SYNERGISTIC HEMOLYSIS IN "NONHEMOLYTIC" *STAPHYLOCOCCUS* SPECIES

by

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TABLE OF CONTENTS

INTRODUCTION

It has been observed that upon re-isolation of certain hemolytic colonies from blood agar plates that these colonies fail to remain hemolytic. Usually these colonies are also coagulase negative. It has been noted that certain colonies of staphylococci appear nonhemolytic at 18 to 24 hours' incubation. Upon further incubation, these same colonies exhibit a small zone of clear hemolysis. Close examination of these plates reveals that hemolysis is seen only around one half of a colony while the red cells of the medium adjacent to the remaining half of the colony are unchanged. It was then noted that an area of apparent incomplete hemolysis or change in the red cells of the medium in some manner was partly responsible for the appearance of the peculiar hemolysis observed. This secondary zone was elaborated around a colony of staphylococci that was also surrounded by a small clear zone of hemolysis. From these observations it appeared that one substance was produced by a nonhemolytic colony and another by a hemolytic colony which were responsible for this peculiar reaction.

In the event of staphylococcal epidemics in hospitals, it is essential that the epidemiology be mapped. This peculiar hemolysis could conceivably explain some of the confusing false negative findings, when studying hemolytic staphylococci.

It was felt worthwhile to investigate the causative agents of this hemolytic phenomenon, and to discern their nature.

REVIEW OF LITERATURE

Five hemolysins of staphylococci have been studied by several workers since the early 1920's. Walbum (1922) described a hot-cold hemolysin acting

on goat cells. Bigger, Boland and O'Meara (1927) demonstrated a similar lysin acting on sheep and human cells. Glenny and Stevens (1935) confirmed this lysin and called it beta hemolysin, which they distinguished from alpha hemolysin. Certain strains of staphylococci produce a rabbit cell hemolysin, distinct from alpha hemolysin, called gamma hemolysin by Smith and Price (1938b). A fourth hemolysin acting on a variety of mammalian bloods, demonstrated by Williams and Harper (1947) was referred to as the delta hemolysin. A fifth lysin, designated epsilon by Elk and Levy (1950) is apparently only produced by coagulase negative strains. A peculiar "satellite zone" of destruction of blood cells was described as "l'hemophagie staphylococcique" by Muller (1927a), and confirmed by Burnet (1928) and Packalen (1938, 1941). There is apparently only one reference in the literature (Gray and Engley, 1961) regarding synergistic hemolysis by staphylococci.

There is no serious disagreement among the workers pertaining to the functions of the staphylococci hemolysins. However, there is confusion in the literature among authors describing similar hemolysins under different names. Most authors agree upon the existence of alpha, beta, and delta hemolysins as being separate and distinct lysins. The properties of alpha, beta, and delta hemolysins will be presented to provide a basis for comparison of the phenomenon studied in this work.

A medium composed of acid casein hydrolysate, glucose, vitamine B_1 and nicotinic acid, when incubated in the presence of CO_2 in a slowly rotating container, is capable of supporting the production of alpha hemolysin (Favorite and Hammon, 1941; Casman, 1940). Marks and Vaughn (1950) stated that alpha hemolysin is not produced anaerobically. Bryce and Rountree (1936) demonstrated that Co_2 greatly enhanced beta hemolysin production. Bigger (1933)

showed that inorganic salts in the medium have little significance. Carbon dioxide favors lysin production, but a fermentable substance such as glycerol plus a phosphate buffer in veal infusion medium with peptones added has the same effect. Also magnesium in trace amounts is needed for hemolysin production, but calcium strongly inhibits formation of hemolysins (Bigger, 1933). McIlwain (1938) also showed the inhibitory activity of calcium. He also demonstrated that the agar molecule, less the calcium, increases hemolysin production. Gladstone (1938) determined that the lack of glucose in the medium impaired hemolysin production and that the most important constituents of the medium were the five amino acids arginine, glycine, proline, valine, and phenylalanine. A medium that is suitable for alpha hemolysin production is equally as good for beta and delta hemolysins.

Each hemolysin in pure form on blood plates can be recognized by its characteristic appearance. The alpha hemolysin exhibits a zone of complete lysis which has a hazy indefinite margin around the colonies of staphylococci on sheep and rabbit blood agar (Marks and Vaughn, 1950; Elk and Levy, 1950). Alpha hemolysin lyses the red cells of rabbits and sheep (Glenny and Stevens, 1935; Elk and Levy, 1950; Marks and Vaughn, 1950; Bryce and Rountree, 1936; Williams and Harper, 1947). It does not lyse human or horse red cells (Marks and Vaughn, 1950). The alpha hemolysin is lethal to mice and causes necrosis in guinea pig skin, whereas beta hemolysin is not lethal to mice and causes a flush in the skin of guinea pigs (Glenny and Stevens, 1935). Glenny and Stevens found that some strains of staphylococci produce only alpha lysin, but none were found to produce beta without alpha, and both alpha and beta hemolysins independently produce anti-hemolysins in horses. Alpha lysin is produced in greatest amount in CO_2 (Casman, 1940), and alpha hemolysis is

inhibited when in a zone of beta lysing organisms (Williams and Harper, 1947).

Bigger, Boland and O'Meara (1927) described a hot-cold lysis in certain staphylococci. Bigger (1933) stated that the hot-cold and zone effects during lysis are due to interfering substances in an unfavorable medium, prolonged cultivation or too alkaline reaction. Levine (1938) confirmed the work of Bigger, et al., but stated that the hot-cold phenomenon is not due to the presence of "abnormal toxins" nor to interfering substances in the course of growth, but is an expression of one of the inherent properties of this particular type of hemolysis. This hot-cold hemolysis appears to be the same as the beta hemolysin of Glenny and Stevens. Strains of staphylococci which are beta hemolytic produce a maximum of hemolysin in growth conditions optimum for alpha production. Carbon dioxide greatly enhances the beta lysin production (Bryce and Rountree, 1936). However, Williams and Harper (1947) found that beta lysin is produced almost equally well in or out of CO_2.

The hemolytic zone of beta-lysing staphylococci appears as a central zone of clear hemolysis surrounded by a wide zone of darkening or of partial hemolysis. This zone of partial hemolysis turns to complete hemolysis upon cooling to room temperature or at 4°C (Williams and Harper, 1947; Marks and Vaughn, 1950; Glenny and Stevens, 1935). The beta hemolysin lyses the blood cells of sheep but not of rabbits, and it is not lethal for mice and guinea pigs (Glenny and Stevens, 1935). Smith and Price (1938a) agreed with Glenny and Stevens, but also stated that beta hemolysin is lethal for rabbits upon intravenous injection. Flaum and Forssman (1936) stated that neither alpha nor beta hemolysins were lethal for rabbits.

The above-mentioned authors agree that the beta hemolysin is more stable to heat than is alpha. Bryce and Rountree (1936) found that beta lysin activity would not diminish after 15 minutes at 60°C, and is somewhat resistant to formalin, but after loss of the hemolytic activity by formalization, it then acts as a toxoid. Smith and Price (1938a) and Kojima (1939) concur. Williams and Harper (1947) found that beta hemolysis is best produced at pH 7.6 on sheep blood plates and in tubed liquid broth. Elk and Levy (1950) stated that beta hemolysin is associated with animal strains and are less common in human strains.

Smith and Price (1938b) described a rabbit cell hemolysin, distinct from the alpha hemolysin and lethal for rabbits, but not mice or guinea pigs. They called it gamma hemolysin. The gamma hemolysin was found to lyse red cells of both rabbit and sheep. It is produced in media suitable for alpha and beta hemolysin production and is enhanced by the presence of CO_2. Gamma hemolysin is completely destroyed by heating at 55°C for 30 minutes. All of the modes of action of the gamma hemolysin are inactivated by anti-gamma hemolysin and normal serum.

Williams and Harper (1947) found evidence of a fourth hemolysin produced by staphylococci, which they called delta hemolysin. Delta hemolysin is produced only when the plates were incubated in an atmosphere containing CO_2 on a medium suitable for alpha and beta hemolysin production. They found it was not produced in strains without alpha or beta hemolysin present, nor in coagulase negative strains of staphylococci. The delta hemolysin generally produces a narrow zone of clear hemolysis with a blurred edge (Elk and Levy, 1950). The lysin is dermonecrotic and lytic for sheep, human, horse and rabbit erythrocytes (Marks and Vaughn, 1950). Williams and Harper found that rabbit, sheep, human, horse, mouse and guinea pig bloods were lysed.

Both of these authors have shown that delta hemolysin acts synergistically with beta hemolysin. Elk and Levy (1950) state that the production of delta hemolysin is closely associated with pathogenicity in both human and animal groups. They were also able to demonstrate that some strains appeared to be pure delta hemolysin producers. Marks and Vaughn (1950) described delta hemolysin as resembling the fatty acids in some of its properties, as it is thermostable and neutralized by normal serum proteins at 37°C, and by its action on the red cells belonging to a wide range of species.

Muller (1927a, b, 1929a, b) described a hemolytic peculiarity which appears as clear hemolytic spots around certain strains of staphylococci. This zone appears after five to eight days incubation on blood agar plates which contain normal serum (plasma). The hemolytic spots fail to appear on blood plates which were made with washed red cells. Muller (1927a) concluded that this hemolytic phenomenon is a result of a triggering component contained in normal serum (plasma). He suggests that the component may be of animate nature. Burnet (1928) and Packalēn (1938, 1941) confirmed Muller's observations. Packalēn suggested that the areas of secondary hemolysis are the visible manifestations of invisible virus colonies deriving from the staphylococcal mother colonies.

The discrepancies between hemolysins in culture filtrates and plate hemolysin patterns of staphylococci were traced by Elk and Levy (1954) to: (1) frequent bacterial variation in respect to alpha, beta, and delta lysin production; (2) interaction between the hemolysins and (3) differing sensitivities of the tube and plate method. The three hemolysins (alpha, beta and delta) behave as independent characters. Coagulase positive staphylococci produce all three, either independently or in combination. Any other

pattern represents a mutational loss.

Table 1 summarizes a few of the properties of alpha, beta, and delta hemolysins.

EXPERIMENTAL PART I

DEMONSTRATION AND EXAMINATION OF THE PHENOMENON OF INDUCED HEMOLYSIS IN NONHEMOLYTIC STRAINS OF STAPHYLOCOCCUS SPECIES BY HEMOLYTIC STRAINS OF STAPHYLOCOCCUS SPECIES

Materials and Methods

The phenomenon of induced hemolysis of nonhemolytic staphylococci is best observed on blood agar plates. The production of the secondary zone by hemolytic staphylococci and the effect of the zone on nonhemolytic strains were examined.

Cultures. Nineteen cultures of staphylococci, isolated from dairy products, were obtained from Professor V. D. Foltz, Department of Bacteriology, Kansas State University (Table 2). Of these, six coagulase positive cultures produced a small zone of clear hemolysis and a larger secondary zone around the colony. The remaining cultures produced no secondary zone, were nonhemolytic, and coagulase negative. Each culture was then inoculated onto proteose peptone agar slants in screw capped tubes and incubated at 37°C for 24 hours. These slant cultures were stored in the refrigerator as stock cultures. Working cultures were prepared by sub culturing from the stock cultures.

Culture Media. Proteose peptone agar was used as a base for 5 percent blood agar plates. The proteose peptone base was prepared as follows:

Table 1. Comparison of some of the properties of the alpha, beta and delta hemolysins of Staphylococcus species.

Property of hemolysin	Hemolysin			
	alpha	beta	delta	gamma
Toxoid	yes	yes	yes	yes
Species of blood lysed	rabbit, sheep	sheep	rabbit, sheep human, horse, mice, guinea pig	rabbit, sheep
Zone appearance	complete with indefinite edge	central zone of clear, wide discolored zone	narrow zone of complete, with blurred edge	clear
Synergistic or inhibited	inhibited by beta	synergistic with delta	synergistic with beta	---
Species to which lethal	mice	possible rabbit	none	rabbit
Dermonecrotic	yes	yes	yes	---
CO_2 required	enhanced	no effect or enhances	enhanced or obligate	enhanced
Produced as pure lysin	yes	yes	yes	---
Effect of cooling	none	clear lysis in discolored zone	none	none
Heat stability	labile	stable	stable	labile
Antigenic	yes	yes	yes	yes

Table 2. Characteristic of cultures used. All cultures were isolated from fluid milk and milk by-products.

Culture number	Prof. Foltz culture No.	Hemolysis	Coagulase	Mannitol	Anaerobic growth
1	400	-*	-	-	+**
2	401	-	-	-	+
3	402	-	-	-	+
4	448B4	-	-	-	+
5	449	-	-	+	+
8	450	+	+	+	+
9	463	-	-	-	+
10	493	-	-	+	+
11	497	-	-	-	+
12	500	+	+	-	+
13	503	-	-	+	+
14	822	-	-	-	+
15	826	-	-	-	+
16	840	-	-	-	+
17	863	+	+	+	+
18	868	+	+	+	+
19	882	+	+	+	+
20	898	-	-	-	+
21	905	+	+	+	+

* Negative reaction to test or observation.
** Positive reaction to test or observation.

Proteose peptone (Difco) ----------	20 gms
Sodium chloride ----------	5 gms
Beef extract (Difco) ----------	3 gms
Agar agar (Difco) ----------	20 gms
Distilled water ----------	1000 gms
Adjust the medium to pH 7.0	

Sheep, human, and rabbit blood were used in making the final medium. Proteose peptone blood agar plates were made by adding 5 percent washed and/or unwashed blood cells for plating purposes. To 95 ml of melted proteose peptone agar at 45°C was added 5 ml of sterile, defibrinated blood. The medium was mixed thoroughly and poured into petri dishes in about 12 ml amounts. The solidified medium was then ready for use.

Blood. Defibrinated sheep blood was obtained from aseptic bleeding of sheep from the jugular vein into sterile flasks containing glass beads with continuous circular shaking of the flask. Rabbit and human blood were collected using the above technique, except that a heart and arm puncture were used, respectively. Samples of all bloods collected were cultured to check the sterility of the lots. The use of blood in its normal state will be considered as unwashed, defibrinated blood, hereafter.

Washed cells were made from whole blood, using strict aseptic techniques. The volume of blood in a test tube suitable for centrifugation was marked with a marking pen or pencil. Physiological saline was then added to the tube of cells and the tube centrifuged for 15 minutes at 2,000 rpm. to pack the cells. The supernatant was removed and another volume of saline added. The tube was mixed, centrifuged and decanted. This procedure was repeated until the supernatant was clear. (Usually three washings were adequate.) Saline was then added to the tube of packed cells to bring the level of solution to the mark of the original volume of blood. This suspension of cells was then used to make 5 percent washed blood agar plates.

Plasma. The human plasma used for the coagulase test was obtained from Professor V. D. Foltz.

Coagulase Test. A loop of culture from a blood agar plate was dispersed in a test tube containing 0.5 ml plasma. The tube was placed in a 37°C water bath and observed for clot formation at 2, 4 and 24 hours. The appearance of a firm clot within 24 hours is considered positive.

Each of the nineteen cultures of staphylococci was inoculated onto whole sheep blood agar plates and incubated at 37°C. The plates were observed up to 96 hours for any hemolysis that might occur around each of the colonies. A coagulase test was run on each culture after 24 hours' incubation, and a Gram stain of each culture was made simultaneously to check the purity of the cultures.

Twelve blood agar plates were used for each hemolytic culture. Six of these plates contained washed sheep blood agar and the remainder contained unwashed sheep blood agar. Each hemolytic culture was inoculated in the center of the blood plates by filling the loop with the culture, gently touching the surface of the plate and rotating the loop in a circular manner until the loop was empty. In a circle with a radius of about 1.5 cm from this inoculum, a nonhemolytic culture was spot inoculated. Initially, each hemolytic culture was encircled by two or three nonhemolytic cultures. The nonhemolytic cultures were rotated so that each of the 13 nonhemolytic cultures was inoculated close to each hemolytic culture. All plates were incubated at 37°C and observed for induced hemolytic activity at 18, 48, 72, and 96 hours.

A series of blood plates, both 5 percent washed and unwashed sheep blood, were inoculated centrally with cultures Nos. 8, 12, 17, 18, 19, and 21. At a

point approximately 1.5 cm in a circle around the central inoculum, nonhemolytic cultures were spot inoculated. The nonhemolytic cultures were rotated so that each culture was incubated in close proximity to each hemolytic culture. These plates were incubated in a 10 percent CO_2 atmosphere at 37°C for 72 hours, at which time the presence of the secondary zone encircling hemolytic colonies and induced hemolysis of nonhemolytic cultures were noted. Any effect of CO_2 was noted.

To observe the phenomenon of induced hemolysis of nonhemolytic cultures on other than sheep blood agar, washed and unwashed rabbit and human blood were used for preparing blood plates. These plates were inoculated and incubated at 37°C for 24, 48, and 96 hours to demonstrate induced hemolysis of nonhemolytic cultures as described for sheep blood plates.

To obtain evidence that the substance in the secondary zone is soluble, a small piece of agar (1 sq. cm) was taken from the secondary zone of each hemolytic culture and transferred to another blood agar plate. A nonhemolytic culture was inoculated at four points, varying in distance, around the square of agar. A second square of agar was placed on another blood plate to serve as a sterility control. The plates were incubated at 37°C and observed at 24 and 48 hours for induced hemolysis of the colonies.

To see if the agent responsible for the secondary zone was produced on other than blood agar plates, each hemolytic culture was centrally inoculated onto Staphylococcus Medium No. 110 plates and onto nutrient agar plates. These plates were incubated at 37°C. Blocks of these media were transferred to blood agar plates as described above and observed for 24, 48, and 72 hours for the appearance of a secondary zone around the transplanted agar squares, and for an induced zone of clear hemolysis around the nonhemolytic colonies.

The 11 nonhemolytic colonies which became hemolytic when allowed to grow in contact with the secondary zone of a hemolytic colony were transferred to blood agar plates to determine whether they would remain hemolytic. These 11 cultures were serially transferred to new blood plates after 24 hours' incubation for a total of four transfers.

Coagulase tests were run on each of the 11 nonhemolytic cultures at the time each showed induced hemolysis. The coagulase test was repeated on each culture as it lost its hemolytic activity upon repeated transfers.

Nonhemolytic strain No. 5 was inoculated onto blood plates and the remainder of the nonhemolytic cultures were inoculated around No. 5 in groups of three cultures not more than 4 mm apart. The cultures were rotated so that each culture would be incubated close to each other culture. The plates were incubated at 37°C and observed up to 96 hours for hemolysis, which would be induced by two or more nonhemolytic cultures.

An attempt to show a possible importance of the presence of these two factors on a diagnostic blood plate was undertaken. Five one-hundredths ml and 0.1 ml of a mixture of one loop of a hemolytic and nonhemolytic broth culture in 3 ml of sterile broth was placed on separate blood plates. An L-shaped sterile glass rod was used to spread the inoculum over the entire surface of the plates. The plates were incubated at 37°C for 24 hours and observed for any hemolysis. An area of 2 sq. cm was marked off on the bottom of the plates prepared with the 0.05 ml inoculum. All colonies in this 2 sq. cm area were numbered and subcultured. Each of these colonies was then tested for hemolysis on sheep blood agar plates and each was checked for coagulase activity.

Results

Six of the 19 cultures (Nos. 8, 12, 17, 18, 19, and 21) that were inoculated onto whole sheep blood agar plates were hemolytic, producing a small zone of clear hemolysis 1 to 2 mm outward from the edge of the colony. All these cultures except No. 12 exhibited a larger secondary zone of change in the red blood cells. This secondary zone extended outward from the colony in concentric rings (Fig. 1) with a radius of approximately 1 to 1.5 cm after three days' incubation on washed sheep blood. These six cultures were coagulase positive (Table 3).

The remaining 13 cultures were nonhemolytic and coagulase negative. By Gram stain all 19 cultures tested were morphologically pure cultures.

Induced hemolysis of nonhemolytic colonies by the secondary zone produced by hemolytic colonies was observed. At 18 hours' incubation, there was a small zone of clear hemolysis around each hemolytic culture. There was a larger secondary zone extending outward, as shown in Fig. 1, from all but hemolytic culture No. 12. The "nonhemolytic" cultures were nonhemolytic. However, the secondary zone had extended outward far enough to be within a few mm of a nonhemolytic culture, and at this point (Fig. 2), there was a half moon of clear hemolysis. Upon further incubation, the secondary zone continued to move closer to the nonhemolytic culture. The closer the secondary zone moved to the "nonhemolytic" colony, the larger the zone of clear hemolysis became around the nonhemolytic colony. At 96 hours' incubation the secondary zone had encompassed the nonhemolytic colony, producing a clear zone of hemolysis around many of the 13 cultures (Table 4). Some of the nonhemolytic cultures (52%) were induced to show hemolysis; others (48%) were not induced to show hemolysis.

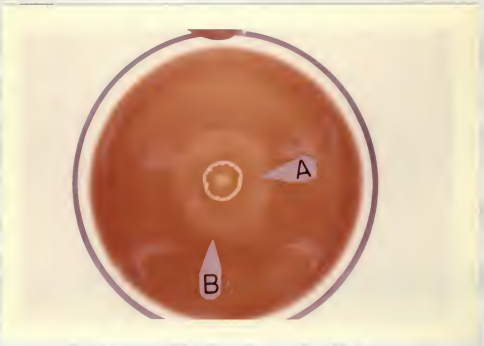

Fig. 1. Staphylococcus species spotted on washed sheep blood plate after 96 hours' incubation, showing secondary zone production. A small zone of clear hemolysis is seen immediately around the central colony. The color of the red cells seems to be more intense in the secondary zone (A), at the periphery of the secondary zone (B) and at the junction of the two zones.

Fig. 2. Induced hemolysis of a nonhemolytic Staphylococcus species colony (pointer) by the secondary zone of a hemolytic Staphylococcus species after 18 hours' incubation. The nonhemolytic colony at 4 o'clock shows the very first signs of induced hemolysis and the third colony at 8 o'clock has not been induced at this incubation time.

Table 3. Hemolytic and coagulase activity of Staphylococcus species tested.

Culture	Hemolysis	Coagulase
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
8	+	+
9	-	-
10	-	-
11	-	-
12	+	+
13	-	-
14	-	-
15	-	-
16	-	-
17	+	+
18	+	+
19	+	+
20	-	-
21	+	+

As the secondary zone approaches a nonhemolytic colony and then surrounds the colony, one sees that the clear hemolysis that occurs (induced hemolysis) always begins at a distance away from the nonhemolytic colony first (Fig. 2). This induced hemolysis progresses toward and encircles the nonhemolytic colony only as the secondary zone moves toward and around the colony (Fig. 3). This induced hemolysis is confined to the area of the secondary zone around a nonhemolytic colony. The appearance of induced hemolysis in this manner suggests that there is a combining of two factors, one from each culture. By themselves the factors can not cause hemolysis, but when together they can and do cause hemolysis. Also as hemolysis always occurs away from the nonhemolytic colony first, and in the medium, it would suggest that each

Table 4. Production of induced hemolysis by nonhemolytic cultures after contact with the secondary zone produced by hemolytic cultures.

No. of nonhemolytic culture	Hemolytic culture No.					
	8	12	17	18	19	21
1	+*	-**	+	+	+	+
2	+	-	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	-	+	+	+	+
9	-	-	-	-	-	-
10	+	+	+	+	+	+
11	-	-	-	-	-	-
13	-	+	-	-	-	-
14	-	-	+	+	+	+
15	-	-	-	-	-	-
16	-	-	-	-	+	-
20	-	-	+	-	-	-

* Positive showing induced hemolysis of the nonhemolytic colony after it has incubated in contact with the secondary zone of hemolytic cultures.

** Negative, showing no induced hemolysis.

substance is soluble.

The results of induced hemolysis of nonhemolytic cultures on plates incubated under 10 percent CO_2 are presented in Table 5. The secondary zone from hemolytic cultures appeared smaller at 72 hours under CO_2 than when incubated aerobically for the same time. There was no clear zone of hemolysis seen around the hemolytic colonies as contrasted by the same cultures on aerobic plates. Cultures Nos. 9 and 15 did not show any induced hemolysis; however, all other nonhemolytic cultures showed induced hemolysis when in contact with the secondary zone of the hemolytic cultures.

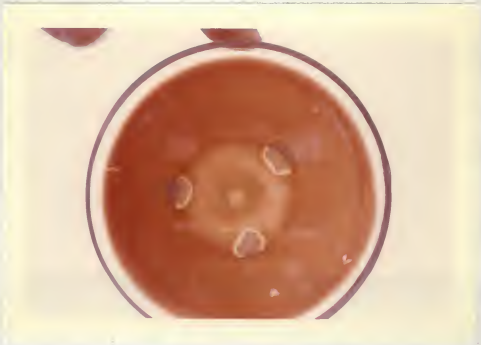

Fig. 3. Induced hemolysis of a nonhemolytic Staphylococcus species by the secondary zone of a hemolytic Staphylococcus species after 72 hours' incubation. Note the clear zone of induced hemolysis around the inner periphery as contrasted by no hemolysis seen around the outer periphery of the nonhemolytic colonies.

Table 5. Production of induced hemolysis on blood plates incubated in an atmosphere of 10 percent CO_2 of nonhemolytic cultures after contact with the secondary zone produced by hemolytic cultures.

No. of nonhemolytic culture	Hemolytic culture No.					
	8	12	17	18	19	21
1	+*	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+
9	-**	-	-	-	-	-
10	+	+	+	+	+	+
11	-	-	-	-	+	-
13	+	+	-	-	-	-
14	+	-	+	+	+	+
15	-	-	-	-	-	-
16	+	-	-	-	+	-
20	-	-	+	+	+	-

* Positive, showing induced hemolysis of the nonhemolytic colony after it has incubated in contact with the secondary zone of hemolytic cultures.

** Negative, showing no induced hemolysis.

The appearance of induced hemolysis on rabbit and human blood plates varies from the results seen when using sheep blood plates. There was no evidence of the production of the secondary zone by hemolytic cultures when using rabbit blood. However, all cultures, hemolytic and nonhemolytic, except No. 20, showed some degree of clear hemolysis around the colony on rabbit blood. The secondary zone did appear around five of the hemolytic cultures on human blood. However, this zone was smaller and slower in appearing than on sheep blood and there was no zone of clear hemolysis seen around these colonies. The "nonhemolytic" cultures on human blood exhibited a clear zone of hemolysis around seven of the 13 cultures at 24 hours' incubation and before the secondary zone had approached the colony. The remaining

six cultures did not show induced hemolysis after contact with the secondary zone at 96 hours' incubation.

The coagulase activity of these cultures after 24 hours' incubation on rabbit and human blood plates remained as originally described for the cultures. Table 6 shows the comparison of these properties as seen on human, rabbit and sheep blood agar plates.

Transplanted squares of agar from the secondary zones of hemolytic Staphylococcus species colonies to a new blood plate produced the secondary zone around the squares of agar. At 24 and 48 hours' incubation, the control showed no contamination. The control and the test squares of agar were both encircled by the secondary zone from which the agar squares came. This secondary zone is identical to the secondary zone produced by the hemolytic culture. There appeared a zone of clear hemolysis around each of the "nonhemolytic" colonies next to the agar square. The clear hemolysis was contained in the boundaries of the secondary zone that appeared outward from the agar squares and around the nonhemolytic colonies. The induced hemolysis is the same as seen when a hemolytic and nonhemolytic culture are on the same blood plate. It is presumed from this test that the substance produced by the hemolytic culture is soluble, as it diffuses through the medium and retains its ability to cause induced hemolysis of nonhemolytic cultures.

Transplanted squares of agar adjacent to colonies of Staphylococcus species on Staphylococcus Medium No. 110 and nutrient agar plates were transplanted to new blood agar plates to see if the agent responsible for the secondary zone is produced when the cultures are cultivated on other than blood agar.

Table 6. Comparison of staphylococci which produced hemolysis and secondary zones on sheep blood agar when inoculated onto rabbit and human blood agar plates.

Culture No.	Clear hemolysis			Secondary zone			Coagulase		
	Sheep	Rabbit	Human	Sheep	Rabbit	Human	Sheep	Rabbit	Human
1	-*	+**	+***	-	-	-	-	-	-
2	-	+	+	-	-	-	-	-	-
3	-	+	+	-	-	-	-	-	-
4	-	+	+	-	-	-	-	-	-
5	-	+	+	-	-	-	-	-	-
8	+	+	-	+	-	+	+	+	+
9	-	+	-	-	-	-	-	-	-
10	-	+	+	-	-	-	-	-	-
11	-	+	-	-	-	-	-	-	-
12	+	+	-	-	-	-	+	+	+
13	-	+	-	-	-	-	-	-	-
14	-	+	-	-	-	-	-	-	-
15	-	+	-	-	-	-	-	-	-
16	-	+	-	-	-	-	-	-	-
17	+	+	-	+	-	+	+	+	+
18	+	+	-	+	-	+	+	+	+
19	+	+	-	+	-	+	+	+	+
20	-	-	-	-	-	-	-	-	-
21	+	+	+	+	-	+	+	+	+

* Denotes a negative reaction.
** Denotes a positive reaction.
*** The secondary zone appeared much slower on human blood agar agar plates than on sheep blood agar plates.

The secondary zone appeared on the incubated blood plates onto which squares of medium from nutrient agar and Staphylococcus Medium 110 plates were transferred. The secondary zone appeared identical with those described on blood agar plates in that nonhemolytic cultures became hemolytic upon contact with the secondary zone, and that induced hemolysis of nonhemolytic cultures occurred identically with the results seen in Table 4. This suggests that the necessary substances, when in contact with one another, cause hemolysis and are not dependent upon blood agar for their origin. The agents are produced by certain strains of hemolytic and nonhemolytic staphylococci under optimum growth conditions and appear to be products of metabolism with these strains.

All 11 of the nonhemolytic cultures which showed induced hemolysis upon contact with the secondary zone remained hemolytic for two transfers on blood agar plates. At the third transfer, all cultures but No. 1 had lost the induced hemolytic activity; culture No. 1 was nonhemolytic at the fourth transfer. The results in Table 7 indicate that the induced hemolysis acquired by nonhemolytic cultures is not a dominant nor stable genetic phenomenon, as hemolysis is lost after four transfers in all cultures tested. This newly acquired property appears to be a transient property that is easily diluted from the culture population upon successive cellular division.

Coagulase tests run on the 11 nonhemolytic cultures after induced hemolysis occurred, and while this property was disappearing on repeated transplanting of the culture to blood agar plates, revealed no correlation between coagulase and hemolysis. However, cultures Nos. 11, 13, and 20, which were coagulase negative before induced hemolysis occurred, were coagulase positive when the culture showed induced hemolysis. Upon the loss of the newly acquired

hemolytic property, the cultures remained coagulase positive (Table 8). The remaining 10 cultures tested showed no change in their coagulase ability with the changing hemolytic property. This would suggest that no correlation exists between the two properties, coagulase and hemolysis.

Table 7. Comparison of stability of induced hemolytic activity of nonhemolytic cultures on repeated transfers of the culture.

Nonhemolytic culture No.	Number of transfers			
	1	2	3	4
1	H*	H	H	NH**
2	H	H	NH	NH
3	H	H	NH	NH
4	H	H	NH	NH
5	H	H	NH	NH
9	NH	NH	NH	NH
10	H	H	NH	NH
11	H	H	NH	NH
13	H	H	NH	NH
14	H	H	NH	NH
15	NH	NH	NH	NH
16	H	H	NH	NH
20	H	H	NH	NH

* Hemolysis.
** No hemolysis.

When two or more nonhemolytic colonies were inoculated 2 to 4 mm apart on a blood plate and incubated at 37°C for 96 hours, no hemolysis was seen around any of the nonhemolytic groups. This lack of hemolysis shows that the necessary substances are not produced by two nonhemolytic cultures. But one must have present the substance from a hemolytic culture, plus that of a nonhemolytic culture to induce clear hemolysis by nonhemolytic cultures.

All of the colonies on the blood plate which was inoculated with 0.05 ml of a mixture containing hemolytic and nonhemolytic Staphylococcus appeared hemolytic as seen in Fig. 4. Of the 400 hemolytic colonies picked from the

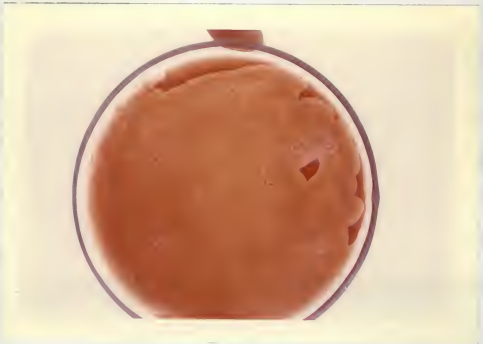

Fig. 4. A washed sheep blood agar plate showing hemolysis around every colony of Staphylococcus species. Of 400 colonies taken from this plate and studied in pure culture, 280 were coagulase negative and non-hemolytic species of staphylococci. The remaining 120 were coagulase positive and hemolytic species of staphylococci.

Table 8. Comparison of the coagulase activity of "nonhemolytic" cultures with the induced hemolytic change of the culture.

Nonhemolytic culture No.	Coagulase before induced hemolysis	Coagulase after induced hemolysis	Coagulase after loss of hemolysis
1	-*	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
9	-	-	-
10	-	-	-
11	-	+**	+
13	-	+	+
14	-	-	-
15	-	-	-
16	-	-	-
20	-	+	+

* Negative.
** Positive.

2 sq. cm area on the blood plate in Fig. 4, 280 (70%) were nonhemolytic on blood agar plates and were coagulase negative when tested in pure culture. The remaining 130 (30%) colonies were hemolytic on blood agar plates and were coagulase positive.

EXPERIMENTAL PART II

THE SYNERGISTIC HEMOLYSIS TEST AND ITS USE IN THE GENERAL CHARACTERIZATION OF THE HEMOLYTIC AND NONHEMOLYTIC SUBSTANCES THAT CAUSE INDUCED HEMOLYSIS IN NORMALLY NONHEMOLYTIC CULTURES

Materials and Methods

For brevity in writing, hereafter, the hemolytic substance causing the secondary zone around hemolytic colonies will be referred to as factor Z.

The nonhemolytic counterpart produced by nonhemolytic cultures will be referred to as factor T.

To determine the properties of factors Z and T, it was necessary to obtain the factors in solution from cultures and to devise a test to show the presence of these factors. Such a test was devised, using the lysis of sheep red cells as an indicator.

Cultures. The same cultures are used in Part II as were used in Experimental Part I.

Culture Media. Tryptone broth was used as the medium for the production and collection of factors Z and T in solution. Tryptone broth was prepared as follows:

Bacto Tryptone	15.0	gms
Nicotinic acid	0.00123	gms
Thiamine hydrochloride	0.00005	gms
Sodium chloride	8.5	gms
Glucose	2.5	gms
Distilled water	Q. S. 1000	ml
Adjust to pH 7.2		

The medium was tubed in 4 ml quantities in screw-capped tubes, sterilized at 121°C for 15 minutes, and stored in the refrigerator until ready for use.

Sheep Cells. Washed 5 percent sheep red cell suspensions were used as an indicator to test for the presence of factors Z and T. Sheep red cells were prepared by washing the cells with 0.85 percent saline until the supernatant was colorless. Usually three washings were adequate. The washed cells were made to the original volume with saline. This suspension was then diluted properly to obtain the 5 percent concentration of washed blood cells for testing purposes. (This is actually about a 2.5 percent suspension of packed red cells.)

Preparation of Cultures. The cultures were inoculated into the tubed tryptone broth. The inoculated tubes were incubated at 37°C in an atmosphere

of 10 percent CO_2 and 90 percent air for five days. Large metal anaerobic jars were used as containers for incubation. The inoculated tubes were slanted and the caps were loosened to allow maximum gas exchange.

Preparation of the Supernatant. Each broth culture was centrifuged at 2600 rpm. in a refrigerated centrifuge set at 10°C for 40 minutes. The cells and supernatant were separated by using a pulled glass pipet with a suction bulb attached. The supernatants were placed in 15 ml sterile bottles, labeled and placed in the refrigerator for storage.

Detection of Factors Z and T in Supernatant. It was suggested earlier in this work that factors Z and T are soluble. If this be true, then the two factors, when combined, will cause lysis of red cells in a tube. Using this hypothesis a test was devised to check for the presence of factors Z and T by a tube hemolysis test as follows: four test tubes were labeled 1 through 4. Tube 1 was the test and tubes 2, 3, and 4 were controls for lysing activity of factor Z alone, lysing activity of factor T alone, and red cell stability, respectively. To tube 1 was added 0.5 ml of supernatant containing factor Z and 0.5 ml of supernatant containing factor T. To tube 2 was added 0.5 ml of supernatant containing factor Z. To tube 3 was added 0.5 ml of supernatant containing factor T. To tubes 2 and 3 were added 0.5 ml of sterile tryptone broth, and 1.0 ml of tryptone broth was added to tube 4. To all four tubes was added 0.5 ml of 5 percent suspension of washed sheep red cells. Hereafter, this test will be referred to as the synergistic hemolysis test (SH).

Six replications of the SH test were set up using supernatants prepared under aerobic conditions. Each set of tests was placed in a 37°C water bath and a timer started. One set of tests was removed from the water bath at 10

minute intervals up to one hour. Lysis of the red cells was noted in tube 1, and the control tubes (2 to 4) were observed for any change in the red cell appearance. The test is positive (factors Z and T are present) if lysis occurs in tube 1 while tubes 2, 3, and 4 remain unchanged.

To determine any effects of a CO_2 atmosphere on the production of factors Z and T, two cultures, one producing factor Z and the other factor T, were inoculated into tryptone broth and incubated in an anaerobic jar with a lighted candle. The jar was sealed and thus resulted approximately a 10 percent CO_2 atmosphere. A second inoculum of the same cultures was incubated in a similar jar which contained a measured amount of sodium bicarbonate. Sulfuric acid was added to the sodium bicarbonate and the jar sealed, giving a 20 percent CO_2 atmosphere. At the end of the incubation period, the cultures were removed and the supernatants prepared and labeled. The SH test was run on each supernatant to determine the lysing activity of the factors Z and T.

The supernatants of each hemolytic culture were tested with the supernatants of each of the nonhemolytic cultures to determine the presence of factors Z and T, respectively. The SH test was used and controlled for lysing activity of factor Z, factor T, and for red cell stability. This procedure was repeated three times.

To determine if a mixture of two supernatants containing the same factor could stimulate lysis, the SH test was performed. Two factor Z supernatants were put together in the SH test and the lysing activity noted. Two factor T supernatants were tested in the same manner. Both tests were controlled as described previously for the SH test.

To determine the relative amounts of factors Z and T produced, the supernatants were titrated by a serial dilution method. A series of twelve test tubes were set up in a rack in duplicate. To all tubes was added 0.25 ml of 0.85 percent saline. To tube 1 of one series of test tubes was added 0.25 ml of supernatant containing factor Z. To tube 1 of the second series of tubes was added 0.25 ml of supernatant containing factor T. Both series of tubes were serially diluted by mixing thoroughly tube 1 and transferring 0.25 ml to tube 2 and so on, discarding 0.25 ml from tube 10. Tubes 11 and 12 were controls for factor activity and red cell stability, respectively. To the two series of tubes was added 0.5 ml of supernatant containing the opposite factor. To all tubes was added 0.5 ml of a 5 percent suspension of red cells. The tube of greatest dilution showing complete lysis of the red cells is the titer. The dilutions of the tubes were 1-2, 1-4, 1-8, 1-16, etc. up to 1-1024 in tube 10.

The effect of temperature of incubation on the production of the two factors was determined. One tube of tryptone broth was inoculated with a hemolytic culture and one tube of tryptone broth was inoculated with a non-hemolytic culture for each temperature of incubation. One tube each of the inoculated cultures was incubated under a 10 percent CO_2 atmosphere at 11°C, 20°C, 25°C, and 28°C for five days. Factor production at 37°C had been previously determined. The supernatants were prepared as before, labeled and kept separate. The SH test was used to determine if the factors Z and T were produced, by testing each supernatant at each temperature level for factor Z with a supernatant known to contain factor T. Each supernatant was then tested for factor T by using a supernatant known to contain factor Z. Each factor was controlled for lysing activity and the red cells were

controlled.

The heat stability of factors Z and T was determined by placing supernatants containing these factors into 56°C and boiling water baths. Into each of 2 test tubes was placed 0.5 ml supernatant containing factor Z. Into another set of test tubes was placed 0.5 ml of supernatant containing factor T. All four tubes were put into a 56°C water bath for 30 minutes, then removed and cooled to room temperature. The SH test was run on each set of tubes. To each tube No. 1 of the SH test was added 0.5 ml of the untreated opposite factor (i.e., factor T added to factor Z). The remaining second tube of each factor served as controls for factor lysing activity. The untreated factor added to tube 1 was also controlled for lysing activity. Red cells were added to all tubes and each tube checked for immediate lysis of the red cells.

Into each of 4 test tubes was added 0.5 ml supernatant containing factor Z and into another set of tubes was added supernatant containing factor T. All tubes were placed in a boiling water bath. Two tubes containing each factor were removed from the water bath after 5 minutes. The remaining tubes were removed after 15 minutes in the water bath. The corresponding untreated opposite factor was added to one of the tubes at 5 minutes and 15 minutes after they had cooled. The second tube at each time interval was the control tube for factor lysing activity. The tests were controlled as above. Red cells were added to each tube and observed for immediate lysis.

Tryptone broth medium was adjusted to pH 7.2 before inoculation with the hemolytic and nonhemolytic cultures. The pH of the supernatant of each culture was determined with bromthymol blue indicator. The difference of pH of the medium before and after culture growth was noted.

To observe any effect of initial pH in the production of factors Z and T, media were prepared with pH adjusted to pH 6.4, 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8. A hemolytic culture was inoculated into one tube of medium at each pH and a nonhemolytic culture was inoculated into a second tube of medium at each pH. All tubes were incubated at 37°C for five days in a 10 percent CO_2 atmosphere. After incubation the tubes were removed and the supernatant of the cultures at each pH were prepared. Each supernatant was adjusted to pH 7.2 to ensure red cell stability. As needed dilute sodium hydroxide and hydrochlorid acid were used to neutralize the supernatants. The SH test was run on each supernatant. Untreated factor T was added to supernatants of hemolytic cultures and untreated factor Z was added to supernatants of nonhemolytic cultures. The tests were controlled for treated and untreated factor lysing activity and for red cell stability. The lysing activity of each test was noted.

The factors Z and T were treated with a change in pH to observe any effect on their lysing activity. Supernatants containing factor Z were distributed in 1 ml aliquots into each of eight test tubes. This procedure was repeated with a supernatant containing factor T. A tube of each supernatant was adjusted to pH of 5.0, 5.5, 6.0, 6.5, and 7.0 with hydrochloric acid and to pH 7.5, 8.0, and 8.5 with sodium hydroxide. The tubes were then allowed to stand for three hours, at which time each tube was adjusted to pH 7.2. The SH test was run on each tube. The corresponding opposite untreated factor was added to the proper tube of treated supernatant. The test was controlled for factor activity using supernatant from the treated tubes, and from untreated tubes. The red cell stability was determined by using uninoculated medium which was similarly treated, to observe any effect that

might be due to changing of pH, and untreated medium as normally employed in the SH test. Lysis of the red cells was observed in each tube.

Saturated sodium sulfate was added to supernatants containing factor Z, to supernatants containing factor T, and to tryptone broth, to see if the factors would precipitate or remain in solution. To 0.5 ml of supernatant containing factor Z in a test tube was added 7.5 ml of saturated sodium sulfate. Supernatant containing factor T was similarly treated. The tubes were centrifuged to separate the precipitate from solution. The SH test was run on the protein-free supernatant and on the resuspended and dissolved (in saline) proteins in the precipitates of the treated T and Z supernatants. The untreated opposite corresponding factor was added to tube 1 in the SH test. The test was controlled for factor lysis in each case and for red cell stability.

To 0.5 ml of supernatant of each factor was added 7.5 ml of saturated ammonium sulfate to cause precipitation of the proteins. The tubes were mixed and centrifuged, and the supernatant and precipitate separated. The SH test was run on the supernatant and protein fraction of each factor. The tests were controlled for Z and T lysing activity in each fraction and for red cell stability. The reason for using saturated ammonium sulfate in addition to the sodium sulfate procedure is that saturated ammonium sulfate has a greater ionic strength than does saturated sodium sulfate and a larger spectrum of proteins will be precipitated.

The surface tension was determined on supernatants containing factor Z, factor T, a mixture of factors Z and T (50-50), on the media, and on distilled water. The DuNouy Tensiometer was used to measure the surface tensions. The Tensiometer was calibrated with 500 mg and 250 mg weights to determine the mg per division on the scale. The samples were then placed in the Tensiometer

and the surface tension tested several times, until a constant reading was obtained. The surface tension was then calculated for each sampling by the following equation:

$$\gamma = \frac{f}{4R} F$$

f - maximum force registered on the torsion-balance scale.
F - correction factor due to shape of liquid held up; this was determined as 0.990 from a table of correction factors.

The radius (R) of the ring used was calculated from the given circumference of the ring (4.00).

Results

Upon removal of the SH test from the 37°C water bath at 10 minute intervals to determine the presence of factor Z and T in the supernatants as shown by lysis of red cells in tube 1, the control tubes showed no apparent change in the red cells at any of the time intervals. These tubes were centrifuged and the supernatant of all the control tubes was clear and showed no coloring from lysed red cells.

Lysis was observed in tube 1 of the SH test in all six time intervals upon removal from the 37°C water bath. After 10 minutes some lysis was seen. Maximum lysis, but not complete lysis, was observed at the 20 and 30 minute tests and continued to 60 minutes. Table 9 compares the degree of lysis at the time the tests were removed from the water bath.

Immediate and complete lysis was seen upon the addition of the red cell suspension to the SH test on the supernatants incubated under a 10 percent CO_2 atmosphere (Fig. 5). Immediate and complete lysis was also seen on supernatants produced under a 20 percent CO_2 atmosphere. The controls for

factors Z and T and red cell stability remained unchanged, showing no lysis.

Table 9. The time needed at 37°C for the appearance of red cell lysis by factors Z and T in supernatants from aerobic cultures.

Tube and contents	Time in minutes					
	10	20	30	40	50	60
1 (Factor Z + T)	++*	+++	+++	+++	+++	+++
2 (Factor Z)	-**	-	-	-	-	-
3 (Factor T)	-	-	-	-	-	-
4 (Red cells)	-	-	-	-	-	-

* Lysis of red cells occurred, the number of + indicates the approximate degree of lysis, ++++ is complete lysis.
** No lysis observed.

The effect of CO_2 atmosphere on the lysing activity of combined factors Z and T is shown in Table 10. The lysing activity of both factors is enhanced when the cultures are incubated under a CO_2 atmosphere.

Table 10. Comparison of aerobic and CO_2 atmospheres during incubation upon the lysing activity of factors Z and T.

Atmosphere at incubation	Degree of lysis
Aerobic	Incomplete at 20-30 minutes
10% CO_2	Immediate and complete
20% CO_2	Immediate and complete

In testing each hemolytic supernatant with each nonhemolytic supernatant the controls remained unchanged in all cases, showing that factors Z and T alone are not capable of lysing red cells in the tube and that the red cells are stable. The results of the three replications of the SH test on these supernatants are identical. Immediate and complete lysis of the red cells

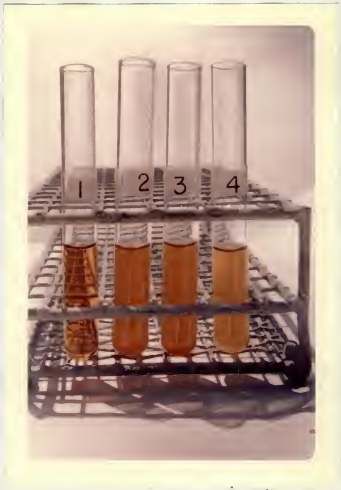

Fig. 5. Synergistic hemolysis test showing complete lysis of 5 percent washed sheep blood cells in tube 1. Tube 1 contains factor Z (hemolytic supernatant) and factor T (nonhemolytic supernatant). Tubes 2, 3, and 4 are controls containing factor Z, factor T, and red cells, respectively.

is seen in tube 1, which contains the supernatants of factor Z plus factor T. In every case, lysis is seen in 100 percent of the 78 combinations of the supernatants as shown in Table 11.

Table 11. Induced hemolysis observed by using supernatants of hemolytic broth cultures with supernatants of non-hemolytic broth cultures using the SH test.

No. of non-hemolytic culture	Hemolytic culture No.						Controls*		
	8	12	17	18	19	21	Z	T	RBC
1	+**	+	+	+	+	+	-***	-	-
2	+	+	+	+	+	+	-	-	-
3	+	+	+	+	+	+	-	-	-
4	+	+	+	+	+	+	-	-	-
5	+	+	+	+	+	+	-	-	-
9	+	+	+	+	+	+	-	-	-
10	+	+	+	+	+	+	-	-	-
11	+	+	+	+	+	+	-	-	-
13	+	+	+	+	+	+	-	-	-
14	+	+	+	+	+	+	-	-	-
15	+	+	+	+	+	+	-	-	-
16	+	+	+	+	+	+	-	-	-
20	+	+	+	+	+	+	-	-	-

* Controls; Z, control for lysis of red cell by factor Z alone. T, control for lysis of red cell by factor T alone. RBC, red cell stability control in the SH test.
** Immediate and complete lysis of red cells in the SH test; both factor Z and T must be present for this reaction.
*** No lysis of red cells observed.

Two supernatants, each containing the same factor, were put into tube 1 of the SH test to see if this combination could stimulate red cell lysis. There was no lysis of the red cells observed in the SH test containing mixtures of the same factor from different cultures. The two factors (Z plus Z, or T plus T) are not additive.

Titration of the supernatants Z and T was performed by serial dilution of the supernatants to determine the concentration of the respective agents

in the broth cultures. The titer of supernatant containing factor Z is 1-512, represented by complete lysis of red cells in tube 9, but not in tube 10. The titer of supernatant containing factor T, by similar determination, was 1-256.

The effect of temperature of incubation is seen by complete lysis of the red cells in tube 1 of the SH test in supernatants of hemolytic cultures, which had incubated at 20°C, 25°C, and 28°C. Complete lysis was also seen in supernatants of nonhemolytic cultures incubated at the same temperatures. This shows that factor Z and factor T, respectively, are produced between 20°C and 28°C (production of the factors at 37°C had previously been established). No lysis of red cells was observed in the SH test from any supernatant of cultures which were incubated at 11°C. Controls for lysing activity of each factor and red cell stability remained unchanged upon the addition of the red cells. Table 12 summarizes the effect of incubation temperature on the production of factor Z and T.

To observe if either factor Z or T is inactivated by elevated temperature, supernatants of these factors were placed in 56°C and 100°C water baths. The tubes were removed from the 56°C water bath after 30 minutes. The SH test performed on each of the supernatants and complete lysis of the red cells was seen in tube 1, containing treated factor Z and treated factor T tested separately in tube 1. The controls remained unchanged.

Table 12. Induced hemolysis observed in supernatants of hemolytic and nonhemolytic cultures incubated at different temperatures.

Factor being detected plus untreated opposite factor	Incubation temp. to produce factor				
	11°C	20°C	25°C	28°C	37°C
Factor Z	-*	+**	+	+	+
Factor T	-	+	+	+	+

* Factor not produced.
** Factor produced.

Upon removal of the tubes containing factor Z after 5 minutes and 15 minutes in the boiling water bath the SH test was performed. Tube 1 of the SH test showed immediate and complete lysis of the added red cells; the controls were unchanged. Upon removal of tubes containing factor T from the boiling water bath, complete and immediate lysis was also seen after 5 and 15 minutes. The controls remained unchanged after addition of red cells. This shows that factors Z and T are stable to heating to 56°C for 30 minutes, and to 100°C for 15 minutes. This would suggest that the two factors are not protein in nature, nor their action enzymatic.

The change in the pH of the medium as a result of growth of the organisms was determined to see if there was sufficient change in pH to cause lysing of the red cells. The average pH of the supernatant from hemolytic cultures was determined to be pH 7.0, which is a drop of 0.2 from the original pH of the medium. There was a spread of pH from 6.8 to 7.1 in the six hemolytic supernatants. The average pH of nonhemolytic supernatants was pH 6.8, which is a drop of 0.4. The pH spread in the nonhemolytic supernatants was from 6.6 to 7.0. The drop in pH as seen in the supernatants above does not appear to be responsible for the lysing of red cells in the SH test. If lysis were caused by lowering the pH of the medium, then lysis would be observed in the control tubes of the SH test (2 and 3).

Table 13. Effect of varying the pH of the medium and the production of factor Z and T.

Factors in supernatant	pH range							
	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8
Treated Z plus untreated T	+*	+	+	+	+	+	+	+
Treated T plus untreated Z	+	+	+	+	+	+	+	+
Controls	-**	-	-	-	-	-	-	-

* Factor was present and showed immediate lysis of red cells in the SH test when untreated opposite factor was added.

** No lysing activity in the SH test.

Medium which was adjusted to varying pH was inoculated with hemolytic and nonhemolytic staphylococci to observe any effect of pH on the production of factors Z and T. Supernatants of both factors Z and T produced at each pH level showed immediate and complete lysis of the red cells when the SH test was run. The controls for factors Z and T lysing activity and for red cell stability remained unchanged. The organisms produced the two lysins between pH 6.4 and 7.8 as seen in Table 13.

To determine if the activity of factors Z and T was retarded or enhanced by pH, the supernatants were adjusted to varying pH and allowed to stand. The supernatants were then readjusted to pH 7.2 and the SH test performed on each. Treatment of the factors Z and T with a change in pH resulted in a negligible effect of pH on the factors. There was immediate and complete lysis in the SH test by factors Z and T after treatment at each pH level. The factors are not destroyed by hydrogen ion concentration between 5.0 and 8.5 (Table 14), nor does the lysing activity appear to be increased at any of the pH levels.

Separation of the proteins from the supernatants by treating the supernatants with saturated sodium sulfate and saturated ammonium sulfate was performed. The SH test was run on the protein-free supernatants and on the redissolved protein fraction of each supernatant. Upon completion of the SH test, immediate and complete lysis of red cells was observed in the protein-free supernatant tested from the supernatants of factor Z and factor T. No lysis of red cells was observed in the protein fraction of the treated supernatants of factor Z and factor T. Table 15 shows the comparison of lysing activity in the two fractions tested.

Table 14. Treatment of factors Z and T by acid and base for 3 hours to observe any effect of pH on the factors.*

Factors in supernatant	pH at which factors were treated							
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Treated Z plus untreated T	+**	+	+	+	+	+	+	+
Treated T plus untreated Z	+	+	+	+	+	+	+	+
Controls	-***	-	-	-	-	-	-	-

* Each treated sample readjusted to pH 7.2 before addition of red cells in the SH test.

** Immediate and complete lysis in tube 1 in the SH test, when untreated factor Z or T added to opposite treated factor in supernatant.

*** No lysis of red cells (controls of factor Z and T lysins and red cell stability, all untreated and treated).

Table 15. Supernatants containing factors Z and T, respectively, treated with saturated sodium sulfate to determine if the lysing activity of the factors is in the precipitated protein fraction or in the protein-free supernatant.

Factor tested	Protein fraction	Protein-free fraction
Z	-*	+**
T	-	+

* Negative, no lysis observed.

** Positive, complete and immediate lysis seen tube 1 of the SH test.

The results of the SH test on the two fractions, separated by ammonium sulfate, were identical with the results seen in Table 15, using sodium sulfate as a precipitating agent. This evidence strongly suggests and confirms the conclusion seen in inactivation of the factors by temperature, that

neither factor Z nor factor T is protein in nature.

The results of surface tension tests on the five samples tested are seen in Table 16. The surface tension of distilled water was 60, and that of the medium was 57.2. The surface tension of supernatant containing factor Z was 54.4, a little lower than that for the medium. The surface tension of factor T supernatant was essentially the same as water and a little higher than for the medium. The surface tension of the solution of 50:50 supernatants of factor Z plus factor T was slightly lower than that of water and essentially the same as that of the medium. There is no great difference seen in any of the samples tested for surface tension.

Table 16. Surface tension determination on supernatants containing factor Z and factor T, compared with that determined for medium and distilled water.

Sample	Reading of scale	Mg. represented	γ
Water	56.9	421	60
Medium	54.7	404.8	57.2
Factor Z	51.5	381	54.4
Factor T	58.2	430.7	61.4
Factor Z + T	54.5	403.3	56.4

DISCUSSION AND CONCLUSIONS

A peculiar hemolytic phenomenon occurring in otherwise "nonhemolytic" staphylococci is described. This "induced hemolysis" of nonhemolytic colonies is seen when these colonies are adjacent to, or are in the secondary zone produced by certain hemolytic strains of staphylococci.

What causes the "induced hemolysis" of nonhemolytic colonies? There seems to be several possible explanations to this phenomenon. The obvious is that both the hemolytic and nonhemolytic staphylococci each produce a

substance that by itself can not lyse red cells, but when combined can and do lyse red cells. From results obtained in this work one sees that this postulation is quite reasonable. It has been shown that each culture produces in solution a substance that when combined causes complete lysis of the red cells. This expression was also observed on blood agar plates, as induced hemolysis always occurred a distance away from the nonhemolytic colony, suggesting the combination of two soluble factors in the medium. Supernatants of hemolytic and nonhemolytic cultures, when combined in the SH test, show immediate and complete lysis of the red cells. The respective supernatants do not lyse red cells by themselves, as seen in the controls of the SH test. Transplanted agar squares from the secondary zone of hemolytic cultures produce the secondary zone of the "mother colony" and stimulate induced hemolysis of nonhemolytic cultures.

A second explanation of the phenomenon is the possibility that nonhemolytic staphylococci "take" the secondary zone agent into their cells, reorganize the substance and excrete it in another form that will lyse red cells. The results of this work do not substantiate this theory completely. The complete lysis of red cells from supernatants free of bacterial cells in the SH test contradicts this theory. However, it is shown that the induced hemolytic phenomenon is retained by the "nonhemolytic" cultures up to four transfers of these cultures onto blood agar plates.

A third theory is that this phenomenon is a synergistic reaction of two lysins. It is known that beta and delta hemolysins act synergistically, and that beta hemolysin inhibits the action of alpha hemolysin. The hemolytic cultures used in this work lyse the red cells of sheep and rabbits and produce the secondary zone in human blood agar plates. The appearance of the hemolytic

zone resembles that of the beta hemolysin, and this zone is intensified by cooling. However, beta hemolysing staphylococci do not lyse the erythrocytes of rabbits or humans.

The nonhemolytic staphylococci showed clear hemolysis on rabbit blood agar plates (some strains were also hemolytic on human blood agar plates), but did not exhibit any lysis of sheep red cells. From the comparison of the lysing activities of the cultures used in this work one would conclude that induced hemolysis does not result from alpha-beta, beta-delta, delta-alpha synergism.

The initial postulation seems to be substantiated by the results as the most plausible explanation of the phenomenon.

The character of factor Z and factor T appears almost identical. The factors are different in two respects. Factor Z is produced in solution in greater quantity than is factor T. Factor T appears to be of higher molecular weight or less soluble than factor Z as indicated by its lack of diffusability through agar medium. Both factors are greatly enhanced by 10 percent or 20 percent CO_2 during incubation. Both factors are produced in medium varying in pH from 6.4 to 7.8, and neither factor is inhibited or apparently enhanced by treatment of medium to pH 5.0 to 8.5. Neither factor is precipitated by saturated sodium or ammonium sulfate, which would eliminate somewhat the possibility of a protein configuration of the two factors. Both factor Z and factor T are stable to heating for 15 minutes at 100°C, and each factor is produced by its respective culture when incubated in a temperature range of 20°C to 37°C. Factor Z and factor T do not appear to be proteinaceous nor enzymatic in nature.

The presence of induced hemolysis of nonhemolytic staphylococci cultivated on blood agar plates containing hemolytic staphylococci (see Fig. 4) could confuse the identification of possible pathogenic staphylococci. One could very possibly, under these circumstances, confuse the "induced hemolytic" staphylococci for a more serious pathogen and consequently report a nonpathogenic strain as the etiologic agent of a disease in an individual. The seriousness of this problem would be the overlooking of a pathogenic strain of staphylococci completely by erroneously selecting a really nonhemolytic organism.

It is felt that a more detailed study of the configuration of the two factors and investigation into the possible clinical importance of these types of staphylococci, is worthwhile.

SUMMARY

1. Synergistic hemolysis of nonhemolytic staphylococci by the secondary zone of hemolytic staphylococci is described.

2. The synergism results from substances produced by each respective culture that do not lyse erythrocytes by themselves. When these two substances are combined, lysis of erythrocytes occurs.

3. The synergistic hemolysis (SH) test for detecting factors Z and T is described.

4. Factors Z and T are soluble.

5. Presence of 10 percent or 20 percent CO_2 during incubation greatly enhances the production of factor Z and factor T.

6. Both factors are heat stable, surviving 100°C for 15 minutes.

7. pH from 6.4 to 7.8 apparently has no effect on the production of the factors.

8. The factors after production are stable in a pH range of 5.0 to 8.5.

9. The factors Z and T do not appear to be proteins or enzymes.

10. The importance of the presence of the synergistic reaction in clinical microbiology is discussed.

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SYNERGISTIC HEMOLYSIS IN "NONHEMOLYTIC" *STAPHYLOCOCCUS* SPECIES

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The synergistic hemolysis of "normally nonhemolytic" staphylococci on blood agar incubated in, or adjacent to, the secondary zone of certain hemolytic staphylococci is described. The synergism appears to result from the combining of two factors produced. One factor (Z) is produced by hemolytic staphylococci that elaborate a secondary zone. The second factor (T) is produced by nonhemolytic staphylococci. The production of the two factors is substantiated by (1) the absence of clear hemolysis around nonhemolytic colonies, and in the secondary zone of hemolytic colonies and (2) by evidence of clear hemolysis appearing as the secondary zone reaches within a few millimeters, and then surrounds the nonhemolytic colonies.

The two factors, Z and T, are obtained in the supernatants of broth cultures of hemolytic and nonhemolytic staphylococci, respectively. The detection of the factors in the supernatants was determined by the synergistic hemolysis test (SH). Complete lysis of the red cells in the SH test was observed only in tubes containing the combined factors. No lysis of the red cells was observed in the control tubes; factor Z alone, factor T alone, and red cell stability control. The SH test proved a reliable method for determining the activity of supernatants containing the factors.

The presence of 10 percent or 20 percent CO_2 during incubation of the hemolytic and nonhemolytic cultures greatly enhanced the production of factors Z and T. Both factor Z and factor T are heat stable, surviving 100°C for 15 minutes without any appreciable loss of activity. Neither factor is precipitated when treated with saturated sodium or ammonium sulfate. Hydrogen ion concentration between pH 6.6 to 7.8 and 5.0 to 8.5 does not appear to exert any effect on the two factors, either during incubation or upon the supernatants containing the factors, respectively. Factor Z is produced in greater

quantity and appears to be smaller in molecular weight or more soluble than is factor T.

The confusing of colonies showing induced hemolysis by "nonhemolytic" staphylococci and truly hemolytic colonies cannot be overlooked. It was possible to show on a blood plate containing a mixture of both hemolytic and nonhemolytic cultures that all colonies exhibited a zone of clear hemolysis. In selecting 400 colonies from such a blood plate, 280 were nonhemolytic on subsequent studies. The remaining 120 colonies were hemolytic. A similar situation on a diagnostic blood plate could very likely result in the failure to identify a possible pathogenic staphylococcus.

It is suggested that factor Z and factor T are not proteins or enzymes. In addition, there is evidence that this phenomenon may interfere with the identification of truly hemolytic staphylococci.