

Identification of Staphylococcal Hemolysins by an Electrophoretic Localization Technique

RIAZ-UL HAQUE

Department of Microbiology, University of Illinois Medical Center, Chicago, Illinois

Received for publication 3 October 1966

ABSTRACT

A technique for identifying and characterizing staphylococcal hemolysins by first separating them electrophoretically in barbital-buffered agar gel (pH 8.4) at 5 ma/cm for 2 hr and then determining their hemolytic activities by exposing them to human, horse, rabbit, and sheep erythrocytes is described. The α -hemolysin produced by a White variant of the Wood 46 strain of *Staphylococcus aureus* migrated 18 mm towards the cathode, and it lysed horse, rabbit, and sheep erythrocytes, whereas a Clear variant of the Wood 46 strain of *S. aureus* produced a lysin which migrated similarly to the α -hemolysin but lysed only rabbit cells. This latter lysin was tentatively named α_1 -lysin. This strain of *S. aureus* also produced β -hemolysin which migrated 36 mm towards the cathode and lysed sheep cells. β -Hemolysin produced by some strains of *S. aureus* showed considerable tailing during electrophoresis, whereas β -hemolysin produced by other strains of *S. aureus* migrated as a well-defined peak. A lysin migrating 11 mm towards the anode was probably δ -lysin. It was, however, not produced in sufficient concentration when the cultures were grown in semisolid medium.

The procedures currently used for the identification of staphylococcal hemolysins are not satisfactory. Cultures of *Staphylococcus aureus* frequently produce several hemolysins which often interact with each other as well as with other diffusible products of *S. aureus* (2, 3, 6, 7). Furthermore, the cultures are seldom homogeneous. Variants are often present which frequently produce hemolysins other than those produced by the parent population (5). These variants may also produce additional enzymes and thus further influence the activities of the hemolysins produced by the parent population. Because of these and other similar problems neither the patterns of lysis produced by cultures of *S. aureus* on various species of blood-agar plates nor the hemolytic titers obtained by titrating culture filtrates against various species of erythrocytes can be interpreted with reliability in terms of the types of hemolysins produced by the culture. Primarily because of the undefined specificity of antiserum, its use does not eliminate these problems.

The complications due to interactions among various products of *S. aureus* can be eliminated provided the hemolysins are first separated from each other as well as from other diffusible prod-

ucts of *S. aureus* and then identified by determining their hemolytic activities. Electrophoresis of staphylococcal culture filtrates in agar gel offered a simple means of accomplishing the desired separation. Consequently, the method of electrophoretic localization for the identification of various hemolysins was developed and is reported here. The method is equally useful for identifying other biologically active products of *S. aureus* as well as those of other organisms.

MATERIALS AND METHODS

Cultures of *S. aureus*. As far as possible, the strains previously used by various investigators for the production of different hemolysins were employed in this study. The Wood 46 strain of *S. aureus* used for the production of α -hemolysin was obtained from the Communicable Disease Center, Atlanta, Ga. Two variants, designated as Wood 46 White and Wood 46 Clear, were isolated from this culture and were supplied by C. Gross of this department.

The Foggie strain of *S. aureus* used for the production of δ -hemolysin by Yoshida (8) was obtained from G. P. Gladstone, Sir William Dunn School of Pathology, University of Oxford, England. Strain 146P, used for the production of δ -hemolysin by R. A. Murphy of this department (M.S. Thesis,

University of Illinois, Chicago, 1966), was isolated from a urine specimen at the University of Illinois Research and Educational Hospital and was supplied by him. Strain 681 has been previously described (4). It was obtained from a mastitic cow and produced large quantities of β -hemolysin.

Production of culture filtrates. A 20.0-ml quantity of sterile Brain Heart Infusion (BHI) containing 0.3% agar (both from Difco) was inoculated with a 0.5-ml portion of a 5-hr-old BHI culture of the test organism. The medium was then poured into standard petri plates and was incubated at 37 C for 48 hr under 10% CO₂ in a CO₂ incubator. The semisolid culture was then centrifuged at $1,000 \times g$ for 20 min, and the supernatant fluid was filtered through a membrane filter (0.22 μ) by use of a Micro Syringe Filter Holder (both from Millipore Filter Corp., Bedford, Mass.). The cell-free filtrates when not in use were stored at -20 C.

Separation of various hemolysins. The hemolysins present in culture filtrates were separated from each other by electrophoresis in agar gel using barbital buffer (pH 8.4). A stock solution of barbital buffer containing 0.165 M sodium barbital (Merck & Co., Inc., Rahway, N.J.) and 0.046 M hydrochloric acid in deionized distilled water was first prepared. A 1% solution of Ionagar No. 2 (Colab Laboratories, Inc., Chicago Heights, Ill.) in a 1:2 dilution of the stock barbital buffer was then prepared by autoclaving the mixture for 15 min. Approximately 40 ml of this solution was poured in a 150-mm sterile petri plate and was allowed to solidify. The purpose of this base layer was to obtain a perfectly horizontal surface. Care was therefore taken not to disturb or relocate the petri plates. After the agar had solidified, a sterile glass plate, 4 $\times$ 3.75 inches (10.2 $\times$ 9.5 cm), which had previously been siliconized with Siliclad (Clay-Adams, Inc., New York, N.Y.), was gently placed on the surface of the agar. The plate was then completely covered with 40.0 ml of the barbital agar. This layer of agar was also allowed to solidify without disturbing or relocating the petri plates.

The glass plate along with its agar gel was freed from the surrounding gel with a scalpel, and eight circular wells 0.5 mm in diameter were cut across the center of the plate (Fig. 1). A specially designed agar gel cutter (Fig. 2) was used for this purpose. It consisted of a knife assembly which could be moved back and forth on two horizontal rods 18.6 cm long but could be stopped at any one of the eight predetermined points. These points were located in an 8.25-cm length of the horizontal rod and were alternately spaced 13.5 and 9.5 mm apart. The knife assembly consisted of a teflon bar 19.5 cm long and 2.8 cm thick with a 0.8 $\times$ 8.7 cm rectangular cut-out in the center. A knife blade 8.6 cm long held in a "T"-shaped knife holder was suspended through this opening. The edges of the knife holder were supported on metal spring assemblies. Two holes (0.6 mm in diameter) were cut in the center of the knife holder; they extended from the top to the bottom of the holder and were located at a distance of 0.25 mm from each side of the knife blade. These holes served as guides for punching the circular wells in the agar gel. The

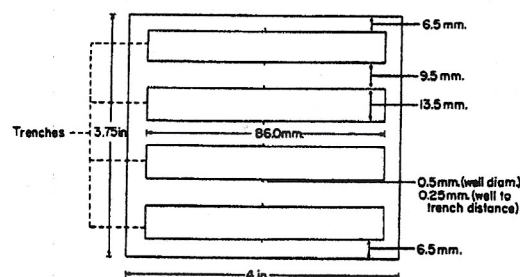


FIG. 1. Agar plate for electrophoresis showing the location of sample wells and trenches for blood-agars.

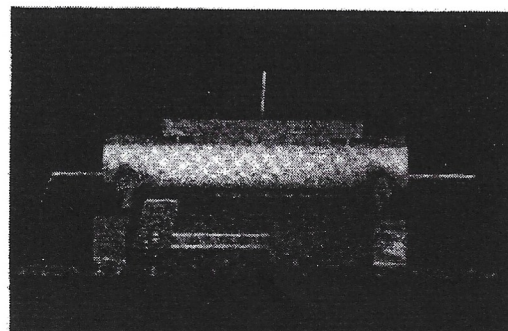


FIG. 2. Cutter for agar gel.

knife assembly and the horizontal rods were mounted on a rectangular piece of Plexiglas which also served as a stage for placing the glass plate containing the agar gel. The plate was placed under the knife assembly in such a way that its length was parallel to the knifeholder and its width was centered directly under the eight marked positions on the horizontal rods. The plate thus extended 6.5 mm past either end of the marked positions. Two "L"-shaped pieces of Plexiglas, one fixed and the other movable, located diagonally from each other on the Plexiglas stage, served to position the plate.

In order to punch the wells shown in Fig. 1, the agar plate was placed in the cutter and was secured in place by sliding and securing the movable piece of Plexiglas. The knife assembly was then moved to the first position, and a circular punch 0.5 mm in diameter and 0.5 inch (1.27 cm) long was inserted through the hole of the knife holder near the outer edge of the plate. The other end of the punch was attached to a vacuum line which facilitated removal of the agar gel cut out by the punch. The knife assembly was then moved to the second position, but now the punch was inserted through the hole away from the outer edge of the plate. This process was repeated at the six remaining marked positions; the two holes in the knife holder were used alternately.

Each of the eight wells was then filled with the culture filtrate by use of a Wright's pipette drawn to a fine capillary. The plate was then inverted and placed in the electrophoresis apparatus (Fig. 3). Two such

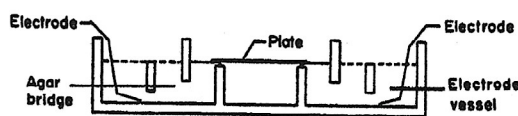


FIG. 3. Apparatus for electrophoresis.

plates could be accommodated in the apparatus at one time. The electrode chambers of the apparatus were connected with their respective agar bridges through a slit cut in the lower portion of the partition. The agar bridges were prepared by filling the electrode chambers and the chambers for the agar bridges with the same barbital-buffered agar used in making the plates and allowing it to harden. Agar from the electrode chambers was later removed by cutting with a sharp metal spatula. Platinum wire electrodes were then placed in the electrode chambers and the chambers were filled with a 1:4 dilution of the stock barbital buffer. Heathkit Regulated Power Supply model IP-32 (Heath Co., Benton Harbor, Mich.) was used as a current source.

The culture filtrates were electrophoresed for 2 hr at a constant current supply of 45 ma per plate. After electrophoresis, four strips of agar (13.5 × 86.0 mm) parallel to the line of migration and 0.25 mm from the wells were removed (Fig. 1). To accomplish this, the plate was again placed in the cutter for agar gel. The knife assembly was positioned at each of the eight locations, and the gel was cut by depressing the knife holder. The plate was then placed in the petri dish and the trenches were separately filled with human, horse, rabbit, and sheep erythrocyte agar. The basal agar medium was prepared by dissolving 1.5% agar (Difco) in Hemagglutination Buffer (Difco), pH 7.0, to which was added 0.001 M magnesium sulfate. The agar was distributed in 10.0-ml quantities in screw-cap tubes and was sterilized by autoclaving. The agar was then melted and cooled to 45 C, and a 0.3-ml portion of thrice-washed and packed erythrocytes was mixed with it. The plates were incubated at 25 to 28 C for 12 to 48 hr when the hemolytic reactions were recorded.

The hemolytic activities of the culture filtrates were also determined without subjecting them to electrophoresis. For this purpose, the trenches for blood-agar were first cut and filled with various species of erythrocyte agars. The circular wells were then filled with culture filtrates. These plates were also incubated at 25 to 28 C for 12 to 48 hr when the reactions were noted.

RESULTS

The hemolytic factors present in the culture filtrates were sufficiently separated from each other after 2 hr of electrophoresis. Each hemolysin had apparently localized in a separate area and caused lysis of various species of erythrocytes, thus indicating the nature of its biological activities.

The culture filtrate of the White variant of the Wood 46 strain of *S. aureus* when tested without

electrophoresis lysed rabbit, sheep, human, and horse erythrocytes. After electrophoresis, this culture was found to contain two hemolytic factors. One of these factors migrated 18 mm towards the cathode and lysed rabbit, sheep, and horse erythrocytes, whereas the other hemolysin migrated very poorly towards the cathode; however, it did extend 11 mm towards the anode, and, in the concentration present in the filtrates, it lysed only rabbit erythrocytes (Fig. 4). These hemolysins were designated α -hemolysin and rabbit cell lysin, respectively.

The culture filtrate of the Clear variant of the Wood 46 strain of *S. aureus* when tested without electrophoresis lysed rabbit and sheep erythrocytes. After electrophoresis, however, this culture filtrate was found to contain two hemolysins (Fig. 5). One of these hemolysins migrated 18 mm towards the cathode and lysed rabbit cells only. The other hemolysin produced a well-defined peak 36 mm towards the cathode and caused "hot-cold" lysis of sheep cells. This second hemolysin was therefore designated β -hemolysin. The factor causing lysis of rabbit cells alone could not be identified with α -hemolysin. It did not lyse any other species of cells, even after 7 days of incubation. Examination of concentrated culture filtrates by electrophoresis also showed this lysin to hemolyze only rabbit cells. This hemolysin, therefore, has biological activity different from that of the α -hemolysin produced by the White

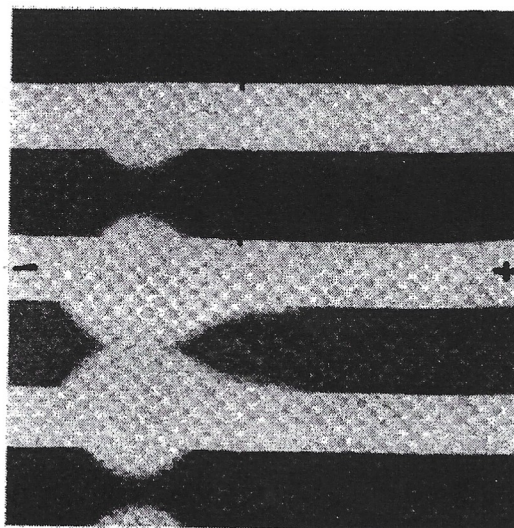


FIG. 4. Electrophoretic localization pattern of the hemolysins present in the culture filtrate of the White variant of the Wood 46 strain of *Staphylococcus aureus*. From top to bottom, human, horse, rabbit, and sheep erythrocytes.

variant of the Wood 46 strain of *S. aureus*; nonetheless, because of similarity of electrophoretic behavior, it was designated α_1 -lysin.

The culture filtrate of the 681 strain of *S. aureus* also showed the presence of two hemolysins. One of these hemolysins was the β -hemolysin (Fig. 6), whereas the other hemolysin was probably identical with the α -hemolysin of the White variant of the Wood 46 strain of *S. aureus*.

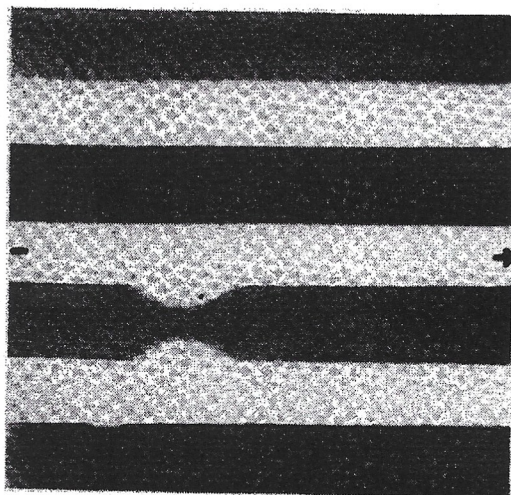


FIG. 5. Electrophoretic localization pattern of the hemolysins present in the culture filtrate of the Clear variant of the Wood 46 strain of *Staphylococcus aureus*. From top to bottom, human, horse, rabbit, and sheep erythrocytes.

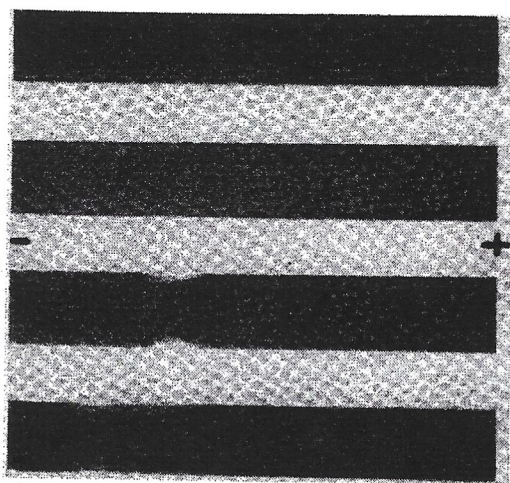


FIG. 6. Electrophoretic localization pattern of the hemolysins present in the culture filtrate of the 681 strain of *Staphylococcus aureus*. From top to bottom, human, horse, rabbit, and sheep erythrocytes.

It, however, did not hemolyze horse cells. β -Hemolysin showed considerable tailing.

The filtrate of the Foggie strain of *S. aureus*, when examined without electrophoresis, lysed all four species of erythrocytes. Upon electrophoresis, however, it was found to contain at least two hemolysins (Fig. 7). One of these hemolysins migrated 18 mm towards the cathode and hemolyzed rabbit, sheep, and horse erythrocytes and thus corresponded with the α -hemolysin of the White variant of the Wood 46 strain of *S. aureus*. The other hemolysin lysed sheep cells in a "hot-cold" manner. This hemolysin was therefore considered similar to β -hemolysin. The Foggie strain of *S. aureus* produced large quantities of this hemolysin. Besides these two hemolysins, there also appeared to be a third peak of hemolytic activity on sheep blood agar. This central peak may represent tailing from the large amount of β -lysin present in the culture filtrate, or it may be a separate hemolysin. Longer periods of electrophoresis may be needed for further separating these hemolysins. None of the hemolysins, however, lysed human cells.

The filtrate obtained from the 146P strain of *S. aureus* also hemolyzed all four species of erythrocytes when examined without electrophoresis. Upon electrophoresis, however, the culture was found to contain α - and β -hemolysins (Fig. 8). The quantity of β -hemolysin present in this culture filtrate was much less than that present in the filtrate of the Foggie strain of *S. aureus*. β -Lysin migrated as a distinct peak and showed no tailing.

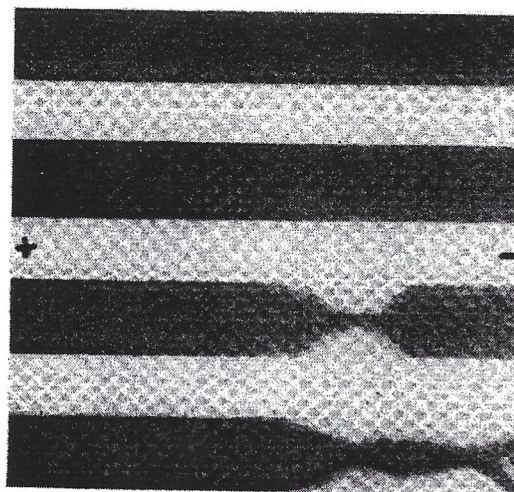


FIG. 7. Electrophoretic localization pattern of the hemolysins present in the culture filtrate of the Foggie strain of *Staphylococcus aureus*. From top to bottom, human, horse, rabbit, and sheep erythrocytes.

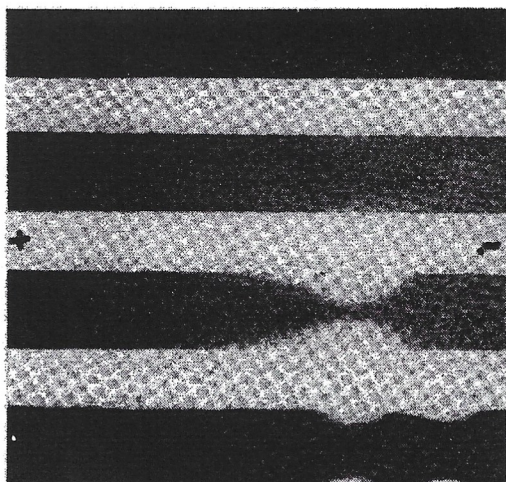


FIG. 8. Electrophoretic localization pattern of the hemolysins present in the culture filtrate of the 146P strain of *Staphylococcus aureus*. From top to bottom, human, horse, rabbit, and sheep erythrocytes.

DISCUSSION

The electrophoretic localization technique of determining the hemolytic activities of various hemolysins of *S. aureus* described in this report has revealed that the hemolytic activities of various hemolysins are vastly different from those hitherto described in the literature. The Wood 46 strain of *S. aureus* has been extensively used for the production of α -hemolysin, and this hemolysin has been invariably described as being active mainly against rabbit and sheep erythrocytes and not against horse erythrocytes. Only concentrated preparations have been shown to be active against horse cells, and these, in fact, were more active against human than horse cells. The order of susceptibility of various erythrocytes to α -hemolysin has been reported by Bernheimer (1), who considered it to be least active against horse cells. Since these studies were conducted with variously "purified" preparations of α -hemolysin, it is very likely that the hemolysin molecule had undergone some derangements as a result of the procedures used for its purification. Furthermore, the procedures used in the past for determining the hemolytic activity of α -hemolysin did not separate it from the influences of other factors present in the crude or partially "purified" preparations. The presence of variants producing β -hemolysin in the culture could be one such factor which would tend to inhibit the activity of α -hemolysin. Such interactions were perhaps operating within the Wood 46 strain of *S. aureus* and may have contributed to the belief that α -hemolysin is not active against horse erythrocytes.

The electrophoretic localization technique has revealed that the major hemolytic component of the culture filtrates of the White variant of the Wood 46 strain of *S. aureus* was active against rabbit, sheep, and horse erythrocytes. It is suggested that only this lysin be called α -hemolysin and that the criteria revealed by the electrophoretic localization technique be used to identify it.

The finding that certain hemolysins present in the culture filtrates of different strains of *S. aureus* had similar electrophoretic migrations but different hemolytic activities poses the question regarding the identity of these hemolysins; for instance, the hemolysin produced by the Clear variant of the Wood 46 strain of *S. aureus* localized in the area of the α -hemolysin, but it hemolyzed only rabbit cells. The absence of lysis of sheep and horse cells was not due to a low concentration of this hemolysin, because concentrated preparations of this hemolysin as well as extended periods of exposure of cells to this hemolysin also failed to cause lysis of sheep and horse cells. These findings emphasize the separate identity of this hemolysin. Various investigators in the past, while attempting purification of α -hemolysin, have used only rabbit erythrocytes to detect the presence of α -hemolysin at various stages of purification. It is very likely that some investigators who have reported a low initial activity of their hemolysin preparations against rabbit cells were perhaps working with this hemolysin rather than with α -hemolysin. Presently, the hemolysin produced by the Clear variant of the Wood 46 strain of *S. aureus* is identified as α_1 -hemolysin. Attempts are now being made to elucidate its antigenic relationship with the α -hemolysin.

The identification of δ -hemolysin of *S. aureus* by conventional procedures is very doubtful because of the synergism known to exist between δ - and β -hemolysins as well as between β -hemolysin and various other products of *S. aureus*. Lysis of sheep and human cells could thus be due to factors other than δ -hemolysin. The culture filtrates of the Foggie strain of *S. aureus* used by Yoshida (8) and the 146P strain of *S. aureus* used by Murphy (M.S. Thesis, University of Illinois, Chicago, 1966) for the production of δ -hemolysin did not reveal the presence of δ -hemolysin by the electrophoretic localization technique. Instead, they contained both α - and β -hemolysins. The latter hemolysin was produced in such abundance by the Foggie strain of *S. aureus* that some other factor, e.g., lipase, which is also produced by this strain in large quantities, may have synergistically lysed human cells when the filtrate was tested without electrophoresis.

The quantity of β -hemolysin produced by the

146P strain of *S. aureus* was much smaller than that produced by the Foggie strain of *S. aureus*. This strain, however, did produce lipase which may have synergistically lysed human cells when the culture filtrate was tested by the conventional procedures.

Under specially selected cultural conditions, however, both of these cultures did produce δ -hemolysin which migrated poorly towards the cathode; however, it extended 11 mm towards the anode and lysed human, horse, and rabbit erythrocytes. The cells of sheep were very resistant but were apparently hemolyzed to some extent after a long period of exposure. Evidence for the production and identification of δ -hemolysin will be presented elsewhere.

The electrophoretic migration of the "rabbit cell lysin" present in the culture filtrates of the White variant of the Wood 46 strain of *S. aureus* was similar to that of δ -lysin of *S. aureus*. It is likely that this lysin was also δ -hemolysin, but its concentration was too low to cause lysis of human and horse erythrocytes. Currently, we are examining concentrated preparations of this hemolysin, and we intend to report its possible identity with δ -hemolysin of *S. aureus* in the future.

β -Hemolysin of *S. aureus* is perhaps the only hemolysin which had been reliably identified in the past by the plate or the tube titration procedures of identifying the hemolysins of *S. aureus*. The electrophoretic localization technique, however, has revealed that this hemolysin is perhaps produced by a larger number of cultures than have been detected by the conventional procedures. The Clear variant of the Wood 46 strain of *S. aureus*, as well as the 146P strain of *S. aureus*, did not reveal the presence of the β -hemolysin when tested by the conventional procedures, but did show its presence when tested by the electrophoretic localization technique.

β -Lysin present in the culture filtrates of the Wood 46 Clear variant and the 146P strain of *S. aureus* migrated as a well-defined peak, whereas the β -lysin produced by the Foggie and the 681 strain of *S. aureus* yielded extensive tailing during migration. Although this phenomenon was not investigated, it is suggested that the tailing may represent different molecular species of β -lysin.

The electrophoretic localization technique presented here is a beginning towards systematic analysis of the culture filtrates of *S. aureus*. Detection of small quantities of products should, however, require the use of concentrated preparations. Analysis of such preparations would perhaps reveal new diffusible products of *S. aureus* which may play an important role in the pathogenesis of *S. aureus*. One caution, however, is in order. Unless products which act synergistically are also biologically active themselves, they may not be detected by the electrophoretic localization technique. Procedures should therefore be developed to detect those products which are only active synergistically. Otherwise, the analysis of the culture filtrates of *S. aureus* will only be incomplete.

ACKNOWLEDGMENT

This investigation was supported by grant 2-41-33-36-3-51 from the Trust Graduate Research Funds of the University of Illinois.

LITERATURE CITED

1. BERNHEIMER, A. W. 1965. Staphylococcal alpha toxin. *Ann. N.Y. Acad. Sci.* 128:112-123.
2. CHRISTIE, R., AND J. J. GRAYDON. 1941. Observations on staphylococcal haemolysins and staphylococcal lipase. *Australian J. Exptl. Biol. Med. Sci.* 19:9-16.
3. ELEK, S. D., AND E. LEVY. 1954. The nature of discrepancies between haemolysins in culture filtrates and plate haemolysin patterns of staphylococci. *J. Pathol. Bacteriol.* 68:31-40.
4. HAQUE, R., AND J. N. BALDWIN. 1964. Purification and properties of staphylococcal beta-hemolysin. I. Production of beta-hemolysin. *J. Bacteriol.* 88:1304-1309.
5. HAQUE, R., AND J. N. BALDWIN. 1964. Types of hemolysin produced by *Staphylococcus aureus*, as determined by the replica plating technique. *J. Bacteriol.* 88:1442-1447.
6. MARKS, J., AND A. C. T. VAUGHAN. 1950. Staphylococcal δ -haemolysin. *J. Pathol. Bacteriol.* 62:597-615.
7. WILLIAMS, R. E. O., AND G. J. HARPER. 1947. Staphylococcal haemolysins on sheep-blood agar with evidence for a fourth haemolysin. *J. Pathol. Bacteriol.* 59:69-78.
8. YOSHIDA, A. 1963. Staphylococcal δ -hemolysin. I. Purification and chemical properties. *Biochim. Biophys. Acta* 71:544-553.

EXPERIMENTAL DATA

Staphylococcus aureus strain..... Date.....

Activities Tests: 1. Hemolysin M.....H..... R..... S.....

2. Lipase:.....

Nature of Sample:.....

3. Egg Yolk:.....

.....

4. DNase:.....

Protein Conc:mg/ml.....

5. Esterase:.....

6. Phosphatase:

7. Gelatinase:

8. Caseinase:

Electrophoretic localization:

--

M

H

+

R

5

Immunoelectrophoresis:

Immunodiffusion:

This image shows a single sheet of white paper with five horizontal black lines. The lines are evenly spaced and run across most of the width of the page. On the right side, between the fourth and fifth lines from the top, there is a small black plus sign (+). The paper has a slightly aged or off-white appearance.

1.
2.
3.
4.
5.
6.
7.

1.
2.
3.
4.
5.
6.
7.