was lost on the column, which does not occur with RNA-core SLS alone. It is possible that there was transfer of SLS from RNA-core to Tween-40 and that the instability of Tween-40 SLS resulted in loss of G-100 Sephadex chromatography. The important point is that the hemolytic moiety of Tween-40 SLS, like all the other SLS-like hemolysins, is transferable to RNA-core, supporting the hypothesis that the hemolytic moieties of all are similar or the same.

Figure 21.

G-100 Sephadex Chromatography of LACH Stabilized by Peak 1 RNA-core and of RNA-core SLS Prepared with Peak 1 RNA-core

- A. LACH Stabilized by RNA-core: 2 ml of MS_m (150 HU/ml) was incubated with 20 OD_{260nm} units of peak 1 core in BBM (3.0 ml total vol) for 30 min at 37C. 1.5 ml of stabilized MS_m (50 HU/ml) + 100 mg sucrose + 0.5 ml blue dextran solution (20 mg/ml) chromatographed together. (○) active hemolysin, (●) blue dextran.
- B. RNA-core SLS: 4 ml of RNA-core SLS (18,000 HU/ml) applied to column. The OD_{260nm} marker (●) is larger RNA fragments which elute at void volume. (○) active hemolysin.

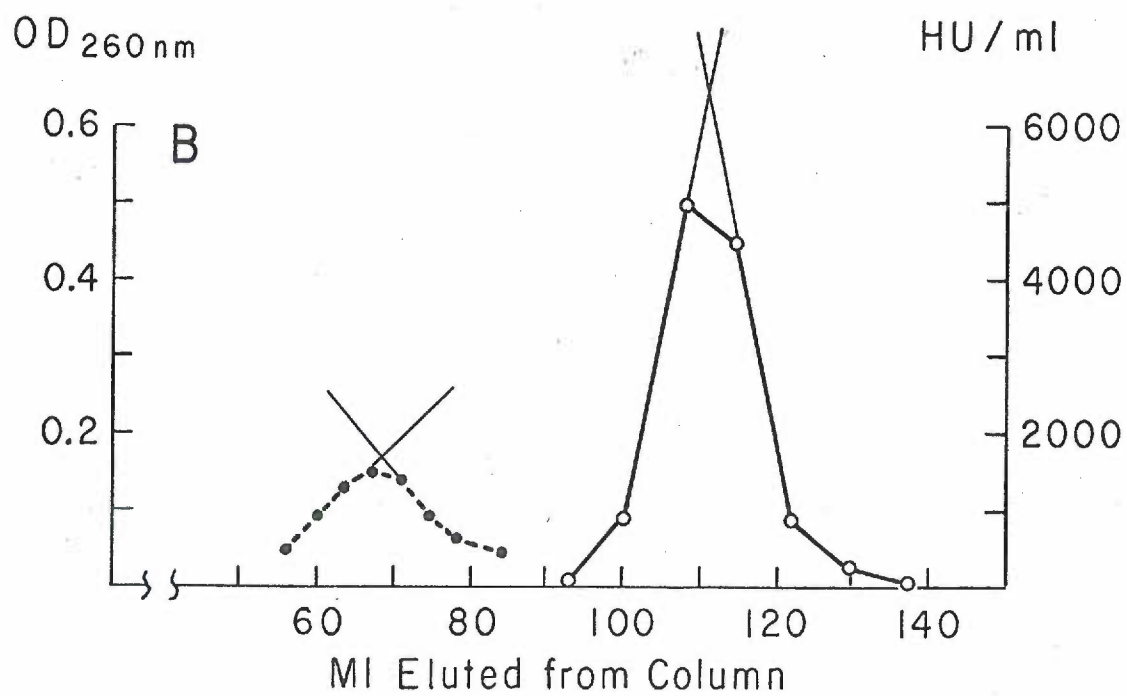
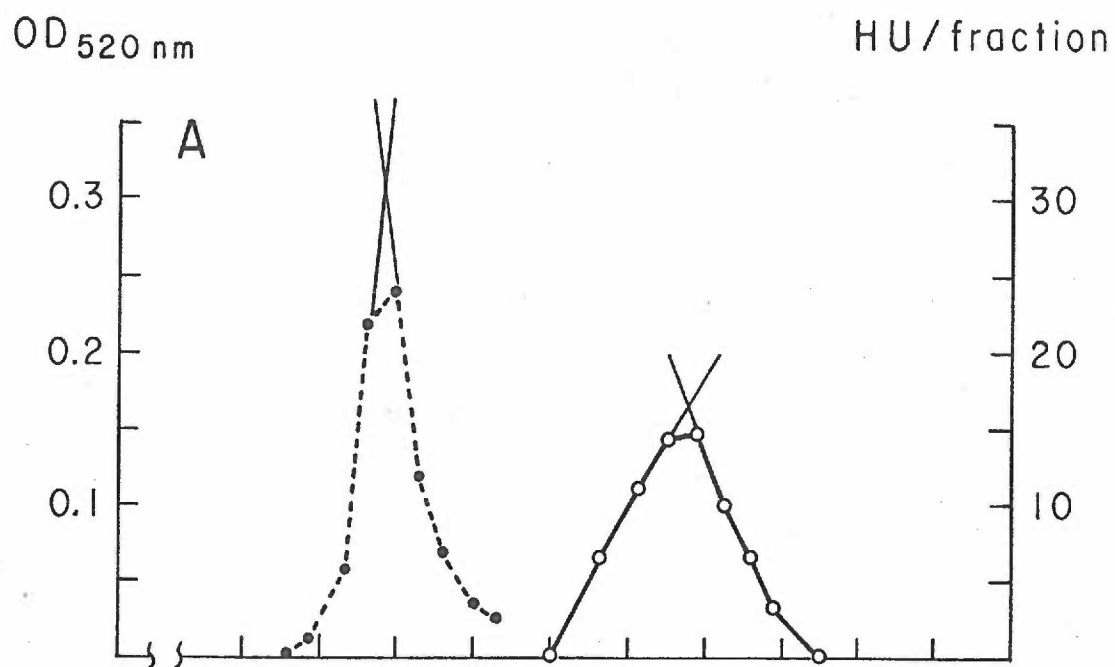
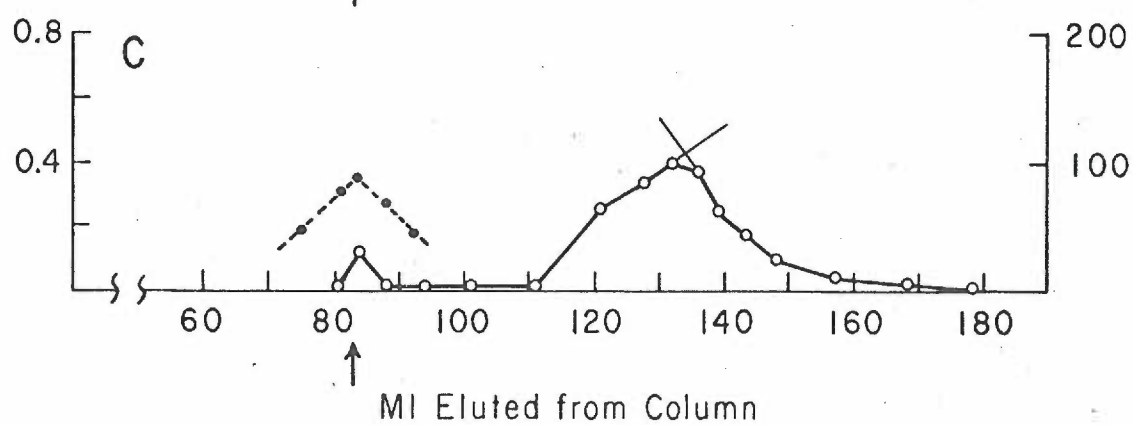
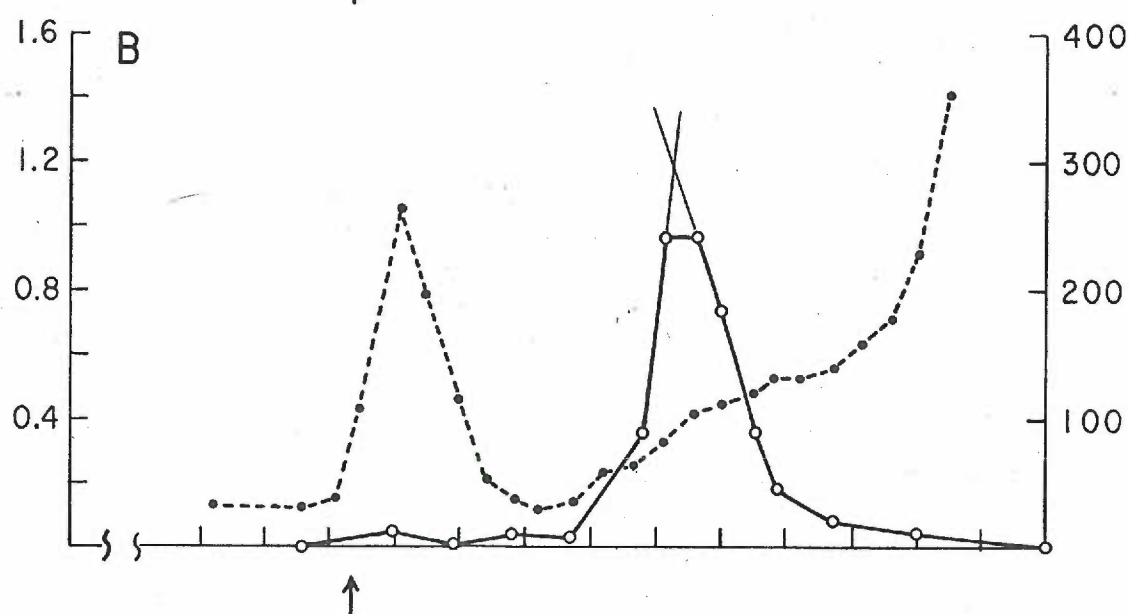
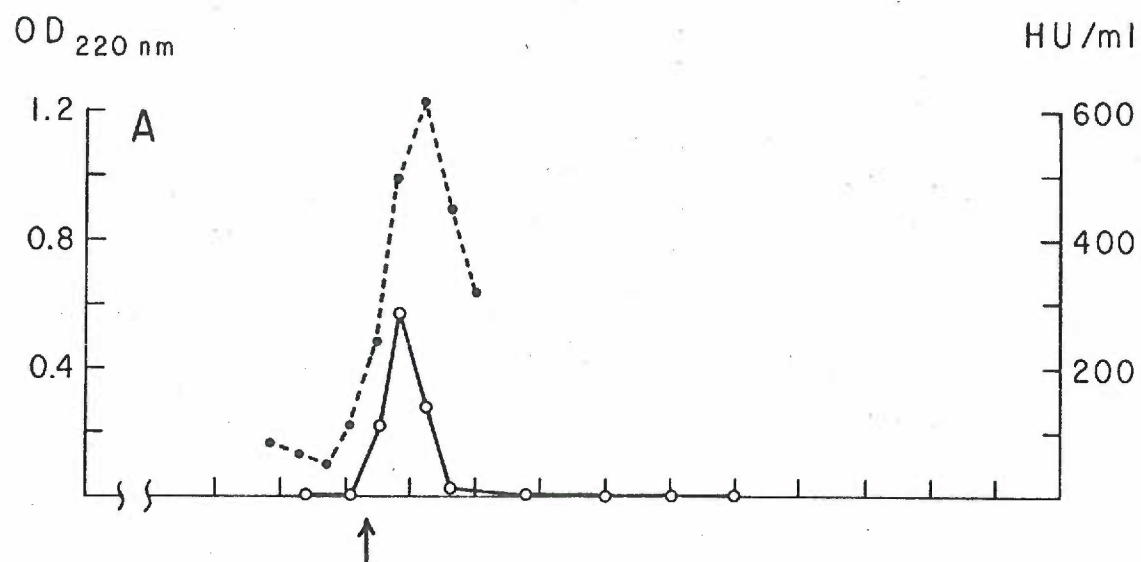


Figure 22.

Chromatographic Behavior of Tween-40 SLS, Tween-40 SLS
Incubated with RNA-core, and RNA-core SLS Incubated
with Tween-40.

- A. Tween-40 SLS: 3 ml of Tween-40 SLS (1100 HU/ml) + 100 mg sucrose applied to G-100 Sephadex column. Tween-40 SLS prepared in resting cell system by incubating cells from 250 ml late log phase culture with 20 mg Tween-40/ml in standard buffer. Eluate assayed for active hemolysin (○). Elution of Tween-40 monitored by change in turbidity at 220 nm (●).
- B. Tween-40 SLS Incubated with RNA-core: 3 ml Tween-40 SLS (900 HU/ml) incubated with peak I core (51 OD_{260nm} units) + 4 mg cysteine for 25 min at 37C and applied to G-100 Sephadex column. (There was no change in titer after incubation.) The Tween-40 SLS was prepared as in part A except with 15 mg Tween-40/ml. (○) active hemolysin.
- C. RNA-core SLS Incubated with Tween-40: 2 ml RNA-core SLS (4600 HU/ml) eluting at 136 ml (V_e) was incubated with 10 mg Tween-40 + BBM to 3 ml total volume and applied to column. Eluate assayed for active hemolysin (○).

Void volume indicated by arrow. OD_{220nm} = dashed line.



Finally, it was shown that sonication not only changed the size of precursor hemolysin during activation in the presence of RNA-core, but also changed the susceptibility to proteolytic enzymes. Although the precursor and activated hemolysin were both susceptible to pronase, only the precursor was susceptible to trypsin (Table 15). Pronase which had been incubated at 37C for 30 min and sonicated 25 min, had no effect on activated hemolysin. Thus, in the experiments with hemolysin precursor, pronase had inactivated the hemolysin precursor before sonication, and not the activated hemolysin afterwards.

Purification of Hemolysin Precursor from Sonicated-cell Supernatant Preparations

Since hemolysin precursor was detected in some sonicated-cell supernatant preparations, it was of interest to determine if large amounts of precursor could be obtained from such a preparation. If so, enough precursor material for purification might be obtained without the use of muralysin, which was troublesome to prepare in amounts necessary to lyse large batches of cells. The characteristics of precursor obtained by sonication could be compared to the characteristics of that obtained by muralysin. Finally, data from experiments conducted on sonicated-cell precursor might help to determine the mechanism by which sonication activated precursor and to determine the location of precursor.

Large amounts of precursor could be obtained by sonicating heavy suspensions of strain C203S (Table 16). The high density of the bacterial suspension apparently prevented most of the activation of

<u>Enzyme Treatment</u>	<u>% Hemolytic Activity Remaining</u>	
	<u>Time of Enzyme Treatment</u>	
	<u>Before Sonication</u>	<u>After Sonication</u>
Untreated	100%	100%
Trypsin (0.4 mg/ml)	14%	97%
Pronase (0.5 mg/ml)	0%	0%

Table 15. The Effect of Pronase and Trypsin on Hemolysin Precursor and Activated Hemolysin (RNA-core Bound Hemolysin).

Precursor was incubated with pronase (0.5 mg/ml) or trypsin (0.4 mg/ml) at 37 C for 30 min at pH 8.0. The preparations were then sonicated at pH 6.5 with 6 mg crude RNA-core and $3 \times 10^{-4} \text{M}$ Mg^{2+} for 25 min.

Activated hemolysin was incubated similarly with trypsin at pH 7.8 and at pH 7.2 with pronase, and assayed at pH 6.5.

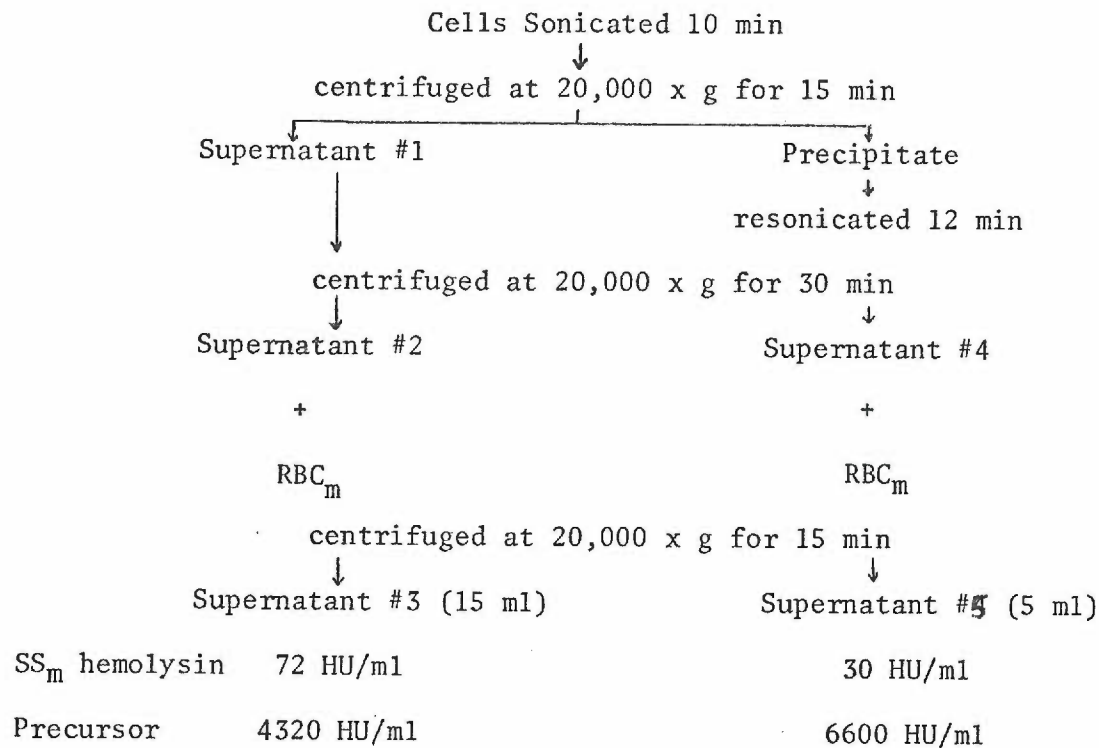


Table 16. Amount of SS_m Hemolysin and Hemolysin Precursor from Sonicated C203S.

Cells harvested from 2.4 liters HI - 0.3% maltose broth, suspended in 15 ml buffer, and sonicated in 7.5 ml batches for 10 min. The precipitate after centrifugation was resonicated in 5 ml buffer for 12 min. All sonications were done at 60 intensity on model 2 sonicator. Supernatant #2 adsorbed with RBC_m from 0.5 ml RBC and supernatant #4 with 1/3 that amount. Precursor assayed by sonicating 0.3 ml supernatants (#3 or #5) with 1 mg core and buffer to 3.0 ml for 20 min. SS_m hemolysin assayed prior to resonication.

precursor. The cellular SLO was stable in these preparations, probably due to reducing substances present in the cell or cell walls (66), but was easily removed by RBC_m adsorption at 0C. The SS hemolysin was not adsorbed, even at 20C.

Purification with streptomycin sulfate to remove the nucleic acids again resulted in large losses of hemolysin precursor, possibly due in part to the removal of protein. For example, in one experiment treatment with 1.0% streptomycin sulfate resulted in a reduction of protein from 11.4 mg/ml to 3.1 mg/ml and a 50% loss in hemolysin precursor. Smaller concentrations of streptomycin sulfate were not useful and decreased the activatable titer of hemolysin precursor (Table 17). Because of the loss of specific activity due to the addition of streptomycin sulfate, this purification step was omitted from further purification procedures.

The hemolysin precursor from the SS preparation was precipitable by acid, as was hemolysin precursor from MS_m preparations. Precipitation of precursor from SS_m by the addition of 0.1 N HCl to pH 4.3 resulted in 100% recovery of the precursor in the precipitate, a small decrease in the OD_{260nm}/OD_{280nm} ratio, and about a 25% increase in specific activity. Precipitation at higher pH values was not as effective.

A marked increase in specific activity was obtained by ammonium sulfate precipitation of the acid-precipitated precursor (Table 18). All recoverable precursor was precipitated between 0-40% SAS concentration. As shown earlier, the majority of precursor from MS_m was also precipitated in this range, but, unlike SS_m precursor, a small fraction was precipitated at higher SAS concentrations.

<u>Streptomycin sulfate treatment</u>	<u>OD_{260nm} 280nm</u>	<u>Mg protein/ml</u>	<u>Precursor HU/ml</u>	<u>Specific activity HU/mg protein</u>
None	1.95	5.6	2916	522
0.25%	1.95	4.6	2580	554
0.50%	1.94	4.4	2040	467
1.0 %	1.59	4.1	1596	388

Table 17. Effect of Streptomycin Sulfate Treatment to Remove Nucleic Acids from an SS_m Preparation on Hemolysin Precursor Titer and Specific Activity.

An SS_m preparation was treated with streptomycin sulfate solution as described in Materials and Methods and centrifuged at 20,000 x g for 30 min. Precursor in supernatant was measured by sonicating 0.5 ml sample with 1 mg crude RNA-core plus buffer to 3.0 ml for 25 min on model 1 sonicator. Protein measured as described in Materials and Methods.

Preparation	Mg protein/ml	HU/ml	Specific Activity HU/mg protein
SS _m (acid precipitate)	3.5	2760	790
25% SAS precipitate	0.44	1476	3350
40% SAS precipitate	0.51	828	1620
60% SAS precipitate	1.0	0	----

Table 18. Purification of Acid-precipitated Precursor from an
SS_m Preparation by (NH₄)₂SO₄.

Precursor precipitated with saturated solution of (NH₄)₂SO₄ as in Materials and Methods and resuspended in buffer. Precursor assayed by sonication of 0.5 ml sample plus 1 mg core plus buffer to 3.0 ml for 25 min on model 2 sonicator at 60 intensity.

When acid-precipitated precursor was incubated with RNase (16 $\mu\text{g/ml}$) and DNase (16 $\mu\text{g/ml}$) plus 0.1 M Mg^{2+} for 30 min at 37C, the solution became cloudy. After centrifugation at 16,000 X g for 15 min, all the activatable hemolysin was present in the sediment (Table 19). The sediment was not soluble in standard buffer, and when dispersed in buffer appeared as opaque, sliver-like bits of material. The titers obtained using the insoluble precursor were probably only approximate, since it was difficult to evenly disperse the precipitate prior to removing samples for assay. EDTA was added in an attempt to dissolve the precipitate. Although this failed, EDTA did markedly increase the activatable titer, perhaps by complexing the excess Mg^{2+} which had been added; EDTA had no effect on the activated hemolysin. Further experiments showed that the incubation of precursor at 37C for 30 min in the absence of Mg^{2+} , DNase, or RNase, caused the solution to become cloudy. Part of the precursor, all of which had previously remained in the supernatant after centrifugation at 16,000 X g for 15 min, was now sedimentable under these conditions.

When purification of large batches of hemolysin precursor was attempted, some of the precursor material became insoluble during several purification procedures. Although good recovery of precursor was achieved after acid precipitation, such precipitation resulted in alteration of hemolysin precursor. After resuspension of the acid-precipitated precursor in buffer, approximately 50% could be sedimented by centrifugation at 20,000 X g for 30 min by which it had previously not been sedimentable, and some of the sediment was insoluble, with

Preparation	Mg protein/ml	Precursor (HU/ml)	Specific activity (HU/mg protein)
Acid-precipitated SS_m	3.0	1620	540
DNase/RNase supernatant	1.4	0	---
DNase/RNase supernatant + EDTA	1.4	108	77
DNase/RNase precipitate	0.7	840	1200
DNase/RNase precipitate + EDTA	0.7	2025	2930

Table 19. Effect of DNase and RNase Treatment of Acid-precipitated SS Preparation.

Sixteen $\mu\text{g/ml}$ of both DNase and RNase were added to an acid-precipitated SS preparation containing 10.4 mg protein + 0.1 M Mg^{2+} at pH 7; the mixture was incubated for 30 min at 37 C. The precipitate was removed by centrifugation at 16,000 X g for 15 min and suspended in 4 ml buffer. All precursor samples assayed by sonication with RNA-core (1 mg crude RNA-core + 0.5 ml sample + buffer to 3.0 ml). EDTA (0.08 M) present in some sonicated preparations.

the appearance described above. Some of the precursor material also became insoluble after freezing, storage at refrigeration temperatures, or precipitation by acetone.

The sedimentability by high speed centrifugation of freshly prepared precursor, from either a SS_m preparation or MS_m preparation, had not been previously examined. SS_m precursor and MS_m precursor were prepared from the same batch of cells, and made to the same volumes. There were 30 HU/ml of active delayed hemolysin in the MS_m preparation and 66 HU/ml of such hemolysin in the SS_m preparation. Precursor was present in both the MS_m (1,800 HU/ml) and SS_m (10,000 HU/ml). After centrifugation at 200,000 X g for 120 min, all precursor recovered from the MS_m preparation was present in the sediment; the majority of SS_m precursor material was also sedimented (Table 20). Both sediments contained insoluble material having the appearance previously described; precursor was present in this insoluble material. On storage at refrigerator temperature for 19 days, all the remaining activatable hemolysin in the uncentrifuged MS_m preparation could be removed by centrifugation at 20,000 X g for 30 min. The sediment contained precursor material in an insoluble form.

When the precursor which remained unsedimented after centrifugation of the SS preparation at 200,000 X g for 120 min was precipitated by acid and then by 40% SAS, a portion of the precursor material was recovered as an insoluble precipitate. It is likely that the potential insolubility is a characteristic of most, if not all, precursor in an SS preparation, as well as in an MS preparation.

Preparation	Before Centrifugation	Precursor, HU	
		After Centrifugation	
		Sediment	Supernatant
SS _m	53,000	29,000	16,000
MS _m	9,550	4,400	0

Table 20. Sedimentation of Hemolysin Precursor in both SS_m and MS_m Preparations by High Speed Centrifugation.

C203S cells (2.4 liters) were grown to late log phase, harvested, and washed as in Materials and Methods. One-half were disrupted by a 10 min sonication with the model 2 sonicator and one-half by incubation with 575 muralytic units of muralysin at 37 C for 30 min. The supernatant from a centrifugation at 20,000 x g for 15 min was adsorbed with RBC_m (from 1.0 ml RBC), further centrifuged at 20,000 x g for 10 min, and the supernatant saved for assay. The preparations were centrifuged at 200,000 x g for 2 hr and the supernatants and resuspended sediments assayed by sonication. Each sonicate mixture contained 0.3 ml sample, 1 mg RNA-core, and buffer to 3.0 ml and was sonicated 25 min at 60 intensity on the model 2 sonicator.

The insoluble precursor material could not be solubilized in sodium phosphate buffer, pH 6.5 or pH 8.7, or in distilled water. It was partially solubilized in 1 N NaOH, but some was also inactivated at the high pH. It was not solubilized in 3.0, 4.5, or 4.8 M NaCl. It could also not be solubilized by incubation with 1 M urea, 1% Tween-40, 14 μ g/ml DNase, or 30 μ g/ml RNase at 37C for 20 min, or by incubation with 3% Tween-40 at 4C for 12 hr. The precursor material particles appeared to be solubilized in 30 mM sodium lauryl sulfate (SDS), but could not be assayed because of the hemolytic nature of SDS. The insoluble precursor material was partially (35%) solubilized (remaining in the supernatant of a centrifugation at 25,000 X g for 30 min) in 3 M urea after incubation at 4C for 12 hr.

Since the precursor material was somewhat soluble in 3 to 4 M urea, especially after prolongation incubation (72 hr) at 4C, such solubilized precursor was subjected to polyacrylamide electrophoresis in 4 M urea as described in Materials and Methods. Most of the protein remained at the origin. There was one large band of protein 1.0 cm past the origin, and a number of bands in an area 1.0 to 1.5 cm beyond that, as indicated by a dense area of dye. Certainly the sedimented or insoluble precursor was not pure, but contaminated by or contained a large number of protein components.

Since precursor material appeared to be particulate in nature, its sedimentation characteristics in a sucrose gradient were examined. The precursor material was both heterogeneous and large in size (Fig. 23). The majority of the activatable hemolysin preceded the bulk of material adsorbing at 260 nm and 280 nm. Some of the precursor

Figure 23.

Centrifugation of Precursor in a Sucrose Density Gradient

Precursor (0.25 ml) from the supernatant (centrifuged at 25,000 x g for 30 min) of a SS_m preparation containing 26,000 HU/ml potential hemolysin was applied to a 5 to 20% sucrose gradient prepared as in Materials and Methods. The preparation was centrifuged at 200,000 x g for 90 min. The fractions (0.2 ml), removed as in Materials and Methods, were assayed by sonicating 0.2 ml samples with 0.3 mg RNA-core plus buffer to 3.0 ml for 25 min at 60 intensity on the model 2 sonicator. (○) activated hemolysin.

OD

△ 260 nm

● 280 nm

HU / fraction

○

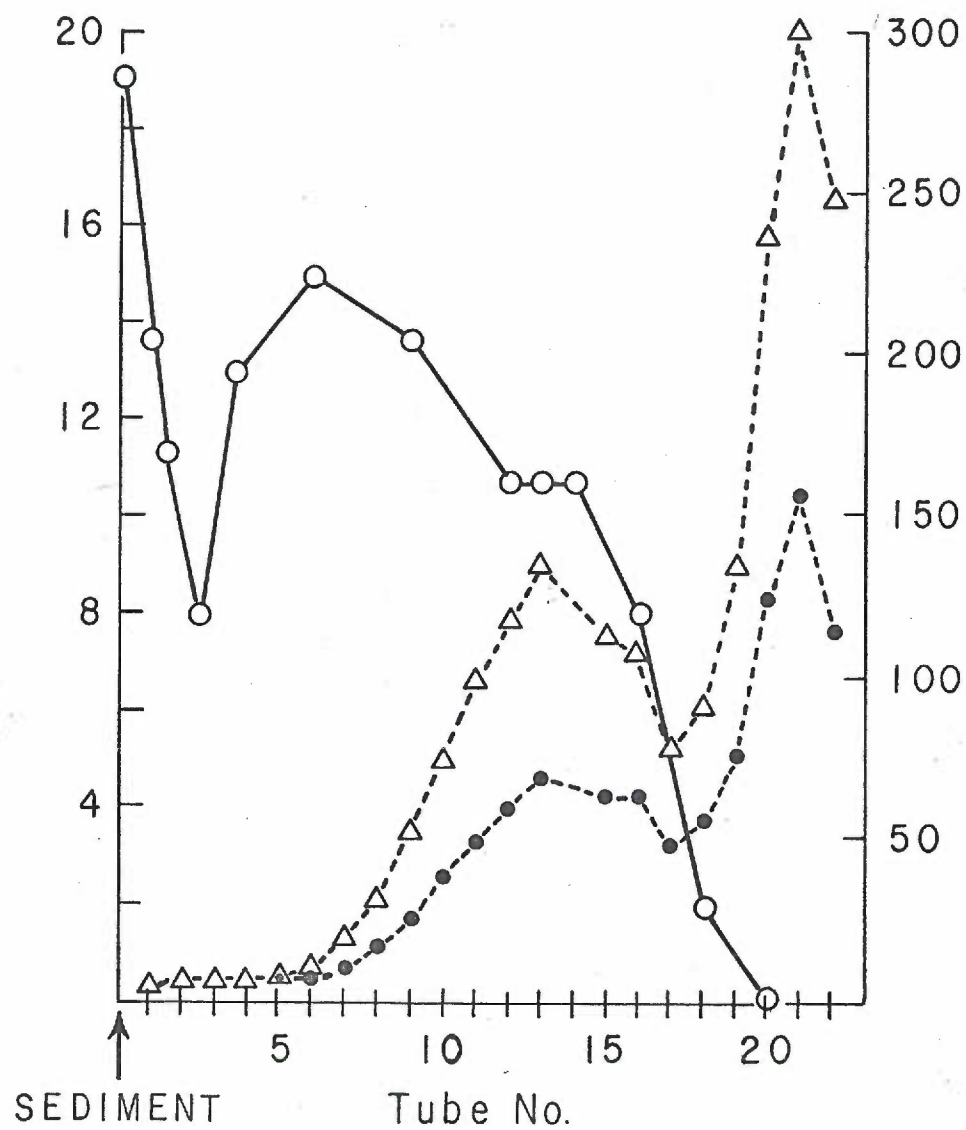
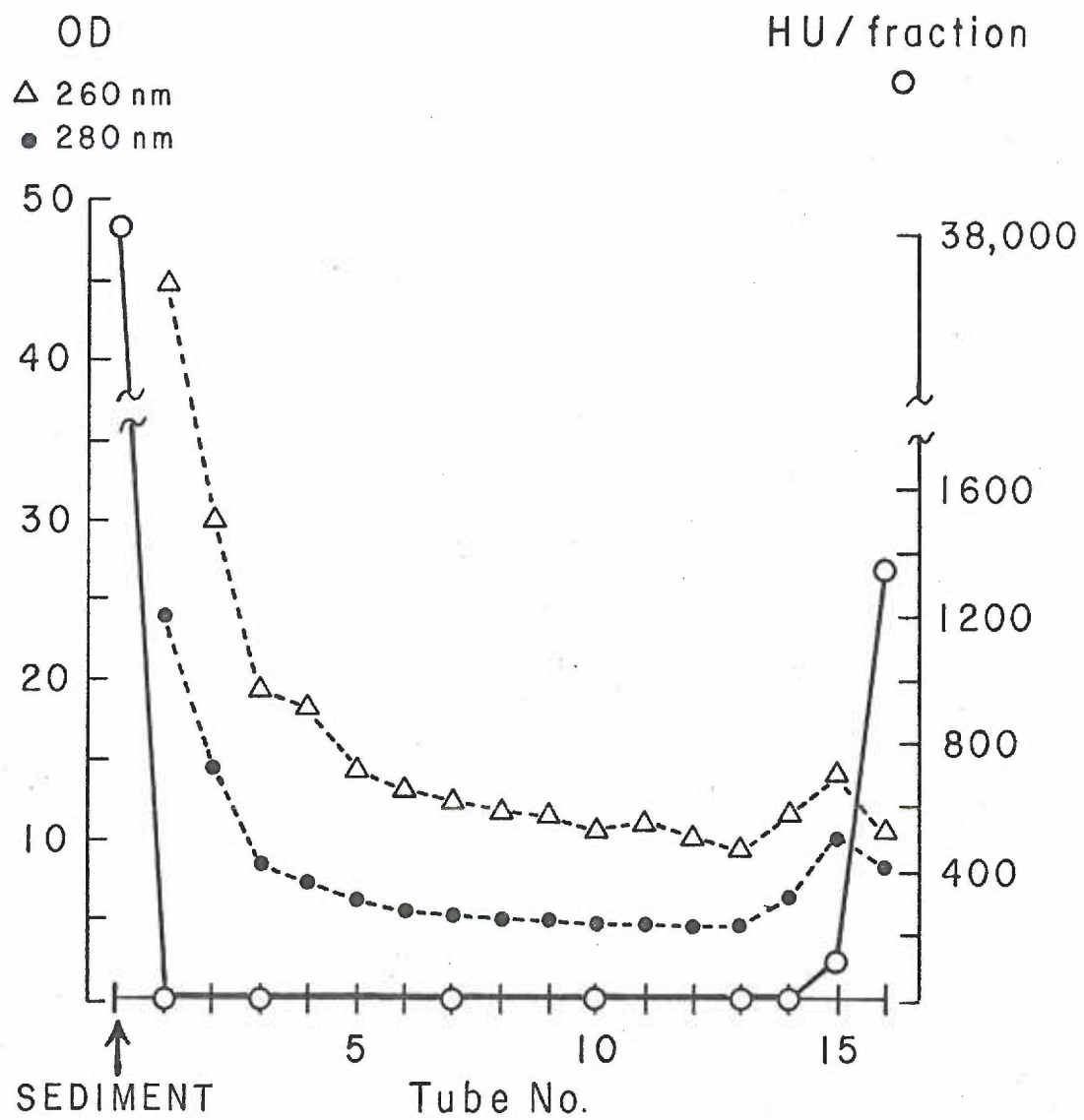


Figure 24.

Centrifugation of Precursor in a CeCl Density Gradient

SS precursor (1.5 ml = 40,100 HU) was mixed with 1.5 ml buffer and 1.875 g CeCl and centrifuged at 30,000 RPM for 48 hr in a SW-39L head in a Beckman Model L Ultracentrifuge. The insoluble sediment was dispersed in 3 ml buffer. Precursor was assayed by sonicating 0.2 ml fractions with 0.3 mg RNA-core plus buffer to 3.0 ml with the model 2 sonicator at 60 intensity for 25 min. Insoluble sediment (0.3 ml) was sonicated with 1 mg RNA-core under the same conditions. (O) activated hemolysin.



sedimented through the gradient and was recovered as insoluble material at the bottom of the centrifuge tube.

The sedimentation characteristics of hemolysin precursor were also examined in a CsCl gradient (Fig. 24). Of the 40,100 potential HU centrifuged, 38,400 HU or 96% sedimented as insoluble material. The remainder (1,500 HU) was at the top of the gradient, where a small amount of active hemolysin was also present.

Hemolysin precursor from the bottom half of a sucrose gradient was recentrifuged at an average of g force of 73,000 for 64 hr in a Cs_2SO_4 gradient (2.350 grams Cs_2SO_4 in 2.95 ml final vol.) and was again recovered as an insoluble sediment. No precursor material was found at the top of the gradient as was present in the CsCl gradient.

Since hemolysin precursor was sedimentable by various centrifugation procedures, it was possible that the first centrifugation to remove unlysed or unbroken cells after muralysin or sonication treatment also removed precursor. Therefore, the sonication sediment was examined for the presence of hemolysin precursor (Table 21). A sonicated suspension was repeatedly centrifuged at 500 X g for 20 min to remove intact cells, and the sediments were examined after Gram staining for the presence of intact cells. After four such centrifugations, less than 10 Gram positive organisms per oil immersion field remained. After two centrifugations at 2,000 X g for 10 min no Gram positive organisms were detected in the stained sediment. The supernatant was then centrifuged at 25,000 X g for increasing periods of time and the sediments and supernatants examined for the presence of precursor (Table 21). Both sediment #2 and #3 contained insoluble material

Preparation	Time of Centrifugation at 25,000 x g	Fraction Assayed	Vol, ml	Precursor HU/ml - Core + Core	μ g Rhamnose/ml	HU/ μ g Rhamnose
2,000 x g supernatant	60 min	sediment (#1)	4	0	420	392
						1
supernatant #1	60 min	sediment (#2)	1	160	400	ND
		supernatant (#2)	7	1148	ND	ND
supernatant #2	180 min	sediment (#3)	2	ND	1184	24
		supernatant (#3)	5.5	784	ND	ND
						77

Table 21. Amount of Precursor and Rhamnose in Sediments and Supernatants of an SS Preparation.

Late log-phase cells (1.6 liters) were harvested and suspended to 7.5 ml in buffer. The mixture was sonicated 20 min at 60 intensity on the model 2 sonicator. The sonicate was centrifuged at 500 x g for 20 min and the sediment containing whole cells discarded. This procedure was repeated 3 times. The supernatant was then centrifuged at 2,000 x g for 10 min, removed from the sediment, and recentrifuged at 2,000 x g for 10 min. The supernatant was then centrifuged at 25,000 x g for 60 min; the resulting supernatant (#1) was recentrifuged as described below. The resulting sediment was suspended in buffer and centrifuged at 25,000 x g for 60 min; the washed sediment (#1) was assayed. Supernatant (#1) was recentrifuged at 25,000 x g for 60 min and the sediment (#2) and supernatant (#2) assayed. Supernatant (#2) was recentrifuged at 25,000 x g for 180 min and the supernatant (#3) and sediment (#3) assayed. Sonications for assay of precursor were done with the model 2 sonicator for 20 min at 60 intensity. Two mg RNA-core were added to appropriate mixtures prior to sonication. Rhamnose measured as described in Materials and Methods.

having the visual appearance of previously described insoluble precursor material. Precursor was present in all the sediments. No precursor was demonstrable in the first sediment unless RNA-core was present during sonication. The largest amount of precursor was present in the third sediment after prolonged centrifugation. Moderate amounts of precursor material could, therefore, be sedimented at 25,000 X g if centrifuged for long enough time periods. Since the first centrifugation to remove unlysed or unbroken cells was usually done at 25,000 X g for 30 min, some precursor probably was lost during this step.

Rhamnose is located predominantly in the streptococcal cell wall (35). The amount of rhamnose in the first and third sediments was therefore measured (Table 21) to further characterize the sediments. The first sediment did contain cell wall material as indicated by the presence of rhamnose. Since the third sediment contained a very small amount of rhamnose, the value presented in Table 21 is only an approximate figure. Although precursor can be sedimented with the cell wall fraction it is not necessarily associated with it, as shown by the ratios of potential hemolytic activity to rhamnose content.

SS Hemolysin: Characteristics and Relationship to Activated Hemolysin and RNA-core SLS

SS hemolysin was formed by activation of hemolysin precursor. Furthermore, since hemolysin activated on sonication with RNA-core, and the product of incubation of SS hemolysin with RNA-core had similar molecular weights, it was possible that the RNA-core-bound activated

during sonication. It was possible that SS hemolysin was an intermediate between precursor and activated hemolysin. The properties of hemolysin precursor and SS hemolysin were, therefore, compared.

Large amounts of high titer SS hemolysin could be obtained in the supernatant of sonicated cells centrifuged at 20,000 X g for 20 min. Prolonged sonication of the 40% SAS precipitate of that supernatant resulted in marked potentiation of titer (Table 22). The potentiation of titer by sonication in the absence of RNA-core was similar to the phenomenon described by Schwab (87).

SS hemolysin and precursor are precipitated in the same ammonium sulfate fractions during purification of a mixture of SS hemolysin and precursor. The percent recovery of SS hemolysin is, however, lower than that of precursor. Further, the SS hemolysin prepared from precursor partially purified by 40% SAS precipitation (Table 22) was also precipitated by 40% SAS. For example, of 42,000 HU of SS hemolysin, 31,000 HU were recovered in the 40% SAS precipitate and only 1,000 HU remained in the 40% SAS supernatant. RNA-core SLS is not precipitated by 40% SAS. The precursor and SS hemolysin are thus similar with respect to SAS precipitation, and differ in this property from RNA-core SLS.

As previously shown (Fig. 20), both SS hemolysin and precursor elute in the void volume of a G-200 Sephadex column. No difference in molecular size was thus demonstrable by this technique.

As was described earlier (Table 17), streptomycin sulfate decreases the potential for activation of precursor. The addition of 2.5% strepto-

<u>Length of Sonication (min)</u>	<u>HU/ml</u>
0	200
10	1600
20	3750
30	4000
40	5400
50	7100
60	8300
75	10,600
90	13,300
120	15,300

Table 22. Preparation of SS Hemolysin by Prolonged Sonication of Hemolysin Precursor

Hemolysin precursor was obtained from an SS preparation of late log-phase cells. The precursor material was precipitated from the SS preparation by 40% SAS, and then sonicated in 4.5 ml vol with the model 2 sonicator at 60 intensity.

mycin sulfate (twice as much as used for precursor) to SS hemolysin resulted in the precipitation of only a small amount of activity. For example, of 400 HU only 35 HU were precipitated by streptomycin sulfate, and 355 HU remained in the supernatant after centrifugation at 10,000 X g for 15 min. The addition of streptomycin sulfate (1.25 or 2.5%) to RNA-core SLS hemolysin resulted in no loss of activity and no precipitation of hemolysin. The SS hemolysin is thus more similar to RNA-core SLS than to precursor with respect to the effect of streptomycin sulfate.

Chloroform precipitates all precursor activity found in the sedimentable portion of the cell after sonication, but does not precipitate SS hemolysin. All the SS hemolysin activity is present in the aqueous layer after chloroform treatment. Similarly, all the activity of RNA-core SLS is present in the aqueous layer after such treatment. The addition of cold methanol or ether partially inactivates precursor, but totally inactivates SS hemolysin. Cold methanol precipitates RNA-core SLS, and ether has no effect on RNA-core SLS. Isobutanol inactivates SS hemolysin, but not RNA-core SLS.

As previously stated, precursor is precipitated by HCl. It is also precipitated by 10% acetic acid. SS hemolysin is similarly precipitated by 10% acetic acid, but RNA-core SLS is not.

The sedimentability of SS hemolysin was compared with that of precursor. After centrifugation at 200,000 X g for 120 min, 297 HU of the original 545 HU of SS hemolysin remained in the supernatant. In the insoluble sediment, 48 HU were measurable. After suspension of the sediment by mixing with an Omnimixer for 4 min at 70 intensity, 180 HU were detected in the sediment. Precursor used to prepare

SS hemolysin, when similarly mixed with an Omnimixer, was not activated. Approximately 40% of SS hemolysin was sedimented which was somewhat less than the 55% of precursor sedimented under the same conditions.

SS hemolysin, like the precursor, appeared heterogeneous on centrifugation in a sucrose gradient and the majority of activity moved away from the bulk of the 260nm-adsorbing material (Fig. 25). These results were similar to those obtained on the sedimentation of precursor in a sucrose gradient (Fig. 23), except that precursor migrated further under the same conditions.

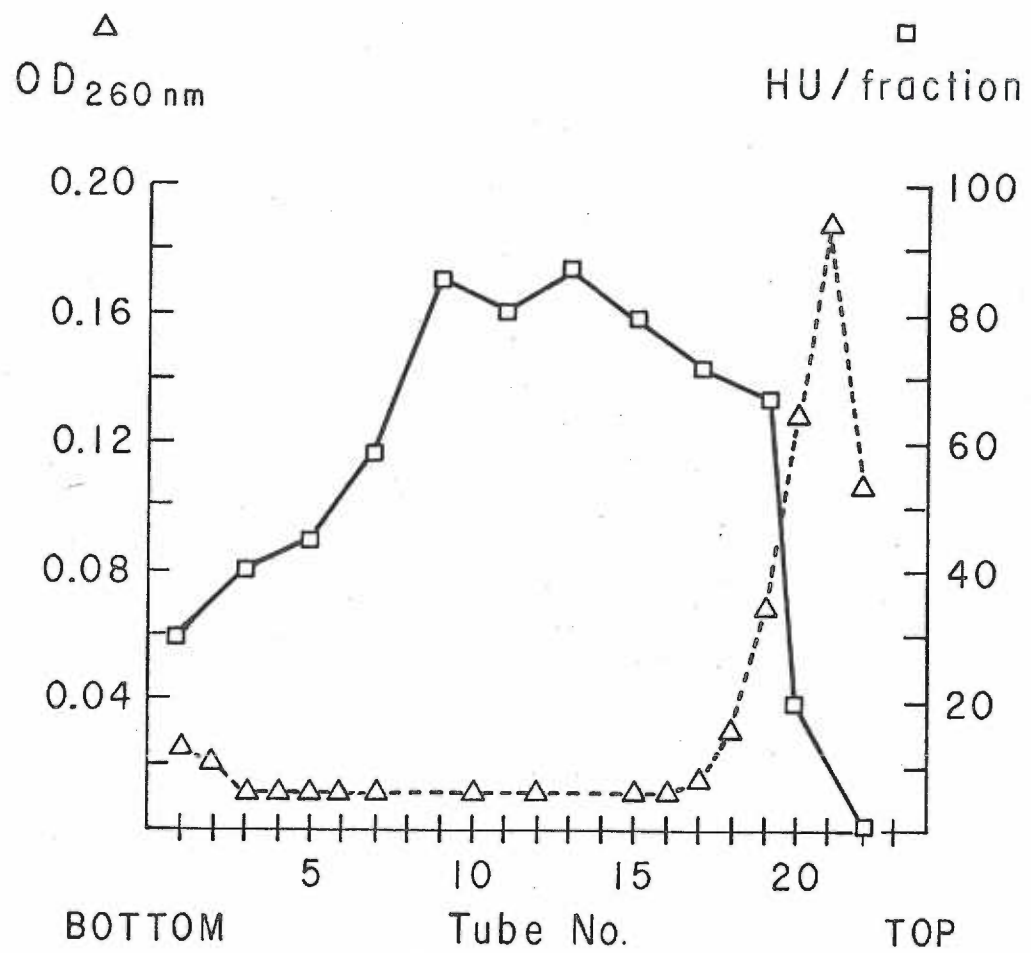
A hypothesis for one of the effects of sonication on precursor during activation is that precursor is fragmented or disaggregated into smaller fragments containing active hemolysin. The present data do not negate such an hypothesis. Precursor and the resultant hemolysin after activation (SS hemolysin) are similar in precipitation by 40% SAS and 10% acetic acid, and in partial sedimentation by high speed centrifugation. Yet they are dissimilar in other ways as shown by the effect of treatment by streptomycin sulfate and chloroform, and by differences in degree of sedimentation in a sucrose gradient. This is as might be expected if the active product was part of the inactive precursor.

If SS hemolysin is an intermediate during the activation of precursor to RNA-core-bound hemolysin, then the hemolytic moiety of SS hemolysin must be transferable to RNA-core in the absence of BBM, which was not present during sonication. The rate of transfer must also be rapid, since RNA-core-bound hemolysin is present after only a few minutes of sonication. As can be seen in Table 23, SS hemolysin is

Figure 25.

Centrifugation of SS Hemolysin in a Sucrose Density Gradient

SS hemolysin (2000 HU/ml) was prepared by a 30 min sonication of precursor sedimentable by centrifugation at 25,000 x g after removal of whole cells from a SS preparation. SS preparation (0.25 ml) was applied to a 5 to 20% sucrose gradient and centrifuged at 200,000 x g for 90 min. The fractions (0.2 ml) were assayed for active hemolysin ($\square$).



<u>SS hemolysin preparation</u>	<u>OD_{260nm} Units of RNA-core</u>	<u>Suspending Medium</u>	<u>HU</u>	
			<u>Prior to 40% SAS</u>	<u>In 40% SAS Supernatant</u>
Untreated	----	----	5940	0
Incubated	10	BBM	5080	5175
Incubated	25	BBM	7250	8460
Incubated	25	Buffer	8400	8660

Table 23. Stabilization by and Transfer to RNA-core of the Hemolytic Moiety of SS Hemolysin.

SS hemolysin was prepared by resonication of an SS preparation from late log phase cells for 2 hr, and precipitation by 40% SAS. SS hemolysin mixed with 10 or 25 OD_{260nm} units of peak 1 core in 3 ml buffer, incubated 30 min at 37 C, and assayed. The mixtures were then treated with 40% SAS as described in Materials and Methods. The supernatant was then assayed.

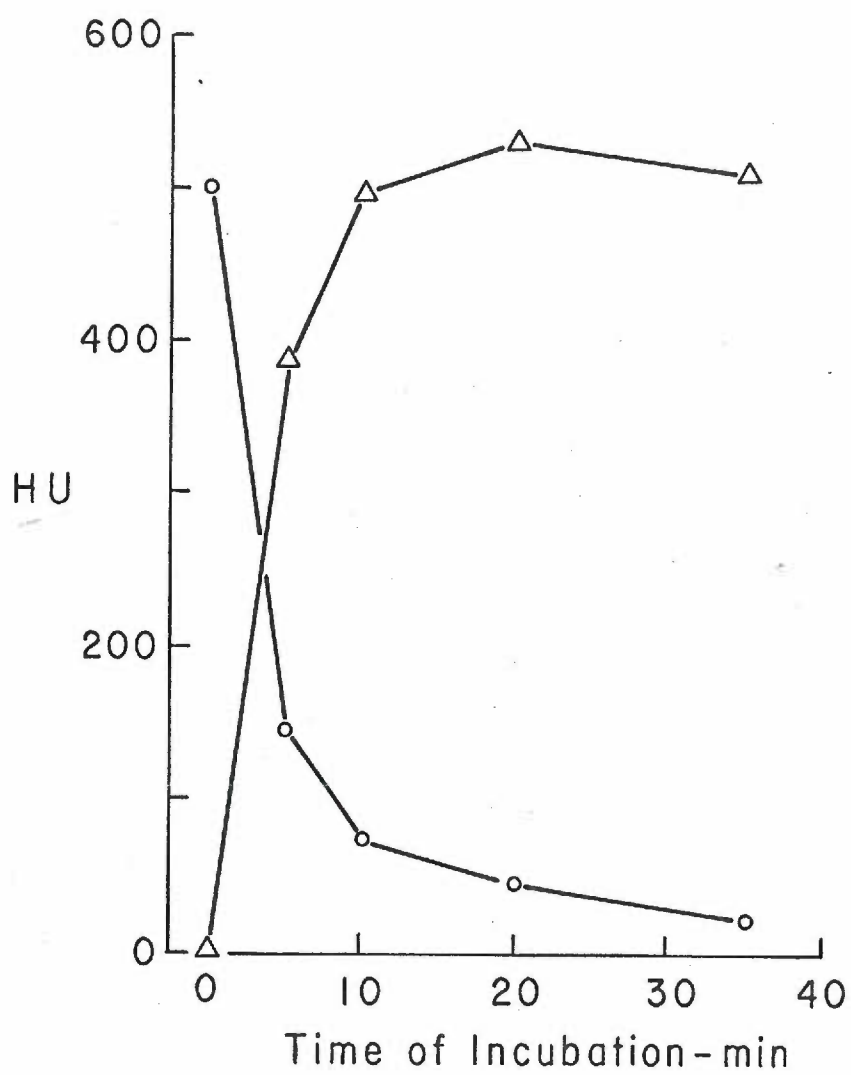
transferred to RNA-core in buffer as well as in BBM. The transfer is demonstrated by movement of the hemolytic moiety from 40% SAS precipitable SS hemolysin to a non-40% SAS precipitable form. The rate of transfer at 37C is very rapid, as shown in Fig. 26. No transfer is demonstrable at OC. Although sonication is done with the sample surrounded by an ice-water bath, the temperature around the probe tip is warmer than OC and may be sufficiently high to promote transfer of the hemolytic moiety of SS hemolysin to RNA-core. This could account for the presence of activated hemolysin (RNA-core-bound hemolysin) after short periods of sonication.

If the mechanism of activation of precursor is due, in part, to the comminution of precursor to smaller subunits, then cellular disruption by means other than sonication might be expected to activate precursor. As an alternative to sonication, C203S cells were disrupted by shaking with Superbrite glass beads, type 100-110, (3M Co.) in the Braun tissue homogenizer for 3 min at approximately OC. After adsorption of the supernatant with RBC_m , very high titer delayed hemolysin remained. The hemolysin was 100% stable after 24 hr at -20C and was 100% stable at OC (ice-water bath) for several hours. At 37C, one HU was inactivated at approximately the same rate as one HU of SS hemolysin. The hemolysin was inhibitable by trypan blue. Very little increase in titer occurred after sonication of the supernatant with RNA-core, indicating that activation had occurred during treatment in the Braun homogenizer. Sonically-released hemolysin and hemolysin released by homogenization were then similar in stability, rate of inactivation, inhibition by trypan blue, and delayed hemolysis.

Figure 26.

Rate of Transfer of the Hemolytic Moiety
of SS Hemolysin to RNA-core

To 4.8 ml SS hemolysin (500 HU/ml) was added 0.25 ml of peak 1 RNA-core (25 OD_{260nm} units). Four replicate tubes containing 1.2 ml each was incubated at 37C for the appropriate time, then cooled in ice 2 min, precipitated by 50% SAS, and centrifuged at 20,000 x g for 15 min. The supernatant (Δ) and sediment ($\circ$) were both assayed for hemolysin.



The activation of precursor by homogenization might explain why Taketo and Taketo (97) obtained what was probably activated precursor (SS hemolysin) from both sonicated and ground Group A streptococci.

One of the known effects of sonication is the shearing of nucleic acid (65). Precursor was incubated with DNase and RNase, under the appropriate conditions, to determine if cleavage of nucleic acid resulted in activation of precursor. No increase in active hemolysin titer occurred, showing that there was no activation of precursor. There was also no loss of potential titer of hemolysin precursor as measured after sonication.

The possibility that sonication destroyed a cellular inhibitor was reexamined. It had previously been shown that SS and MS hemolysins were partially additive, but the effect of the addition of unsonicated MS_m preparation to SS hemolysin had not been tested. When 0.5 ml of an MS_m preparation (83 HU/ml) was added to 0.5 ml of an SS preparation (438 HU/ml) from the same batch of cells, the final titer was 273 HU/ml compared to the expected value of 261 HU/ml. Therefore, no cellular inhibitor in the MS_m preparation inhibited the activated SS hemolysin.

Since the presence of LACH could be due to the action of muralysin on the cell envelope, sedimentable precursor from an SS preparation was incubated at 23C with muralysin, which releases LACH from 203S cells. No activation of precursor was detectable under these conditions. Precursor remaining in solution after centrifugation of an SS preparation at 25,000 X g for 5 hr was also incubated alone, with muralysin, and with muralysin and RNA-core at 37C for 20 min, but no activation occurred. LACH appears to be an active in vivo hemolysin, and not a product of the preparative procedure used.

Characterization of RNA-core SLS and its Hemolytic Moiety: Attempts to Purify the Hemolytic Moiety

The absolute proof that all the SLS-like hemolysins have the same hemolytic moiety would rest on the determination that the structure of the hemolytic moiety of each is the same. The hemolytic moiety referred to is the active portion of the hemolysin, such as a polypeptide, produced by the streptococci. In the 40-year history of SLS no one has ever been able to purify the hemolytic moiety of even one SLS-like hemolysin (39). The central problem is that, once the hemolytic moiety and carrier are presumably separated, all hemolytic activity is lost. The activation of precursor by sonication suggested that, after removal of the hemolytic moiety from RNA-core, the hemolysin might be reactivated by sonication of the hemolytic moiety with RNA-core. The use of hemolysin precursor also offered a new approach to the problem of isolating the hemolytic moiety of SLS. In summary, there were indications that the hemolytic moiety of SLS could be determined using many of the procedures developed in the present research.

There was another reason to attempt to purify RNA-core SLS, even if it could not be completely purified. Previous work (12) had indicated that the hemolytic moiety is less than 3000 molecular weight. Several similarities in the properties of RNA-core SLS and those of certain polypeptide antibiotics also suggested that the hemolytic moiety might be a polypeptide. One investigator has published an amino acid content of RNA-core SLS (57), which also supports the

concept that the hemolytic moiety is a polypeptide. The assumption implicit in his research, and in several statements based on his research made by others, on the nature of the hemolytic moiety was that no other peptides are bound to RNA-core or RNA-core SLS. Neither he nor anyone else has ever determined the number of peptides bound to RNA-core. If there was more than one peptide bound to RNA-core, then the accuracy of the amino acid content (57) and the size of the hemolytic moiety (12) may seriously be doubted.

Three different approaches to purify the hemolytic moiety of SLS were tried. The first was to prepare SLS with one carrier in the resting cell system, purify that carrier-SLS combination, transfer the hemolytic moiety to another carrier, and then to further purify that carrier-SLS combination. The second was to purify the precursor, activate it, purify the SS hemolysin, transfer the hemolysin moiety to RNA-core, and then to purify the RNA-core SLS. The third was to prepare the RNA-core SLS in the resting cell system, to purify it as much as possible, and then to determine how many peptides could be detected in this preparation. If only one was present, it could be further characterized, and possibly reactivated. The first two approaches were based on the premise that the hemolytic moiety from purified carrier¹-SLS or SS hemolysin could be transferred to carrier² or to RNA-core, which could be purified differently so as to remove all contaminating proteins. The third approach was based on the supposition that the SLS hemolytic moiety is a polypeptide and that it is the only peptide bound to RNA-core in the resting cell system.

The first approach involved the preparation of albumin-SLS or Tween-40 SLS and then transfer to RNA-core. The G-100 Sephadex chromatographic behavior of the first two is much different than the last (42). Purification of either of the first two on G-100 Sephadex could separate it from all contaminants of less than 40,000 to 50,000 molecular weight; the transfer of the hemolytic moiety of RNA-core and chromatography on G-100 Sephadex would remove all the contaminants of molecular weight greater than 30,000.

Although the approach appeared theoretically sound, it was not practical on the scale needed to obtain large amounts of RNA-core SLS. First, it was difficult to prepare Tween-40 consistently with different batches of cells. Second, the total yield of Tween-40 SLS was only one-sixth as much as RNA-core SLS under optimum conditions for each. For example, 2.75×10^5 HU Tween-40 SLS could be prepared in the resting cell system with late log phase cells harvested from 5 l growth medium, while 1.7×10^6 HU RNA-core SLS could be obtained from the same amount of similarly harvested cells. Unlike cells grown with RNA-core, cells grown with Tween-40 did not continue to make hemolysin in the resting cell system. The addition of Tween-40 to growing cells inhibited further growth (as measured by change in OD) and resulted in only a small amount of Tween-40 SLS as compared to the amount of RNA-core SLS formed under analogous conditions. Third, the Tween-40 SLS could not be easily concentrated by lyophilization, methanol precipitation, ultrafiltration, or freezing and thawing. Fourth, Tween-40 SLS was much less stable than RNA-core SLS at both refrigeration temperatures and frozen at -20°C . Fifth, poor recovery of the Tween-40 SLS from the

G-100 Sephadex column was obtained. Sixth, when large quantities of Tween-40 SLS were chromatographed, the Tween-40 SLS tailed extensively as it eluted from the column, contaminating the eluate region of RNA-core SLS. Finally, after purification and transfer to RNA-core and further purification of RNA-core SLS, there was only a small amount of hemolysin finally remaining. No hemolysin was obtained by incubating purified bovine serum albumin with strain C203S in the resting cell system.

The second approach involved the use of hemolysin precursor and the transfer to RNA-core of activated SS hemolysin. The precursor material from sonicated cells was used because larger amounts could be prepared from this source than from MS_m preparations. Large amounts of SS hemolysin of high titer could be obtained by prolonged sonication of whole cells grown to late-log phase, or of partially purified hemolysin precursor from late-log phase cells. After sonication of cells harvested from late-log phase growth, 1840 HU/mg cell N or 18,000 HU/liter harvested cells was obtained after sonication for 20 min. The amount of SS hemolysin obtained was increased by prolonging the sonication time of such cells -- 62,000 HU/liter harvested cells was obtained after sonication of another batch of cells for 120 min. Using unpurified hemolysin precursor prepared by a similar procedure to that in Table 16, 200,000 HU/liter harvested cells could be obtained after sonication of precursor for 120 min. This ratio was calculated from data obtained after sonication of a dilute suspension of SS preparation. Sonication of concentrated suspensions of the SS preparations resulted in a lower final titer of hemolysin; e.g. prolonged

sonication of the 40% SAS-precipitated precursor from the sonicated-cell supernatant yielded a maximum of 47,500 HU/liter harvested cells. This calculation was made from data obtained in experiments using concentrated precursor material to prepare SS hemolysin for transfer to RNA-core. The necessity to sonicate dilute suspensions of precursor material for very prolonged times made preparation of even 1×10^5 HU of SS hemolysin a very troublesome and prolonged procedure. The two steps that severely limited this overall approach were the concentration and purification of SS hemolysin by 40% SAS, and the transfer of the concentrated hemolysin to RNA-core. Precipitation by 40% SAS of SS hemolysin derived by sonication of precursor resulted in 25 to 50% loss of hemolytic activity. The transfer of the hemolytic moiety to RNA-core was much less efficient with large amounts of SS hemolysin than with small amounts, even with optimum amounts of RNA-core. Part of the problem may have been due to the large amount of cellular material precipitated with SS hemolysin by 40% SAS. The separation of the cellular material from the transferred hemolysin by reprecipitation of the cellular material by 70% SAS was also not completely successful and some cellular material remained with the transferred hemolysin after chromatography of the 70% SAS supernatant on G-100 Sephadex.

In the third procedure, RNA-core SLS was prepared in the resting cell system. The hemolysin was purified and then examined to determine if one or more peptides were associated with the RNA-core.

The RNA-core used for the preparation of RNA-core SLS was first purified by G-100 Sephadex chromatography. The material in the first

major peak (peak 1) after the void volume (Fig. 2) was as effective as unpurified RNA-core to obtain RNA-core SLS, and was used in the rest of the experiments. The second major peak was not effective in RNA-core SLS production. The peak 1 RNA-core was further purified by successive phenol extractions until no precipitated material formed at the aqueous-phenol interphase. This phenol-extracted RNA-core was as effective as the unpurified RNA-core in promoting RNA-core SLS formation. The RNA-core was considered to be free of contaminating peptides and protein, as it contained no ninhydrin-reacting material.

High titer RNA-core SLS in large quantities (1.7×10^6 HU from cells from 5 l culture) could be prepared in the resting cell system. The hemolysin could be concentrated by methanol precipitation (44), lyophilization, or using ultrafiltration with an Amicon UM-10 membrane. After chromatography on G-100 Sephadex an overall recovery of 80% to 90% was obtained. A sample (30,000 HU) of this RNA-core SLS was run on SDS polyacrylamide electrophoresis. There were three very faint protein bands when the gel was stained, indicating very few protein contaminants. The RNA-core SLS was then further purified on DEAE cellulose by step-wise elution with NaCl between 0.3 M and 0.5 M NaCl. Only one-sixth to one-third of the activity could be recovered after such chromatography. This RNA-core SLS was mixed with 80% acetic acid and then chromatographed on a Biorad Aminex A5 column. The acetic acid was used on the assumption that it might separate peptides from the RNA-core in the same manner that it solubilizes and dissociates aggregates of tyrocidine B, a cyclic decapeptide (112). Such treatment

with 80% acetic acid destroyed the hemolytic activity. There were six peaks of ninhydrin-reacting material (assumed to be peptides) eluted from the column. This indicated that the RNA-core SLS purified as described did not contain only a single peptide, as hoped for. Each of the peptide fractions was mixed with RNA-core and sonicated for various periods of time, but no reactivation of hemolytic activity was obtained. No proof could therefore be obtained that one of the peptides might be the hemolytic moiety.

The effect of various concentrations of acetic acid was explored to determine whether 1) acetic acid at lower concentrations than 80% could be used in purification and 2) 80% acetic acid did remove all peptides from RNA-core. The addition of acetic acid did not inactivate RNA-core SLS obtained from the resting cell system until the concentration of acetic acid reached 50% (Table 24). This was totally unexpected, since Bernheimer and Rodbart had reported (15) that dilute acid inactivates RNA-core SLS. When the effect of acetic acid was tested on RNA-core SLS obtained from cells growing with RNA-core, the results resembled those described by Bernheimer and Rodbart. Fifty percent of the hemolytic activity of such RNA-core SLS was inactivated by 10% acetic acid, and 80 to 90% was inactivated by 20% acetic acid. Attempts to reactivate RNA-core SLS inactivated by acetic acid treatment by sonication after either neutralization of the acetic acid by NaOH, or removal of the acetic acid by lyophilization, were unsuccessful. RNA-core was precipitable from mixtures of acetic acid and buffer by two volumes of absolute ethanol. All the active hemolysin in the 30% acetic acid mixture

<u>% Acetic Acid</u>	<u>HU</u>
0	15,000
10	14,000
20	14,000
30	12,000
40	11,500
50	500
80	0

Table 24. Effect of Acetic Acid on RNA-core SLS Hemolytic Activity

To RNA-core SLS prepared in the resting cell system was added glacial acetic acid in sequential amounts. The solution was mixed and allowed to stand 5 min in an ice bath. A precipitate was formed only on addition of 10% acetic acid; the precipitate was removed by centrifugation at 15,000 x g for 10 min. The RNA-core SLS in the acetic acid mixture was assayed by removing 0.1 ml from the mixture, diluting in 5 ml buffer, neutralizing the solution to pH 6.5 to 7.0 with NaOH, further diluting the mixture to 13.0 ml, and assaying the solution for hemolysin. The values for HU are corrected to the original volume.

was similarly precipitated by ethanol. Therefore, the hemolytic moiety presumably remained attached to the RNA-core while the hemolysin was still active. The RNA-core in the RNA-core SLS-80% acetic acid mixture was examined to determine if all ninhydrin-reactive material had been freed from it. The RNA-core removed from the 80% acetic acid mixture by ethanol precipitation, suspended in buffer, and reprecipitated by two volumes of absolute ethanol contained only traces of ninhydrin-reacting material. Alkaline hydrolysis of the RNA-core as described in Materials and Methods did not free any ninhydrin-reacting material above the previous background level. Although the results did not negate the possibility that the hemolytic moiety was separated from the RNA-core, they did not prove it since 1) the hemolytic moiety in the amount of hemolysin used may have been equivalent to only a trace of ninhydrin-reacting material and 2) there was no way to detect the hemolytic moiety after acetic acid treatment.

RNA-core SLS prepared in the resting cell system differed from the RNA-core SLS prepared from growing cells in ways other than the effect of acetic acid on hemolytic activity. RNA-core SLS prepared in the resting cell system was not inactivated when diluted to approximately 200 HU/ml and stored at 4C for 24 hr or for as long as 7 days. RNA-core SLS prepared from growing cells and diluted to 3500 HU/ml, was 60% inactivated on storage at 4C for 24 hr. RNA-core SLS prepared in the resting cell system was 80-90% recoverable from a G-100 Sephadex column while only 17-45% of that prepared from growing cells was recoverable from G-100 Sephadex. The elution

volumes for each were approximately the same; the slight differences may have been due to the difference in purity of the RNA-core SLS from growing cells which was suspended in growth medium. The difference in stability between the two may be related to the amount of hemolytic moiety bound per RNA-core molecule. With approximately one-twentieth the amount of RNA-core and one-twenty-fifth the amount of cells, the same amount of RNA-core SLS could be obtained from growing cells as from the cells harvested from 5 liters growth medium, and suspended in BBM with RNA-core.

Although high concentrations of urea or NaCl might have been used to separate the hemolytic moiety from RNA-core, they were not used because of the problem of removing them from the peptide mixture. It was pertinent, however, that 7 M urea did not inactivate RNA-core SLS.

RNA-core SLS obtained from either the resting cell system or from growing cells in the presence of RNA-core was stable to ether, chloroform, isoamyl alcohol, isobutanol, and n-heptane extractions. RNA-core SLS was precipitated by both ethanol and methanol. Presumably none of these procedures removed the hemolysin from the RNA-core. The effect of these solvents to remove contaminating polypeptides was not tested.

Eighty percent SAS precipitated part of the activity of RNA-core SLS from the resting cell system. Using 5×10^5 HU of RNA-core SLS from the resting cell system, 20% of the RNA-core SLS activity was precipitated by 80% SAS. The precipitated activity chromatographed on G-100 Sephadex exactly as RNA-core SLS and was inhibited by 13 $\mu\text{g/ml}$ trypan blue. The RNA-core SLS in the 80% SAS supernatant had the same chromatographic behavior as did the precipitated RNA-core SLS.

A series of purification procedures was combined to determine if contaminating peptides could be removed from the RNA-core SLS. RNA-core SLS from the resting cell system was treated with 80% SAS. The precipitate (containing 20% of the activity) was then resuspended in sodium phosphate buffer and treated with 10% acetic acid. The mixture was centrifuged at 15,000 X g for 10 min and the precipitate discarded. The RNA-core SLS was precipitated by two volumes of absolute ethanol, suspended in sodium phosphate buffer, reprecipitated with ethanol, and resuspended in buffer. The active RNA-core SLS was then chromatographed on a G-100 Sephadex column, lyophilized, dialyzed 48 hr against two changes of 0.01 M sodium phosphate buffer, pH 7.0, lyophilized again, and treated with 80% acetic acid. The solution was applied to an Aminex A5 column, washed into the column with 80% acetic acid for 0.5 hr, and then eluted. The eluate was divided into two parts for analysis. One part was analyzed for ninhydrin-reactive material, and the other was hydrolyzed with NaOH and then assayed for ninhydrin-reactive material. The eluate again contained a number of peaks of ninhydrin-positive material. After alkaline hydrolysis of the eluate, no additional ninhydrin-reacting peaks were revealed.

Although determination of the composition and structure of the hemolytic moiety was not possible, it was shown that more than one polypeptide was tightly bound to RNA-core. The previous hypothesis implicit in the work of Koyama (57) and others is, therefore, likely not correct. It was also shown that once the hemolytic moiety of RNA-core SLS has been inactivated presumably by separation from RNA-core by 80% acetic acid treatment, it cannot be reactivated by sonication with RNA-core.

Use of the Glucose Effect to Determine the Temporal Relationship Between Hemolysin Precursor and RNA-core SLS

Evidence supporting the hypothesis that hemolysin precursor is related to RNA-core SLS was obtained by utilizing the effect of glucose on both. As previously shown (Fig. 3), glucose (0.3%) markedly decreased the production of RNA-core SLS. In fact, little if any was measurable prior to the onset of stationary phase, by which time the level of glucose may have fallen to less inhibitory levels (79). The presence of 0.3% glucose in the growth medium also totally inhibited production of all hemolysin precursor (Fig. 14). If hemolysin precursor is the precursor to extracellular RNA-core SLS, inhibition of hemolysin precursor production should prevent production of RNA-core SLS.

The addition of 0.5% glucose to cells growing in HI-03% maltose immediately inhibited further production of hemolysin precursor (Fig. 27). After the addition of glucose, the amount of hemolysin precursor per cell decreased as the cell mass increased. The total amount of precursor stayed relatively constant (170 HU/ml at 0 hr, 140 HU/ml at both 1 and 2 hr). Doubling the amount of glucose to 1.0% had the same effect as 0.5% glucose on precursor levels. A much smaller amount of glucose (0.03%, or $2 \times 10^{-4}M$) caused transient repression of hemolysin precursor production (Fig. 28). It was of interest that cyclic adenosine 3', 5'-monophosphate (cAMP) did not relieve this transient repression when added at a concentration six times that sufficient to relieve transient glucose repression of β -galactosidase in Escherichia coli (80). In some experiments on transient repression

Figure 27.

Effect on Hemolysin Precursor Production of Addition
of 0.5% Glucose to HI-0.3% Maltose Media

Cells were grown in Step 3 as in Materials and Methods. At $OD_{520nm} = 0.4$ (0 time), 0.5% glucose was added to one set of flasks. The cells were harvested and washed at the times indicated and sonicated in 4 ml volumes at 75 intensity on the model 1 sonicator for 20 min. HU/ml corrected to common OD_{520nm} for all cells. ($\blacktriangle$) maltose-grown cells, ($\bullet$) glucose-grown cells, (---) OD_{520nm} .

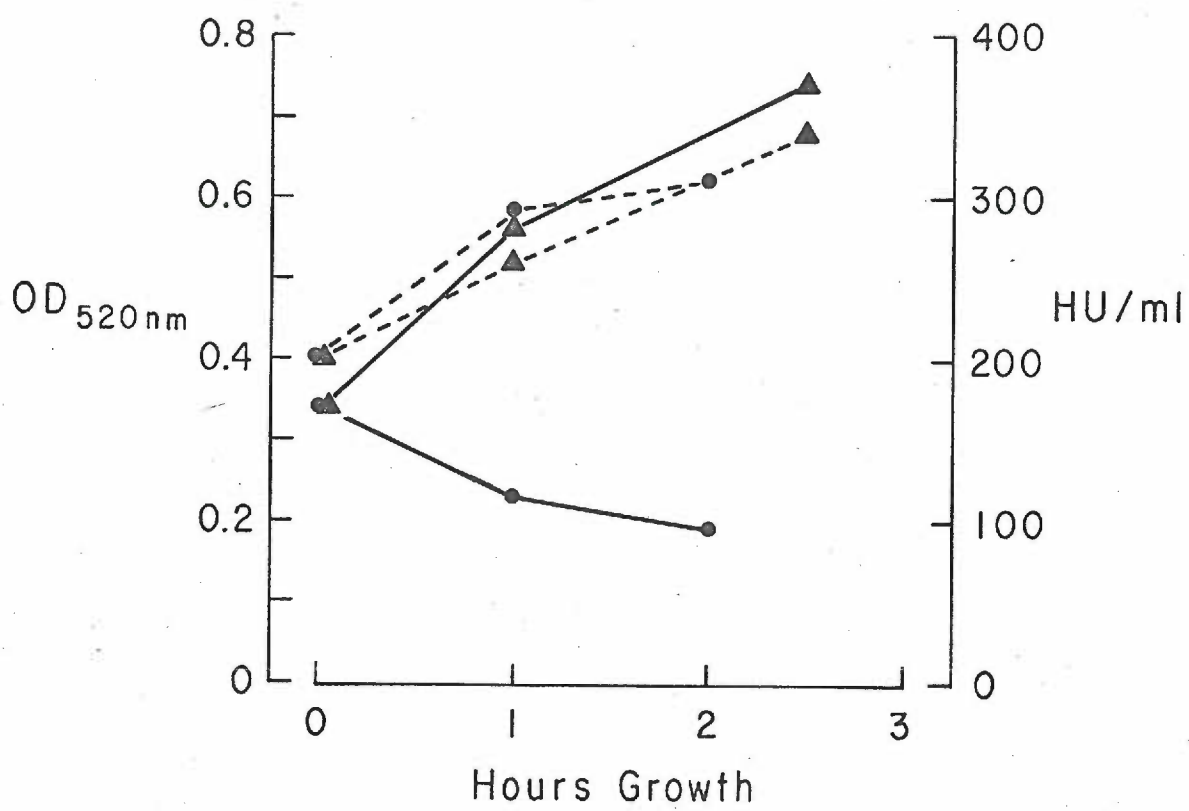
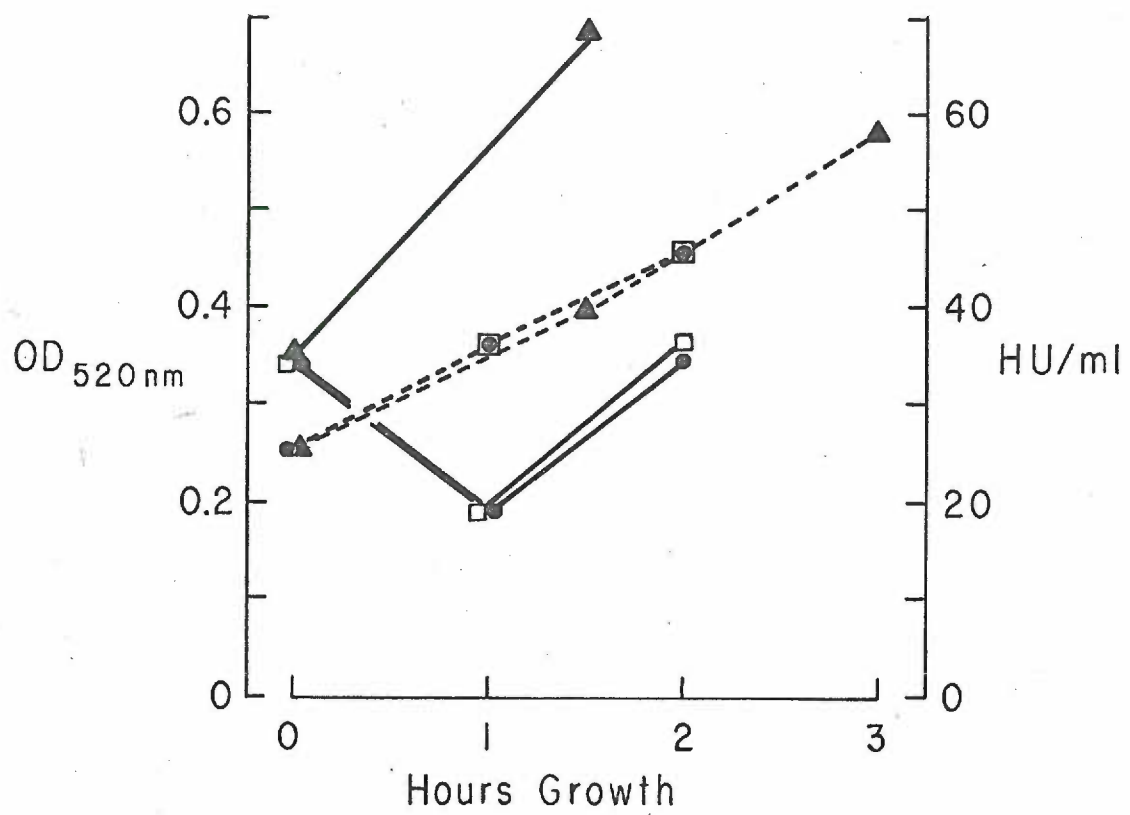


Figure 28.

Effect of Hemolysin Precursor Production of Addition of
 2×10^{-4} M Glucose +/- Addition of cAMP to HI-0.3% Maltose Media

Cells were grown in Step 3 as in Materials and Methods. At
 $OD_{520nm} = 0.26$ (0 time), glucose (2×10^{-4} M) +/- cAMP (4×10^{-7} M)
were added to the appropriate flasks. The cells were further treated
and assayed as in Fig. 27. HU/ml corrected to common OD_{520nm} for all
cells. (Δ) maltose-grown cells, ($\bullet$) glucose-grown cells, ($\square$)
glucose plus cAMP-grown cells, (---) OD_{520nm} .



of hemolysin precursor production, the cells escaping glucose repression rebounded to make as much hemolysin precursor/cell as did cells of the same age to which glucose had not been added. Perhaps cells grown in glucose-containing media begin to make RNA-core SLS in stationary phase (Table 3 and Fig. 3) because glucose has been depleted from the medium and cells are no longer repressed.

Extracellular SLS formation did not cease immediately after addition of 1.0% glucose to growing cells (Fig. 29). There was a gradual diminution in hemolysin formation, but the pattern was not like that for hemolysin precursor. In the resting cell system with HI-0.3% maltose-grown cells, the addition of glucose to cells suspended in BBM also did not immediately inhibit the RNA-core SLS increase in titer (Table 25). This result agreed with that found by others (9) that glucose can be used in the resting cell system. The RNA-core added to growing cells had no effect on hemolysin precursor levels (Table 26), showing that the diminution in extracellular SLS levels after addition of glucose and RNA-core was due only to the glucose. It was remarkable that cells grown in the absence of RNA-core and which had produced only 200 HU/ml of extracellular hemolysin, predominantly SLO in character, had the same precursor levels as cells grown in the presence of RNA-core and which had produced greater than 4000 HU/ml of RNA-core SLS.

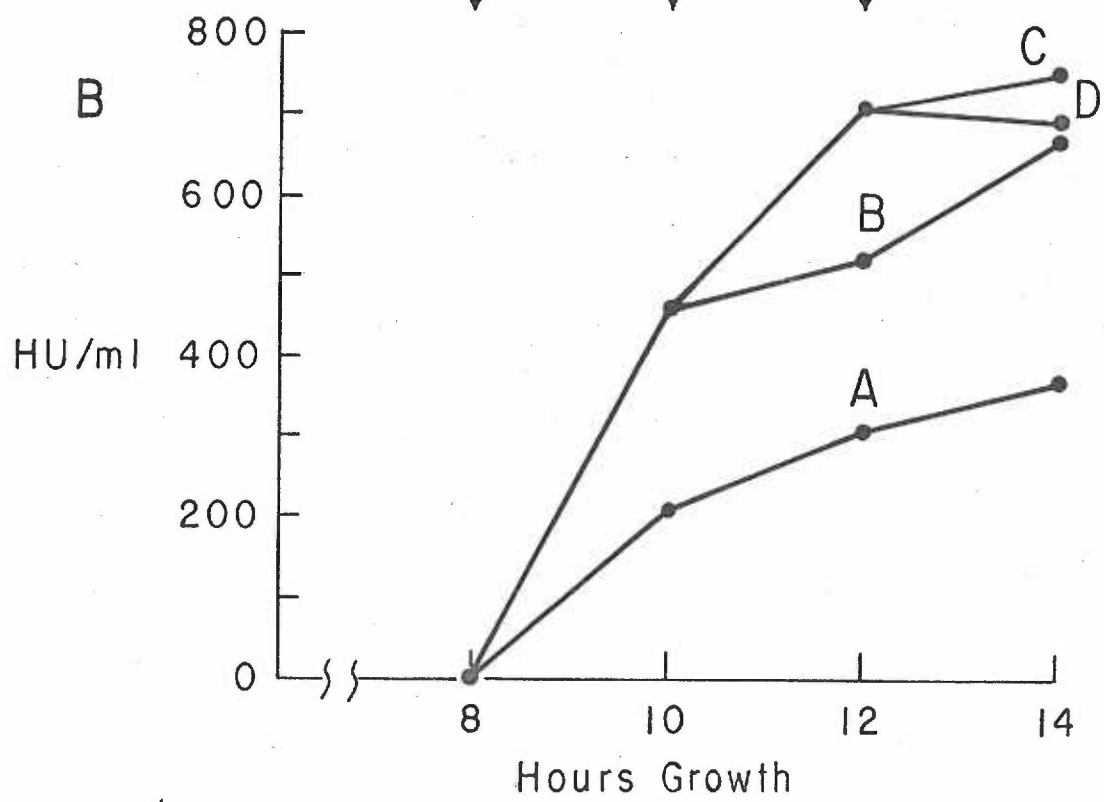
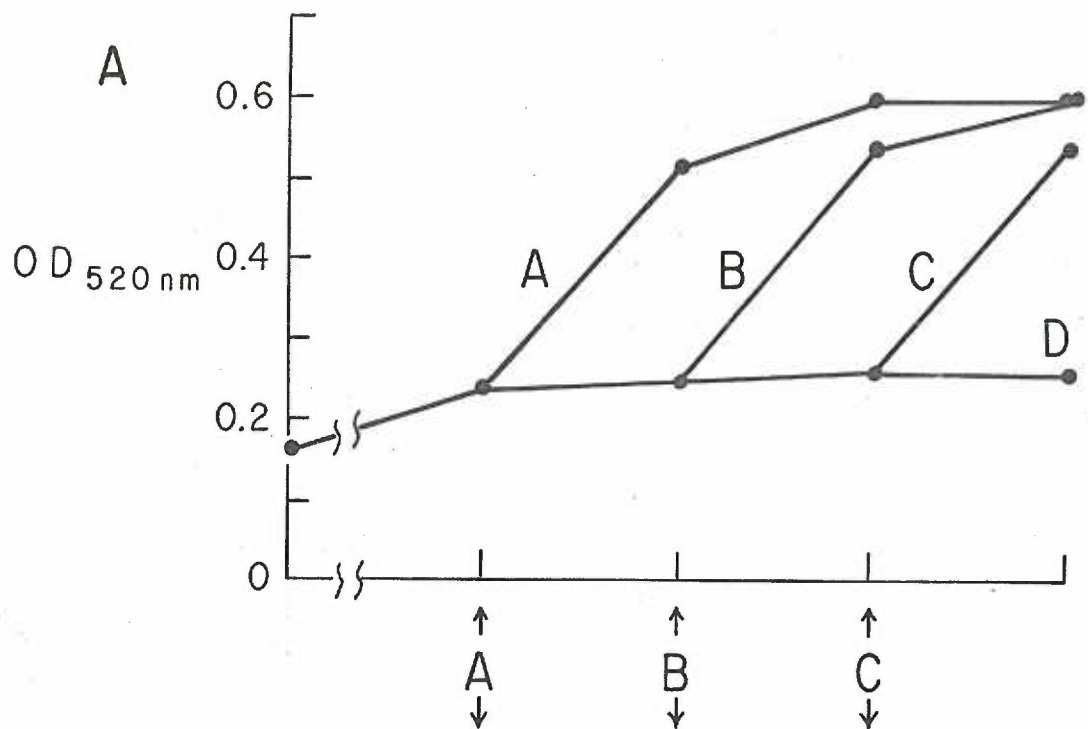
The data strongly support the hypothesis that hemolysin precursor is the precursor to SLS. First, if formation of hemolysin precursor is prevented by glucose, the formation of extracellular SLS is also inhibited. Second, when continued hemolysin precursor production is

Figure 29.

Effect on Extracellular RNA-core SLS Production of
Adding 1.0% Glucose to Cells Growing in HI Broth

Cells grown as in Materials and Methods except Step 3 medium was HI broth. Crude RNA-core (50 mg) added to each flask at 8 hr. Glucose (1.0%) added to the appropriate flask as indicated by the arrows.

- A. Cell growth.
- B. Hemolysin production.



Preparation	HU/ml	
	45 min	45 + 60 min
Cells in BBM	13,400	9,000
Cells in BBM + glucose	10,100	5,850

Table 25. Effect on RNA-core SLS Formation of the Addition of Glucose to Cells in the Resting Cell System.

Cells grown as in Materials and Methods to late log phase in Step 3. After the cells were harvested and washed, cells harvested from 200 ml medium were suspended in 3 ml BBM, pH 7.0, containing 10 mg crude core with or without glucose (3.0%). The cells were incubated 45 min, then removed by centrifugation and resuspended in fresh BBM of the same composition. The supernatant was assayed. The cells were reincubated for 60 min and the supernatant assayed. The HU/ml for each preparation was corrected to a common OD_{520 nm}.

Incubation (hr)	Culture OD _{520nm}	HU/ml			
		Extracellular hemolysin		Hemolysin precursor	
		- core	+ core	- core	+ core
0	0.08	0	0	ND	ND
4.0	0.38	110	1400	ND	ND
4.5	0.48	156	3350	289	241
5.0	0.50	200	4400	132	118
5.5	0.51	260	4400	110	115

Table 26. Effect of RNA-core on Hemolysin Precursor

Peak 1 RNA-core (7.0 mg) was added to one set of flasks containing 100 ml HI-0.3% maltose at 0 time and the cells were grown in Step 3 as in Materials and Methods. Precursor levels were measured by harvesting 50 ml cells grown +/- RNA-core, washing, and suspending them in 5.0 ml buffer; the cell suspensions were then sonicated 25 min at 60 intensity on the model 2 sonicator. ND = not done.

inhibited, the formation of extracellular SLS is diminished. This suggests that hemolysin precursor forms a pool from which extracellular SLS is drawn.

The cessation of hemolysin precursor production after addition of very small amounts of glucose demonstrates the marked sensitivity of precursor formation to changes in the environment. The highly efficient regulation of precursor is also demonstrated by the similarity in precursor levels in cells forming very high levels of RNA-core SLS or in those forming very little. An alternative hypothesis is that there is no difference in precursor levels because SLS is excreted into the medium in the presence or absence of RNA-core, but is stabilized only in the presence of RNA-core.

DISCUSSION

The answers to the original questions asked in this thesis will be discussed and related to the results of other investigators of cellular SLS where appropriate. Since the cellular forms of SLS are more numerous and varied than previously known or expected, an attempt will be made to integrate them into a hypothesis concerning the sequence of events involved in the formation of extracellular SLS. The possible mechanisms of activation of hemolysin precursor will be discussed, and the activated and active forms of SLS compared. The possible relationships of SLS to other bacterial hemolysins and to polypeptide antibiotics will be explored. Finally, the possible role of the hemolysin precursor of SLS in the pathogenesis of poststreptococcal glomerulonephritis will be discussed.

A brief summary of the hemolysins described in this thesis is as follows: MS_m hemolysin or LACH is the labile active cellular hemolysin, exhibiting a delay in hemolysin, released by muralysin treatment of intact Group A streptococcal cells. It is apparently an active in vivo hemolysin. SS hemolysin is the hemolysin, exhibiting a delay in hemolysis, present in the sonicate of cells disrupted by sonication. It is also present after sonication of MS, MS_m, SS, and SS_m preparations. It is apparently not an active in vivo hemolysin, but is formed by the effect of sonication on a latent or precursor hemolysin. Hemolysin precursor is an inactive cellular substance that can be activated by sonication in the absence of RNA-core to SS hemolysin or in the presence of RNA-core to activated hemolysin (RNA-core bound hemolysin).

The importance of growth conditions for optimal production of SLS was illustrated by the production of RNA-core SLS in various media of differing glucose or maltose content. The effect of maltose on RNA-core SLS production is apparently to shift it from stationary phase to log phase of growth. The mechanism for this effect is unknown. In the presence of glucose, RNA-core SLS production does not occur until late in stationary phase, as was also shown by Ginsburg et al. (41) and reported by Bernheimer (9). Perhaps, the production is inhibited by the "glucose effect" (68) until such time as the glucose concentration has been lowered to less inhibitory levels. Differing concentrations of glucose or other potentially repressive substances (94) in the growth medium might then be determining factors for the relationship of SLS production to the growth cycle. Since many of the investigators have used media differing in both glucose content and other nutrients, comparison of experiments of others is sometimes difficult. The advantage of using a medium without added sugar, such as HI, is that the highest HU/cell is formed. The advantage of adding maltose to the growth medium is that RNA-core SLS production can be related to periods in the growth cycle. Cells harvested in late log phase growth produce maximal amounts of RNA-core SLS. There is no such correlation of maximal production to time of growth when cells are grown in BHI, HI-0.3% glucose, or even HI, making it difficult to compare experiments on hemolysin production and to harvest cells at peak hemolysin production. After this work was finished, an abstract of a similar report by the Japanese investigator, Ohtsuki, on the effect of maltose on SLS production appeared in Biological Abstracts (76). He (75) reported that the RNA-core SLS

produced in a medium containing maltose far surpassed that produced in a medium containing glucose, but not produced in broth without added sugar. Bacterial growth was greater in the maltose medium than in plain (broth) medium. He did not show that there was a difference in time of onset of RNA-core SLS production in glucose medium compared to that in maltose medium. The early onset of production in both media may have been due to SLO production. There was no evidence in the tables or figure legends (in English) to show that the titer of hemolysin, reported as RNA-core SLS, was corrected for the presence of SLO. The text (in Japanese) was not translated, so this supposition may not be correct.

The growth conditions also affect the production of hemolysin precursor. The presence of 0.3% glucose totally inhibited precursor production, at least until early stationary phase growth. Prior to this time, production of precursor had reached maximal levels in the HI-0.3% maltose medium. Since this is the first report of the presence of a latent or precursor form of cellular SLS, no direct comparison can be made to other research. However, it appears that the "intracellular" SLS hemolysin (AS hemolysin) reported by Taketo and Taketo (97) is the activated product of hemolysin precursor. Their results indirectly confirm the repression of hemolysin precursor by glucose, since they found very little "intracellular" SLS after breakage of cells grown in 1% glucose. What small amount of hemolysin was present might have been cellular SLO.

After development of an adequate growth system, it was possible to seek an answer to the question of whether an active in vivo cellular SLS-like hemolysin is present in Group A streptococci. The answer is that there is such an hemolysin (LACH), and that it is of low titer and very labile. Like RNA-core SLS, it exhibits delayed hemolysis, is inhibited by trypan blue, is not adsorbed by RBC_m at OC, does not require reducing substances for its activity, and is stabilized by RNA-core. Many experiments were done to determine if this hemolysin was truly an active hemolysin prior to cell lysis. The various substances present in the muralysin preparations were incubated at both 23C and 37C with hemolysin precursor from an SS preparation. No activation of hemolysin precursor could be demonstrated even when RNA-core was present in the mixtures. The neutralization of the reducing agent, 2-mercaptoethanol, by N-ethylmaleimide had no effect on the hemolytic activity of LACH in an MS_m preparation. No results have indicated that LACH is not an active in vivo cellular SLS-like hemolysin.

Why LACH is so very labile, even at OC, is unknown. Perhaps it is inactivated by cellular enzymes, although this would seem less likely to occur at OC or -20C. Another possibility is that it is weakly bonded to a stabilizing or carrier substance and becomes inactive after dissociation. It is also possible that the reverse is true -- that the hemolysin is inactivated due to aggregation of it or possibly a substance to which it is bound. Both of these last two possibilities may occur with other forms of SLS as will be discussed.

The reason for the low titer of LACH is also unknown, but may, in part, be due to the extraction procedure and lability of the hemolysin. All streptococcal cells are not lysed by muralysin treatment nor are all protoplasts enmeshed in sticky DNA lysed by osmotic shock. The failure to release all LACH would lower the titer of hemolysin obtained. Some LACH could be expected to be inactivated during the incubation of streptococcal cells with muralysin, during sedimentation of the cell debris, and during adsorption of SLO. The low titer might, however, reflect cellular control mechanisms to minimize accumulation of a membranolytic or toxic substance (26). LACH might also be an exoproduct or waste product rapidly excreted from the cell.

There are several possible or probable reasons why no investigator has found LACH prior to this time. First, those using sonication or grinding to disrupt cells would have either obscured the presence of LACH with the more stable SS hemolysin or been unable to detect a labile delayed hemolysin because of the presence of cellular SLO. Since there is overlapping inhibition of LACH and cellular SLO by inhibitors, the use of these inhibitors may have obscured the presence of small amounts of LACH. Finally, those investigators using muralysin to lyse cells or prepare protoplasts may not have explored the presence of labile cellular SLS-like hemolysin because of the inability to remove or inhibit SLO.

The question of whether the cellular form of LACH is different from that of SS hemolysin is not as easily answered. SS hemolysin does not seem to be the in vivo active intracellular form of SLS as suggested

by Taketo and Taketo (97) or an active intracellular hemolysin as suggested by Schwab (87), since its formation is due to an effect of sonication on hemolysin precursor. The lability of LACH precluded the assessment of its size by Sephadex chromatography and the low titer prevented determination of its possible particulate heterogeneity in a sucrose density gradient. The use of both procedures would have resulted in data useful to compare the two. The only procedures available were streptomycin sulfate and ammonium sulfate precipitation and sedimentability during high speed centrifugation. The lability of LACH still imposed limits on the conclusions that could be drawn from these procedures. Both SS hemolysin and LACH were similar in that little of either was precipitated or inactivated by 2.5 % streptomycin sulfate. They differ during SAS precipitation. Essentially all SS hemolysin was precipitated by 50% SAS whereas a significant amount of LACH was not. In the 50% SAS supernatant 28% of LACH was recovered, while 14% was in the 50% SAS precipitate. The lability of LACH may have been partly responsible for some of the loss of activity. After centrifugation at 200,000 X g for 2 hr, 30 to 45% of the SS hemolysin activity was sedimented. No LACH was sedimented after centrifugation at 200,000 X g for 1 hr, if a correction of the lability was made. The assumption that all the unaccountable activity was due to the instability of LACH may, of course, not be completely correct. Finally, these hemolysins differ dramatically in their stability at 0C and -20C. There are then a number of differences that suggest that these two hemolysins are different. It is possible, though, that SS hemolysin is composed of

material that included LACH, and that this might account for the apparently larger size of SS hemolysin and its greater stability.

The stabilization of LACH by RNA-core and the fact that this RNA-core-LACH combination had the same elution volume as RNA-core SLS indicates that LACH and RNA-core SLS may have the same hemolytic moiety. If they do, LACH may be an intermediate between the hemolytic moiety being synthesized in the cell and the RNA-core SLS detected extracellularly. The location of some or all of LACH in the periplasmic space would support this hypothesis. The inability to stabilize protoplasts made it impossible to determine the location of LACH as support for such an hypothesis. There is only indirect evidence that the hemolytic moiety of LACH was transferred from a cellular substance to RNA-core. The fact that some LACH is precipitated by 50% SAS indicated LACH was a large substance. If the entire LACH material bound to RNA-core, one would have predicated that the RNA-core LACH complex would have eluted from G-100 Sephadex before RNA-core SLS. Since the two elution volumes were the same, it suggests that transfer of a small substance -- the hemolytic moiety -- occurred rather than the binding of a large substance to RNA-core. The nature of the postulated cellular carrier is unknown.

The demonstration of the presence of a SLS-like hemolysin after sonication confirms the observations of both Taketo and Taketo (97) and Schwab (87). Although the SS hemolysin, like the hemolysin described by Taketo and Taketo, is obtained by sonication, exhibits properties of SLS, and is transferable to RNA-core, it differs from

their hemolysin by one major property. SS hemolysin is completely stable to chloroform extraction, whereas the AS hemolysin of Taketo and Taketo is completely destroyed by such treatment. Whether or not this difference is due to the difference in bacterial strains, growth conditions, buffer, or pH used is uncertain. Such differences may have resulted in 1) a different cellular carrier binding the hemolytic moiety, or 2) a difference in the stability of the bond between carrier and hemolysin. The differences between RNA-core SLS and the "intracellular" hemolysin reported by Schwab (88) might be examined by effects of the carrier substance on the behavior of the hemolytic moiety. Since he did not determine if the hemolysin was stabilized by or transferred to RNA-core, it is impossible to determine if the hemolysin contained the same or similar hemolytic moiety as RNA-core SLS. The reported antigenicity (89) of the "intracellular" hemolysin may have been due to one of two phenomena. First, the antibody may have been formed against the carrier substance and not the hemolytic moiety. This possibility was raised by Schwab who stated that "the inability of the specific inhibitor to affect Streptolysin S' could be due to a reflection of a difference in physical dispersion of the hemolysins or association of the hemolytic activity with antigenic moieties" (89). Second, the antibody inhibited only a small fraction of the hemolysin and then only after prolonged incubation with the hemolysin. The inhibitory effect was enhanced by centrifugation and removal of the sediment or precipitate prior to assay of the mixture. Schwab concluded that the antigen-antibody reaction occurring was an example of "neutralization as a result of steric hindrance by the structure of the aggregate" (89). However,

the normal inactivation of SS hemolysin may be due to aggregation. High speed centrifugation of SS hemolysin is sufficient to remove some of the hemolysin from a solution. The sedimented hemolytic activity is not measurable unless the compacted sediment is dispersed by high speed mixing. The antibody material added by Schwab may have promoted an increased rate of aggregation of SS hemolysin by a means other than formation of complexes of antigen with divalent antibody. The SS hemolysin may have aggregated with itself or adsorbed to and aggregated on the antibody material added. Since Schwab did not state whether the sedimented material was dispersed by high speed mixing, the possibility remains that the hemolysin could have been demonstrated to be active. In summary, the antibody demonstrated by Schwab may have been directed against the carrier or may not have been specific antibody at all.

The presence of single sonically-released hemolysin after sonication depends both on the sonicator and the conditions of sonication. This apparently explains why Schwab (87) found no SLO in his preparations while Taketo and Taketo (97) did. The technique of adsorbing cellular SLO from a mixture containing cellular SLS-like hemolysin makes it possible to disrupt cells with the least amount of subcellular fragmentation, and still obtain a preparation free of active SLO without adding chemical inhibitors.

The presence of a hemolysin precursor activatable by sonication in the MS, MS_m, SS, or SS_m preparations accounts for the increase in titer of these preparations during sonication. There is presently no

evidence to suggest that the hemolysin released from whole cells by sonication is any different than the hemolysin obtained after sonication of the various preparations listed above. All sonically released SS hemolysin and SS hemolysin prepared from MS, MS_m, or SS_m preparations are assumed to be the result of activation of hemolysin precursor. SS hemolysin and activated hemolysin (RNA-core bound hemolysin) are different, however, in that they have different molecular weights, due presumably to their different carriers. The potentiation of titer by prolonged sonication of cell-free cytosol confirms the finding of Schwab (87). He also found that hemolysin was obtainable from cellular debris, but did not wash this debris or determine that it was free of intact cells (87). No SS hemolysin was detected after prolonged sonication of cell-free, washed debris, as was shown in Table 21. Hemolysin precursor was present, however, since activated hemolysin was obtained after sonication of the debris in the presence of RNA-core.

The activation of hemolysin precursor is potentiated by the presence of added RNA during sonication. Whether or not RNA is absolutely required is uncertain. The dependence of the activation of precursor in washed cell debris upon added RNA-core indicates that activation of some precursor material depends upon the presence of RNA. The activation of hemolysin precursor material, which contained only very small amounts of associated RNA after purification of an MS_m preparation, suggests that some of the precursor material may be activated in the absence of RNA or that very little cellular RNA is required. Cellular

RNA may contribute to the activation of hemolysin precursor during sonication of whole cells. Differences in the RNA present at different times during cell growth may also play a role in the degree of activation obtained. This might account for why more activation of hemolysin precursor was obtained with RNA isolated from late log phase cells than with RNA with early log phase cells. Differences in the type of RNA present may be responsible for the difference in the effect of chloroform on the AS hemolysin of Taketo and Taketo (97) from that on SS hemolysin. The fact that chloroform does not precipitate the SS hemolysin, which was demonstrated to be of large weight by G-100 Sephadex chromatography and ammonium sulfate precipitation, is indirect evidence that the carrier of SS hemolysin may be cellular RNA.

The potentiation of activated precursor titer is not dependent upon only RNA-core or streptococcal RNA. Potentiation could also be demonstrated with E. coli RNA. Neither of the mammalian RNA preparations, however was effective. In fact, the rat t-RNA consistently inhibited some of the increase in titer.

The presence of the proper amount of RNA-core during activation of hemolysin precursor was necessary to obtain the maximum titers of activated hemolysin. Determination of the maximum potential titer of precursor during purification was difficult, especially when only small amounts of precursor were available due to 1) the inability to predict the proper amount of RNA-core to use and 2) the necessity to sonicate precursor with several different amounts of RNA-core.

The maximum hemolysin precursor titer also depended upon the length of sonication and the concentration of the sonicate mixture which complicated the determination of the maximum titer. The final titers of dilute solutions corrected to the original volume differed from the corrected titers of more concentrated solutions. Whether or not this is due to the fact that sonication does not fragment material as much in dense and viscous solutions (106) is uncertain.

The determination of the maximum amount of precursor present in the cell then depends 1) upon the length of sonication, 2) the concentration of the sonicated solution, and 3) the amount of RNA or RNA-core present. When all of these factors are considered, there is a significant amount of precursor present in Group A streptococcal cells grown and harvested under the proper conditions. Under optimal conditions of assay, the amount of SS hemolysin activated from hemolysin precursor is approximately equal to the amount of RNA-core SLS measurable after incubation of streptococci in the resting cell system for one hour at 37C. If the assumption is made that sonication of the cells with RNA-core would have increased the titer of hemolysin by 1.5 to 2.0 fold, then there is more hemolysin precursor than RNA-core SLS formed. There may, in fact, be much more potential hemolysin in the form of precursor than is released extracellularly as RNA-core SLS. Contrary to Ginsburg (39), there is a very large amount of potential hemolysin present within the streptococci.

A number of observations indicates that hemolysin precursor aggregates. First, hemolysin precursor material, in solutions which

have been allowed to stand at 4C, sediments at lower centrifugation speeds than were previously used to remove the contaminating cellular debris. Second, after solutions of concentrated hemolysin precursor material are frozen and thawed, the turbidity of the solution is much increased and the precursor can again be sedimented at lower centrifugation speeds than previously used. Finally, the fact that hemolysin precursor material which is sedimented after centrifugation at 200,000 X g for 2 hr is insoluble suggests that it may be tightly bound to itself or like material.

Hemolysin precursor material which appears to be easily denatured, for example by acid or acetone treatment, remains insoluble after denaturation. Perhaps aggregation is related to denaturation. Another possibility is that non-precursor material promotes aggregation of precursor. One possible example of this is the circumstantial evidence that membranes and phospholipids cause the aggregation of staphylococcal α -toxin into an insoluble form (13) and cause the polymerization of staphylococcal leukocidin (114) (4).

The tendency to aggregate or associate with cellular material is apparently a characteristic of bacterial cytolytic toxins (14). The observation that the hemolysin precursor material and/or SS hemolysin apparently aggregate is the first that any form of SLS aggregates. Taketo and Taketo (97) suggested, however, that the large size of AS hemolysin might have been due to aggregation. The observations on the aggregation of cytolytic toxins (hemolysins) have been made on extracellular material. The usual form of staphylococcal α -toxin has an

$S_{20,w}$ of 3.0, but some aggregates during purification to a form having $S_{20,w}$ of about 12. The α -toxin also polymerizes in borate-phosphate buffer to a water-insoluble protein (3) (16) (25). The F and S components of staphylococcal leukocidin both polymerize in low ionic strength solutions (113). Staphylococcal δ -hemolysin may bind to one or more carrier substances (14). The Bacillus cereus hemolysin, cereolysin, undergoes loss of activity during ultracentrifugation, freezing and thawing. It is known to polymerize into an insoluble material (17) (14). The Bacillus subtilis hemolysin, subtilysin, also aggregates (18).

The possibility that precursor aggregates or associates with cellular material limits the conclusions that can be drawn from certain data about its cellular location or form. This is especially true of data from sonicated cell preparations. Based on the differential sedimentability of the hemolysin precursor from SS preparations, the precursor may be associated with the cell membrane fraction. After streptococci are disrupted mechanically in a Mickle disintegrator and the intact cells and cell walls are removed, the cell membranes can be sedimented at 20,000 X g (35). Sonication comminutes the subcellular fractions of Group A streptococci to smaller particles (59) than obtained with the Mickle disintegrator. Prolonged centrifugation at 20,000 X g to 25,000 X g might be expected, though, to sediment some of the membrane material. Prolonged centrifugation of SS preparations does result in sedimentation of hemolysin precursor at 25,000 X g. The fact that very little rhamnose is present in material containing precursor sedimented at 25,000 X g

for 3 hr suggests that cell wall material is not a prominent component of this fraction. The assays for the chemical markers of streptococcal membranes (35) (20-25% lipid or 2% glucose of the total weight) required much more material than was available and were not, therefore, undertaken. Aggregated hemolysin precursor may, however, have the same sedimentation characteristics as the membrane fraction, but not contain membrane. During sonication some change in physical behavior of hemolysin precursor could have been caused by the disruption process itself. The hemolysin precursor may also have become associated with cellular material during the sonication process and then retained some of the physical properties of that cellular material. Evidence which indicated that the particulate nature of hemolysin precursor was not a consequence of sonication was obtained from muralysin-lysed cell preparations. Hemolysin precursor material from such preparations was completely sedimentable.

Proof of the cellular location and possibly the cellular form of Streptolysin S depended upon the stabilization of strain C203S protoplasts. Although a large amount of time and effort was devoted to this problem, stabilization of protoplasts could not be obtained. A number of different procedures to stabilize the protoplasts were tried including many of the published ones used by others. It is pertinent to note that there is little published data on the degree of stabilization of streptococcal protoplasts during the removal of cell wall. Friemer et al (36) stated that one-third of the cells lysed

during removal of cell wall, two thirds of the protoplasts remaining stable in 7% NaCl. One of the reasons for unsuccessful stabilization of all of the strain C203S protoplasts is that it may be impossible to stabilize all protoplasts during removal of cell wall. Another possibility is that the strain C203S cells are structurally different or more osmotically labile after removal of the cell walls and are, therefore, unsuitable for the preparation of protoplasts. There does not appear to be any data on the preparation of protoplasts using this strain. Finally, the cells grown in the HI-0.3% maltose medium may have different structural characteristics than cells, grown in more standard media such as Todd Hewitt or beef heart-proteose peptone broth. Perhaps cells grown in the medium I used are not suitable for the preparation of protoplasts. It is unknown whether production of high titer SLS might influence membrane stability of the producing organism.

The other possible reason for lack of stabilization of protoplasts might have been the presence of a membrano-lytic substance in the muralysin preparation. Although the muralysin was not hemolytic, it is possible that the preparation could have contained material that would disrupt the streptococcal membrane. The Group C streptococcus and phage used were obtained from the Dow Chemical Co. on the suggestion of Dr. S. S. Barkulis who had used this or a similar streptococcus and phage to prepare muralysin (5). Whether or not a membrano-lytic substance was released upon lysis of this Group C streptococcus and was not present in phage lysates prepared by other workers is

unknown. Group C streptococcus and its phage used to prepare muralysin by others (35), were obtained from Dr. Friscetti at the Rockefeller University, but unfortunately the Group C streptococcus could not be cultivated. The use of muralysin prepared from different phage-Group C streptococcal systems was, therefore, not possible. The possibility that muralysin may have indirectly freed small sections of the membrane containing precursor could, therefore, account for the sedimentability of precursor. Unexplained, however, is the fact that ghosts prepared from muralysin-lysed cells contained very little hemolytic activity. Sonication of the preparation with RNA-core did not result in even small amounts of activated hemolysin. The majority of precursor was in the original lysate of the muralysin-treated cells.

Although some investigators (95) (94) (93) have suggested that SLS is formed or released from the cell membrane, this has not been proven beyond doubt. None of the published reports on protoplast ghost fractions rules out the possibility that synthesis may be by remaining intact protoplasts. There is no evidence to suggest that the production of SLS is followed by its association or incorporation into membrane. Whether SLS may be associated with the ribosomes bound to the membrane is also uncertain.

The mechanism of activation of hemolysin precursor remains to be elucidated. One of the known effects of sonication is to disaggregate membranes and solubilize many of the membrane components (86). There is at least one example of potentiation of activity of a membrane-associated substance by sonication. Brief periods of sonication are known to increase the adenosine triphosphatase activity of membrane

preparations from the cortex of rabbit kidney (110). Although this process of membrane disaggregation may be involved in activation of precursor, proof that this is so depends upon isolation of membranes from protoplasts.

The fact that homogenization also activates hemolysin precursor indicates that activation is due to an effect they share in common. Since both sonication and homogenization can shear material such as nucleic acid into smaller fragments, this effect might be involved in activation. Yet treatment of precursor with RNase and DNase in the absence or presence of RNA-core did not activate the hemolysin. The results from the sucrose density sedimentation of precursor and SS hemolysin do suggest that there is some shift in size during activation of precursor. A comparison of Figures 23 and 25 indicates that there is no peak of SS hemolysin corresponding to the initial peak of the precursor in the sucrose gradient. The bulk of the SS hemolysin is also slightly shifted toward the top of the gradient when compared to the bulk of the precursor. Whether or not this slight shift is significant enough to indicate a size difference is uncertain. The fact that SS hemolysin has many of the properties of precursor suggests that the activated material is similar to the precursor material. However, the possible involvement of transfer of the hemolytic moiety from precursor material to another cellular carrier such as RNA is, not excluded by these experiments. The difference in the effect of chloroform on hemolysin precursor and SS hemolysin could support such a possibility. Presently, the fragmentation or disaggregation of

hemolysin precursor exposing active hemolytic sites, perhaps followed by transfer to another cellular substance, is the most probable explanation of activation. The fact that SS hemolysin after sedimentation at 200,000 X g for 2 hours can be "reactivated" by high speed mixing supports the idea that active hemolysin can remain in a latent state, activatable by the freeing of its active site(s). There is no evidence that the hemolysin is freed from a cellular inhibitor as suggested by Schwab (87). It is of interest, however, that the hemolysin of Streptococcus zymogenes is inhibited by cellular teichoic acid (6).

The activation of hemolysin precursor to activated hemolysin (RNA-core bound hemolysin) possibly involves SS hemolysin as an intermediate. Both hemolysins are the result of activation of precursor; both apparently have the same or similar hemolytic moiety. The transfer of the hemolytic moiety of SS hemolysin to RNA-core can occur under the conditions present during sonication. The rate of transfer is also fast enough to account for the presence of all the activity of activated precursor in a RNA-core bound form after sonication of precursor in the presence of RNA-core.

The physical relationship of the SLS-like cellular forms of SLS is demonstrated by their chromatographic behavior on G-100 Sephadex after they have been incubated or sonicated with RNA-core. Precursor activated by sonication in the presence of RNA-core has the same chromatographic behavior as the extracellular RNA-core SLS. The product of incubation of RNA-core and SS hemolysin also has the same elution

volume from G-100 Sephadex as RNA-core SLS. This evidence indicates that hemolysin precursor is a latent form of SLS and can be activated to RNA-core SLS. The labile active cellular hemolysin (LACH) after stabilization by RNA-core is also chromatographically identical to RNA-core SLS. The fact that the hemolytic moiety of Tween-40 SLS can be transferred to RNA-core is further support that all the hemolytic moieties are related. This premise is supported by the work of Ginsburg et al (42) and Taketo and Taketo (98) who evaluated the chromatographic behavior and transferability of the extracellular hemolysins. Taketo and Taketo (97) also presented evidence for the transfer of SS hemolysin to RNA-core.

The temporal relationship of the hemolysin precursor to extracellular RNA-core SLS is indicated by the experiments utilizing the glucose effect on production of both. When glucose is added to the culture media prior to the onset of RNA-core SLS or precursor production, it totally represses their formation. When glucose is added after the onset of production of either, it stops formation of precursor, but only partially inhibits formation of RNA-core SLS. Although the amount of precursor per cell declines, there is a relatively small fall in the total amount of precursor. These results indicate that SLS is first formed as precursor. The hemolytic moiety is then activated and combines with RNA-core to form extracellular RNA-core SLS. Once glucose is added further formation of precursor is prevented, but the majority of that which has already formed remains in a potentially activatable

form. Since RNA-core SLS formation by cells which do not contain precursor is prevented by glucose, the continued formation of RNA-core SLS by precursor-containing cells in the presence of glucose supports the hypothesis that RNA-core SLS is formed from precursor. An alternative hypothesis is that some of the SLS which is formed in the cell is stored in a latent form (hemolysin precursor) and the rest becomes extracellular. Such a hypothesis would require that the regulatory mechanisms for RNA-core SLS change -- that an initially totally repressible substance becomes moderately resistant to repression by glucose -- but that the regulation of hemolysin precursor remains the same. There is presently no evidence for this. The present data support the hypothesis that hemolysin precursor is in fact the precursor to extracellular RNA-core SLS.

It seems probable that hemolysin precursor is activated prior to transfer of the hemolytic moiety to RNA-core SLS. When RNA-core is incubated with precursor, no RNA-core SLS is formed. However, if the precursor is activated by sonication, RNA-core SLS is formed upon incubation of the activated precursor (SS hemolysin) with RNA-core. The presence of LACH in the cell suggests that it may possibly be the activated form which is an intermediate between precursor and RNA-core SLS. Proof of this hypothesis awaits specific labeling of precursor and the demonstration that the label moves into the LACH pool and from there to extracellular RNA-core SLS.

Although the role of BBM (maltose, Mg^{2+} , and cysteine) and RNA-core was not specifically examined, several conclusions about them can be

drawn from this research. First, the stabilization and possible transfer of the hemolytic moiety of the active cellular hemolysin to RNA-core does not depend upon BBM. Secondly, in the in vitro system used, hemolysin precursor is not activated by BBM and RNA-core. Third, maltose is not essential for production of hemolysin precursor or RNA-core SLS by growing cells, nor is it essential for production of RNA-core SLS by resting cells. Fourth, the addition of RNA-core does not appear to lower the cellular amount of hemolysin precursor obtainable from growing cells. If RNA-core does cause an increase in cell loss of precursor, the rate of production of precursor must have increased to keep the cellular amount constant. Fifth, moderate amounts of RNA-core stabilize the hemolytic moiety of SLS. Finally, RNA-core may be necessary for the binding of RNA-core SLS to RBC. RNA-core does have an affect on RBC, since it causes them to adhere to one another. Furthermore, pre-incubation of RBC with RNA-core blocks the hemolytic affect of low concentrations of SS hemolysin and MS hemolysin.

Based on the previous data and work of others, the following sequence may occur during the in vivo formation of SLS: The hemolytic moiety is synthesized in the latent form of hemolysin precursor. The hemolysin precursor may be incorporated in or associated with a sub-cellular fraction (93) (94). Alternatively, the precursor may be synthesized as part of a large aggregated cellular complex. The hemolysin precursor is then activated by cellular mechanisms which may require an energy source (maltose or glucose), Mg^{2+} , and reducing

conditions. Whether the activation process is due to the removal of a cellular inhibitor (87), cleavage of a masking subunit (97), or to disaggregation of an aggregate is presently speculative. The hemolytic moiety of the activated hemolysin, LACH, is then transferred to an extracellular carrier, such as RNA-core, which comes into contact with the streptococcal cell (44) (45) (100). The transfer of the hemolytic moiety probably requires neither glucose, maltose, Mg^{2+} , or reducing conditions. Alternatively, LACH or the hemolytic moiety is excreted from the cell, and is stabilized only when it is immediately protected by a substance such as RNA-core. The transferred and/or stabilized hemolysin can then bind to RBC (31) or other susceptible cells (69) (82) where it causes cytolysis, possibly by disaggregation of the membrane (39).

This scheme does not explain why Taketo and Taketo observed an increase in titer on incubation of their AS hemolysin with RNA-core (97) or why I obtained a similar increase upon incubation of SS hemolysin with RNA-core. Taketo and Taketo suggested that the increase they observed was due to release of hidden hemolysin during incubation. An alternative hypothesis is that the hemolytic moiety when bound to RNA-core has greater hemolytic potential than when bound to the cellular carrier. During the lag phase prior to hemolysis, RNA-core SLS is apparently binding to or interacting with the RBC membranes (31). The final hemolysin titer would be dependent upon the amount of hemolysin which interacts with or binds to the RBC and causes hemolysis before it is inactivated. The higher hemolytic titer would be caused by a

hemolysin stable to thermal inactivation rather than by a more labile one. One of the effects of the transfer of the hemolytic moiety from SS hemolysin to RNA-core is an increase in stability of the hemolysin. This increase in stability could then explain the increase in titer. It would not be necessary to hypothesize the expression of new hemolytic sites during incubation of SS hemolysin with RNA-core. Such a hypothesis is not supported by the experimental data which showed that precursor with a number of potential hemolytic sites was not activated by incubation with RNA-core.

The inability to obtain continued production of RNA-core SLS from cells lysed with muralysin or by sonication may be due to the absence of the proper amounts of amino acids, energy sources, or other necessary conditions. The cellular mechanisms for activation of the remaining precursor were obviously not functional under the conditions used.

The fact that very dilute solutions of RNA-core SLS are very unstable raises a question about the role of RNA-core in the stabilization of the hemolytic moiety. RNA-core SLS prepared in the resting cell system and incubated in concentrated amounts at 37C for 30 minutes retains approximately 80% of its activity. This degree of stability is slightly greater than that reported by Bernheimer (9), who used a different buffer system (1×10^{-1} M acetate, pH 6.5). When the same very high titer solutions of RNA-core SLS are diluted to 1 to 2 HU/ml, 100% of the activity is lost within 10 minutes at 37C. The RNA-core does not stabilize the hemolytic moiety in these dilute suspensions

of RNA-core SLS. This phenomenon has not been previously reported, possibly because no one has examined the behavior of very dilute RNA-core SLS preparations. One hypothesis for the fast rate of inactivation of RNA-core SLS in dilute solutions is that the hemolytic moiety is in equilibrium with RNA-core and remains active only when it can immediately reassociate with RNA-core. In solutions with a high ratio of RNA-core to hemolytic moiety -- in concentrated RNA-core SLS solutions -- the dissociated hemolytic moiety would have a high probability of being able to reassociate with RNA-core. After the dissociation of the hemolytic moiety from RNA-core in dilute solutions, the probability would be greater that the hemolytic moiety would have less chance of reassociating with RNA-core before being inactivated. This hypothesis is supported by the behavior of RNA-core SLS prepared from growing cells. The amount of RNA-core present in 8,000 to 10,000 HU of RNA-core SLS prepared from growing cells is very small. Therefore, the ratio of RNA-core to hemolytic moiety is low. When this hemolysin is diluted to 4,000 HU/ml, 40% of the hemolytic activity is lost at 4C in 24 hours. In contrast, RNA-core SLS prepared in the resting cell system, and then diluted to 200 to 400 HU/ml, is stable at 4C for days to weeks. This loss of activity after dilution may partially account for the large loss of RNA-core SLS prepared from growing cells when it is chromatographed on G-100 Sephadex. Finally, this hypothesis may explain why the RNA-core SLS preparation of Koyama and Egani (61) lost in 12 hours almost all of the hemolytic activity after the bulk of excess RNA-core had been removed during purification.

The difference in behavior of dilute solutions of SS hemolysin from those of RNA-core SLS may be due to the influence of cellular components present in the SS hemolysin suspensions. The presence of such material preventing rapid inactivation of hemolysin at 37C could explain why the product of incubation of SS hemolysin and RNA-core does not exhibit the same lability as RNA-core SLS. It is also possible that the cellular carrier influences the stability of SS hemolysin, or that the inactivation of SS hemolysin might be due to aggregation.

The behavior of dilute RNA-core SLS from the resting cell system or from growing cells is consistent with the possibility that RNA-core SLS may be an aggregation of hemolytic moiety and RNA-core [also an aggregate (57)]. The fact that RNA-core SLS from the resting cell system is not inactivated by 7 M urea and a large number of organic solvents, but is inactivated by 50% acetic acid suggests that the binding of the hemolytic moiety to RNA-core may be similar to that occurring in the aggregate of tyrocidine molecules (112). The aggregates of this peptide antibiotic are dissociated by comparable concentrations of acetic acid and NaCl. The fact that RNA-core SLS prepared from growing cells is inactivated by much smaller concentrations of acetic acid than is RNA-core SLS from resting cells is consistent with the possibility that such RNA-core is a much looser aggregate, more readily susceptible to disaggregation.

An attempt was made to demonstrate that the hemolytic moiety of RNA-core SLS was a polypeptide. Although this attempt failed, the properties and behavior of the hemolytic moiety were consistent with

a small protein or polypeptide. It presumably has peptide linkages since it is inactivated by pronase. RNA-core SLS is not denatured or inactivated by methanol, ethanol, iso-butanol, isoamyl alcohol, n-heptane, ether, chloroform, or 7 M urea which might be expected to denature a large protein. Some of the hemolysin is not precipitated by 100% SAS which is again consistent with a small protein substance. The transfer of the hemolytic moiety to RNA-core does not result in a shift in elution profile of RNA-core; the hemolysin elutes slightly before the first peak of RNA-core which might be expected for a complex of RNA-core and small protein or polypeptide.

How many of the changes in properties of the hemolytic moiety after transfer to the RNA-core are due to the affect of RNA-core is uncertain. The size change has already been discussed. The change in susceptibility to trypsin may be due to either the loss of a trypsin sensitive site of the hemolytic moiety during activation, to the destruction of cellular stabilizing material by trypsin, or to the protection of the trypsin sensitive site by the RNA-core. Taketo and Taketo (97) showed that AS hemolysin was sensitive to trypsin, but that the product of incubation of AS hemolysin and RNA-core was not sensitive to trypsin digestion. This suggests that the resistance to trypsin digestion of the RNA-core SLS is due either to the lack of susceptibility of the carrier to trypsin or the inability of trypsin to reach the trypsin-sensitive site of the hemolytic moiety. The inactivation of SS hemolysin by iso-butanol, methanol, and ether may

be due either to their effect on the cellular carrier or on the hemolytic moiety which is not as well protected as when bound to RNA-core. The fact the SS hemolysin is as stable as RNA-core SLS after chloroform extraction suggests that the effect of iso-butanol, methanol, and ether might be due to their more hydrophilic nature than chloroform. Such an effect might be due to the interaction with polar lipids or a bond between polar lipids and another substance. The observation that after treatment of precursor with ether or methanol, activated hemolysin could still be obtained suggests that the hemolytic moiety is in a protected state.

It does not appear possible to compare the properties of the cellular forms of SLS to those of other cellular hemolysins. There are little or no published data even on the presence of cellular forms of the known extracellular hemolysins. The only other hemolysin reported to have a cellular form is SLO; that observation was made during research on SLS (97).

SLS is one of several hemolysins not inactivated by SH-compounds (14). This group includes the staphylococcal α -toxin, β -hemolysin, δ -hemolysin and leukocidin, Clostridium perfringens α -toxin, Cl. hemolyticum lysin, Cl. septicum lysin, Cl. chauvoei lysin, Bacillus subtilis hemolysin, and the hemolysins of Vibrio cholerae, Escherichia coli, and species of Pseudomonas. The tendency of some of these hemolysins to aggregate has already been discussed in relationship to hemolysin precursor. Both Cl. septicum hemolysin (8) and Streptococcus zymogenes hemolysin (6) have a similar pattern of hemolysin kinetics to SLS.

There is a lag in hemolysis when low concentrations of staphylococcal α -toxins are used (4), but not when moderate or high concentrations are used (4) (25). There is no lag in hemolysis, as observed with SLS, in the hemolysis caused by E. coli hemolysin (117), staphylococcal δ -hemolysin (14), or subtilysin (18). Almost all of these hemolysins with the possible exceptions of staphylococcal δ -hemolysin (14) and subtilysin (18), differ significantly in size from RNA-core SLS. It is important to remember that the size of RNA-core SLS is dependent on the size of the RNA-core. Perhaps, the size of other hemolysins may be dependent on their association with carrier substances (56). If some of these hemolysins are found from hemolysin precursor substances, the most likely ones might be the hemolysin of Streptococcus zymogenes, the δ -hemolysin of staphylococcus, and possibly subtilysin described below.

Subtilysin (14) and hemolysin precursor share many characteristics. Besides the possibility that both aggregate, they share similar acid and heat stabilities. Both are precipitated at acid pH. There is no increase in specific activity (activatable or active) of either when chromatographed on Sephadex gels. Subtilysin is a peptide lipid, and the hemolytic moiety of SLS is presumed to be a peptide or polypeptide. They differ only in enzymatic susceptibilities to proteolytic agents and in solubility in organic solvents. However, what affect the lipid component of subtilysin and the carrier or associated material of hemolysin precursor have on the enzymatic susceptibilities and solubility of the hemolytic moieties is uncertain.

It is pertinent to discuss some of the similarities of SLS and the polypeptide antibiotics since 1) the hemolytic moiety of SLS may be a polypeptide (30) (57), 2) SLS is active against protoplasts or spheroplasts of several bacteria and is, therefore, an antibiotic in the broadest definition of the term (11), 3) there may be a similar pathologic potential for both SLS and certain polypeptide antibiotics, and 4) the form of hemolysin precursor might be related to the cellular complex for synthesis of these antibiotics. Certain of the peptide antibiotics, like Streptolysin S, are hemolytic; these include the polymyxins, the tyrocidines, and the gramicidins (51) (52) (91). The tyrocidines (8) (52) and polymyxins (91) cause rapid hemolysis of RBC while the hemolysis caused by the gramicidins is delayed (51). Like SLS, these hemolysins are inhibited by phospholipids (51) (52) (91). All are synthesized at their highest rate during late log phase prior to sporulation (64); RNA-core SLS in the HI-0.3% maltose medium is also formed maximally during late log phase of growth. These peptide antibiotics are about half the presumed size of the hemolytic moiety of SLS, but other peptide antibiotics such as nisin, synthesized by Streptococcus lactis, are approximately the same size (81). All of these antibiotics possess a cyclic structure and most, but not all, are resistant to proteases and peptidases from animal and plant sources; RNA-core SLS is susceptible to degradation by some proteases. Most of these peptides undergo molecular association. The aggregates of tyrocidine are disaggregated by a similar concentration of acetic acid and NaCl (112) to that which inactivated and presumably separated

the hemolytic moiety of RNA-core SLS from RNA-core. Like RNA-core SLS, the peptide antibiotics are usually resistant to inactivation with organic solvents; however, they also tend to be soluble in these solvents. The hydrophilic nature of RNA-core probably prevents such solubilization of RNA-core SLS. Like the hemolytic moiety of SLS, polymyxin binds to nucleic acids (91). Finally, nisin, like SS hemolysin, is sedimentable (111).

The tyrocidines, gramicidins, and polymyxins are synthesized by mechanisms not involving ribosomes or messenger RNA (81). The proposed mechanism of synthesis (64) is similar to that of fatty acid synthesis (67). Such synthesis involves a stable multienzyme complex on a cellular particle of 2.3 million molecular weight (67). The synthesis of gramicidin involves at least two enzyme complexes with a combined weight of approximately 400,000 (64). Although SLS formation is inhibited by chloramphenicol (115), an inhibitor of protein synthesis involving ribosomes, some or all of the hemolytic moiety may be synthesized by a system similar to that for gramicidin. First, chloramphenicol may inhibit formation of or otherwise interfere with, the cellular mechanisms for activation of hemolysin precursor. Second, chloramphenicol may inhibit formation of that fraction of the hemolytic moiety which depends upon ribosomal synthesis. Such may be the reason for the inhibition of nisin by chloramphenicol (53). The important point to be made is that if synthesis of the hemolytic moiety involved mechanisms similar to those of gramicidin, the hemolysin precursor may be associated with a large cellular enzyme complex. Activation by sonication could then be related to its effect on such a complex.

The fact that these polypeptide antibiotics are toxic to humans suggests possible similar potential for polypeptides produced by streptococci. Of interest is the fact that the polymyxins are very nephrotoxic (91) causing damage to the epithelial lining of the convoluted tubules of the kidney (38). RNA-core SLS also causes damage to the tubules of the kidney (101).

The pathological injuries of major importance caused by Streptococcus pyogenes infection are rheumatic fever and post-streptococcal glomerulonephritis, not renal tubular damage. The research interest in SLS as an important substance in the pathogenesis of these injuries is, however, probably at an all-time low. The decrease in SLS as an important factor in post-streptococcal glomerulonephritis is likely due to several things. First, SLS has not been shown to cause significant, prolonged damage to the renal glomerulus (39). Second, it is difficult to postulate that SLS could reach the glomerulus after production in other parts of the body, since it would avidly bind to membranes causing cytolysis or hemolysis or be inactivated at body temperature. In the instances where intraperitoneal injections of RNA-core SLS have been reported to cause renal tubular damage, sublethal, high-titer injections were required (102). It is doubtful that yeast RNA-core is present to stabilize the SLS produced during localized streptococcal infection; albumin-SLS which might be produced is much less stable than the RNA-core SLS used for injections. It is also doubtful that single injections of large doses of RNA-core SLS approximates what actually occurs during streptococcal infection. Third, SLS production does not correlate with the nephrotoxicity of certain streptococcal strains (83).

The discovery of a latent form of SLS may be of significant importance in the elucidation of the mechanism of certain events in post-streptococcal glomerulonephritis. First, the hemolysin precursor is stable at body temperature and would not be inactivated as quickly as RNA-core SLS. Second, it could reach the glomerulus without binding to erythrocytes or other cells. The previous identification of streptococcal antigens at the glomerulus (105) during infection indicates that precursor could reach the glomerulus. Third, because of its large size precursor would not be filtered by the glomerulus, but could remain at the glomerular membrane. Fourth, if the precursor was activated at the glomerular membrane, the activated hemolysin could cause direct toxic injury to both the glomerular membrane and to erythrocytes and leukocytes coming in contact with the hemolysin. It has been shown that RNA-core SLS under the proper conditions will cause some glomerular damage (102). The unverified finding of Snyder (39) that hemolysin is found in crushed rabbit kidney four days after infection of rabbits with Group A streptococci suggests that active hemolysin may indeed be present in the kidney during infection. This injury could account for the hemoglobinuria (lysed erythrocytes in the urine) and hematuria (intact erythrocytes in the urine) which herald the onset of post-streptococcal glomerulonephritis (20). It could also explain why there is evidence of direct toxic injury to the glomerulus at the time of onset of streptococcal infection in those cases in which post-streptococcal glomerulonephritis will follow (83). After infection with streptococci, certain strains of rabbits also show evidence of acute

toxic glomerular injury, and some of these rabbits develop glomerulonephritis (7). Theories based on antibody-mediated mechanisms cannot explain the early period of membrane damage but could explain the later stages of injury (23). Whether or not the antibody response is against a streptococcal component (105) or against limited areas of glomerular membrane damaged by a particulate toxin is still unproven.

There are at least two possible reasons for the lack of correlation between SLS production and nephrotoxicity of certain Group A streptococcal strains. First, the time of assay and growth conditions used may not have been optimal for SLS production. Second, hemolysin precursor, not extracellular SLS production, may be the important factor in nephrotoxicity. The presence of only minimal hemolysis surrounding colonies of some nephritogenic streptococci on blood plates may be related to decreased conversion by the streptococci of precursor to active hemolysin.

SUMMARY AND CONCLUSIONS

Streptococci grown in medium containing maltose produced high titers of RNA-core SLS during the logarithmic phase of growth. Glucose, however, repressed RNA-core SLS production during this time. The effects of maltose and glucose on hemolysin production were utilized in a growth system used to demonstrate that there were several cellular forms of SLS.

Streptococci contained a sonically-releaseable hemolysin (SS hemolysin) which had many of the characteristics of RNA-core SLS. SS hemolysin appeared to be different, though, than the hemolysin released by muralysin treatment. Part of the difference was due, however, to the presence of cellular SLO in the latter hemolysin preparation (MS preparation). After adsorption of the cell lysate with erythrocyte membranes to remove SLO, an SLS-like hemolysin, LACH, remained in the MS_m preparation. Although SS hemolysin and LACH were similar in several ways including inhibition by trypan blue, stabilization by RNA-core, and in exhibiting delayed hemolysis, they differed in sedimentability and precipitation by ammonium sulfate, and were very dissimilar in stability at 0C.

While LACH is apparently an active in vivo hemolysin, SS hemolysin is formed by the action of sonic energy on a hemolysin precursor; prolonged sonication enhances this activation. The latent hemolysin is a very large, heterogeneous substance as determined by ammonium sulfate precipitation, gel filtration, and sedimentation characteristics. It is easily denatured and tends to aggregate. Part or all of this precursor material is protein in nature since it is precipitated by both chloroform and ammonium sulfate, is inactivated by pronase and trypsin, and is not inactivated by nucleases.

There is a significant amount of cellular hemolysin precursor present. Under optimal conditions, the amount of SS hemolysin activated from hemolysin precursor is approximately equal to the amount of RNA-core SLS measurable after incubation of streptococci in the resting cell system for 1 hr at 37C.

The mechanism of activation of hemolysin precursor is unknown, but may involve disaggregation or fragmentation to smaller subunits or particles. Both sonication and homogenization activate precursor, but high speed mixing does not. The activated SS hemolysin is somewhat less dense than precursor, and has some, but not all, of the characteristics of precursor. The change in size could, however, be due to transfer of the hemolytic moiety to cellular RNA during activation. Activation is potentiated by yeast RNA-core and streptococcal and E. coli nucleic acid. While RNA may not be required for activation of all precursor material, it is essential for the activation of some. Finally, neither SS hemolysin, which is large and heterogeneous in size, nor RNA-core SLS are precipitated by chloroform, which does precipitate precursor material.

An attempt was made to determine the cellular location of the various forms of SLS, but was unsuccessful because the streptococcal protoplasts would not be stabilized. Chemical analysis did demonstrate that sedimentable hemolysin precursor was probably not associated with the cell wall.

The hemolytic moiety of LACH, SS hemolysin, and cellular hemolysin precursor are similar or the same since the hemolytic moiety of each associates with or is transferred to RNA-core under the proper

conditions. The transferred hemolysins, activated hemolysin (precursor activated in the presence of RNA-core), and extracellular RNA-core SLS all have the same molecular weight of about 19,000 as demonstrated by chromatography on G-100 Sephadex. By physical characteristics, the cellular SLS-like hemolysins appear related to extracellular RNA-core SLS.

The hemolysin precursor also appears to be the temporal precursor to RNA-core SLS. Continued RNA-core SLS production occurs after the addition of glucose to the growth medium only when cells contain hemolysin precursor. If hemolysin precursor production is inhibited by glucose prior to any cellular precursor accumulation, RNA-core SLS formation is totally inhibited.

Finally, the behavior of the hemolytic moiety of RNA-core SLS during purification procedures was consistent with its proposed peptide nature. However, after extensive purification of RNA-core SLS, there was apparently more than one peptide bound to RNA-core. The hemolysin could not be reactivated by treatment of the peptides, so whether or not one of these peptides was the hemolytic moiety of SLS could not be proven.

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