

THE CHARACTERIZATION OF STAPHYLOCOCCAL TOXINS

II. THE ISOLATION AND CHARACTERIZATION OF A HOMOGENEOUS STAPHYLOCOCCAL PROTEIN POSSESSING ALPHA HEMOLYTIC, DERMONECROTIC, LETHAL, AND LEUCOCIDAL ACTIVITIES*·†

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Previous studies on the electrophoretic migration of the components of crude staphylococcal toxin (1) led to an attempt to isolate the staphylococcal alpha hemolysin by these methods. The isolation of a homogeneous toxin which possessed alpha hemolytic, dermonecrotic, lethal, and leucocidal activities would provide strong direct support for the "unitarian" theory, which holds that these four activities are actually different expressions of one toxic component. Further, the availability of purified alpha hemolysin would make possible a variety of studies which cannot be satisfactorily carried out using crude staphylococcal toxin.

The importance of isolating and individually characterizing the various staphylococcal toxins has been emphasized by Dubos (2) and by Elek (3). The emphasis upon studying the toxins individually is prompted by the fact that much of the work using crude staphylococcal toxin is difficult to interpret, since there is no way of knowing whether the activity being studied is enhanced, unaffected, or inhibited by other components of the crude mixture.

In the past, many workers have directed themselves to the task of isolating a staphylococcal toxin with varying degrees of success. Some of the chemical methods employed were precipitation by acetic acid (4), ammonium sulfate (5, 6, 10, 15, 16, 20), trichloroacetic acid (21, 22), acetone followed by trichloroacetic acid (7), phosphoric acid (10), methanol (8, 9, 19), ethanol (6, 10, 18, 20), and cellosolve (6, 19). Physical methods such as chromatography (10-12, 15, 16, 18), various electrophoretic techniques (6, 10, 13, 15, 16, 18, 20), and ultracentrifugation (14), have also been applied to the problem of isolating various staphylococcal toxins.

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Many of these workers did not attempt to test the homogeneity of their products. Others applied tests for purity but were unable to demonstrate that their products were homogeneous. However, two of the above groups have successfully applied critical tests for the purity of their toxins. Woodin (15, 16) used gel diffusion and ultracentrifugation to test the purity of his leucocidin components, while Bergdoll *et al.*, (18), and Hibnick and Bergdoll (17), applied the same techniques to purified staphylococcal enterotoxin.

The work presented here describes the continuous flow paper electrophoretic techniques used for the isolation of alpha hemolysin and presents direct experimental evidence for the homogeneity of the product.

Materials and Methods

The methods used for the preparation and continuous flow electrophoretic fractionation of crude toxin, for measuring alpha hemolytic, dermonecrotic, leucocidal, and lethal activities, for detecting anti-alpha hemolysin, and for determining the concentration of biuret-positive and carbohydrate components are presented in the preceding paper (1).

Agar Double Diffusion Techniques.—The method of Ouchterlony (23) was used with slight modification. Noble agar 0.8 per cent (Difco) was used in a solution containing 0.8 per cent sodium chloride and a 1/10,000 concentration of merthiolate. The method of Korngold *et al.* (24), was used to prepare the wells. Following the introduction of antigen and antibody into the wells, the plates were incubated for 24 hours at room temperature and for 48 hours at 4°C before reading.

Microimmunoelectrophoresis.—Schiedeger's method (25) was used with modification. Noble agar (Difco) was used in a veronal buffer at a pH of 8.6, ionic strength 0.02. The antigen was allowed to migrate for 2 to 5 hours at 4 milliamperes (6 standard microscopic slides per experiment). Following migration of the antigen, the antitoxin was added and the slides were incubated for 72 hours at room temperature in a humidified chamber. After washing in veronal buffer, pH 8.6, the slides were stained with amido black (10B—color index, 246).

Ultracentrifugation.—*Standard method:* The toxin was dialyzed for 24 hours against 0.04 M phosphate buffer at a pH of 7.6. The final protein concentration of the dialyzed toxin was 0.6 per cent. This preparation was centrifuged at 59,780 RPM which gave a force of approximately 240,000 *g*. The average temperature during centrifugation was 18.6°C. The bar angle was maintained at 40°, and photographs were taken at 8 minute intervals. *Synthetic boundary method:* The toxin used was the same preparation as above. Schachman's technique (26) using a synthetic boundary cell was employed. The first two photographs were taken at a bar angle of 40° and the remaining photographs at a bar angle of 35°. Photographs were taken at 8 minute intervals. The centrifuge temperature averaged 18.6°C. The movement of the boundary was measured with a microscopic comparator.

Preparation of Crude Antitoxin.—Four adult New Zealand white rabbits were used to produce crude antitoxin. These animals were injected intradermally with 0.1 ml of a 10⁻² dilution of crude toxin in each of three sites every 4th day for a total of five such injections. Ten days after the last injection the animals were bled and the serum was stored at -20°C.

Preparation of Specific Anti-Alpha Hemolysin.—The serum from each of 15 adult New Zealand white rabbits was tested for antibodies to staphylococcal products by use of anti-alpha and anti-beta hemolysin titrations as well as by immunoelectrophoretic analysis. Of the fifteen rabbits tested only three were negative for each of the tests. These three rabbits were immunized using purified alpha hemolysin by the method used to produce crude antitoxin.

Measurement of Anti-Beta Hemolysin.—The procedure used was essentially the same as

that used for the anti-alpha hemolysin (1), except, of course, that 2 units of beta hemolysin were employed, a 2 per cent suspension of sheep cells replaced the rabbit cells, and following the 1 hour incubation at 37°C the tests were refrigerated for 1 hour.

RESULTS

The results of previous studies (1) led us to the use of electrophoretic methods in an attempt to isolate alpha hemolysin. This work indicated that at a pH of 8.6 the alpha hemolysin made up the advancing edge of the components which migrated toward the cathode. We, therefore, carried out a fractionation at pH 8.6 and found, as expected, that the tube showing the strongest biuret reaction also showed peak alpha hemolytic activity. This same eluate produced the largest area of dermonecrosis in the skin of rabbits and possessed the highest lethal activity for mice. When tested by double agar diffusion and immunoelectrophoretic techniques, this eluate produced one band of precipitate against crude alpha antitoxin. However, when this eluate was subjected to ultracentrifugation procedures, it was found to contain two components. One component was apparently of relatively low molecular weight, since it remained at or near the meniscus, while the other component moved readily. To separate these components it was decided to use two different sets of electrophoretic conditions with the expectation that this would result in a homogeneous alpha hemolysin preparation.

An initial fractionation was carried out at a pH of 5.6 using lactate buffer, ionic strength 0.02, 98 milliamperes. This fractionation resulted in the occurrence of peak alpha hemolytic activity in eluate 10. This fraction also showed the highest biuret activity, was lethal for mice, and produced dermonecrosis when injected intradermally into rabbits.

This fraction was dialyzed against distilled water, concentrated by re- evaporation and refractionated using veronal buffer at a pH of 8.6, ionic strength 0.02, and 98 milliamperes. This fractionation resulted in peak biuret activity in eluate 14. This eluate also showed peak alpha hemolytic, dermonecrotic, lethal, and leucocidal activity. Eluate 14 was tested for purity using double agar diffusion, immunoelectrophoretic, and ultracentrifugation methods.

Agar Double Diffusion Techniques.—The antigenic purity of eluate 14 was tested using this technique. Fig. 1 shows the results of testing crude staphylococcal toxin and purified alpha hemolysin against alpha antitoxin of rabbit origin.

It can be seen that at least three lines of precipitate appear between crude staphylococcal toxin (well *a*) and crude staphylococcal antitoxin (well *b*). However, only one line of precipitate can be detected between crude staphylococcal antitoxin (well *b*) and purified alpha hemolysin (well *c*). These results show clearly that only one antigenic component exists in the purified alpha hemolysin preparation in a concentration high enough to give a visible reaction by this relatively sensitive method.

Microimmunoelectrophoresis.—Experience with this method convinced us that with our materials it is superior to agar double diffusion, since we consistently obtained a larger number of lines of precipitate by this method.

Fig. 2 shows the results of testing crude staphylococcal toxin and purified alpha hemolysin against crude alpha antitoxin by immunoelectrophoretic methods. These reagents were the same as those used in the agar double diffusion studies.

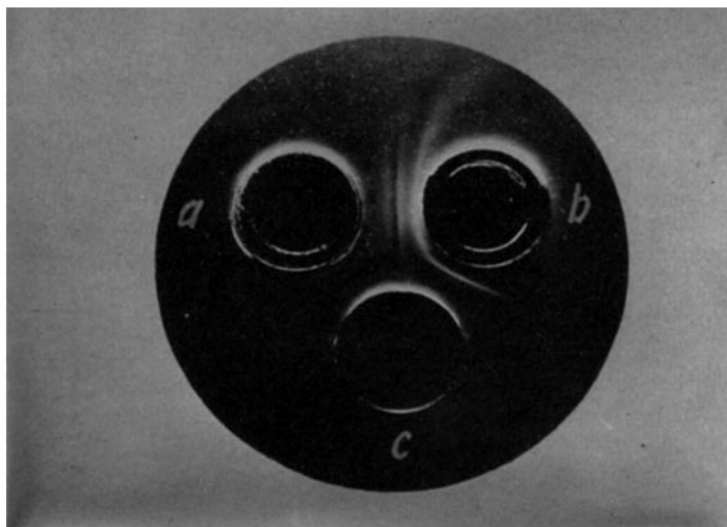


FIG. 1. Well *a*, undiluted crude staphylococcal toxin; well *b*, undiluted crude antitoxin (rabbit origin); well *c*, undiluted purified alpha hemolysin (eluate 14).

An examination of Fig. 2 A reveals that crude toxin contains at least six antigenic components. Fig. 2 B shows that only one detectable antigenic component remains in the purified alpha hemolysin preparation. These results clearly demonstrate that the purification procedures have resulted in a separation of the various components of crude staphylococcal toxin.

*Ultracentrifugation.*¹—The purified fraction (eluate 14) was subjected to ultracentrifugation studies in an effort to detect impurities. The preparation was concentrated by preevaporation and dialyzed for 24 hours against 0.04 M phosphate buffer, pH 7.6. The final preparation had a protein concentration of approximately 0.6 per cent. Fig. 3 (upper three photographs) shows the results of subjecting this preparation to approximately 240,000 *g* using standard methods. Since the sedimentation was slow, it would have been difficult to

¹ We are indebted to Dr. David R. Briggs and Mr. Ronald C. Roberts for their aid in this portion of the work.

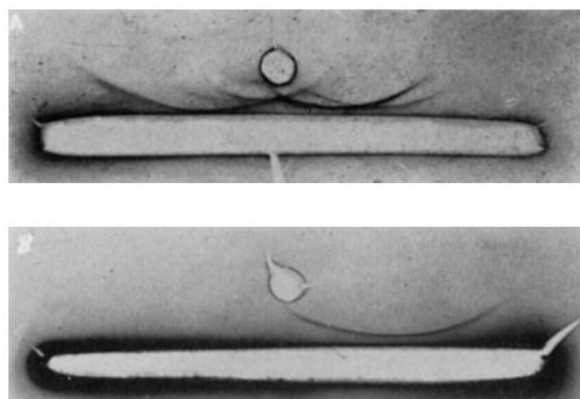


FIG. 2 A. The circular well contained crude staphylococcal toxin, while the elongated moat contained crude staphylococcal antitoxin. 2 B. The well contained purified alpha hemolysin (eluate 14), while the moat contained the same crude staphylococcal antitoxin used in A.

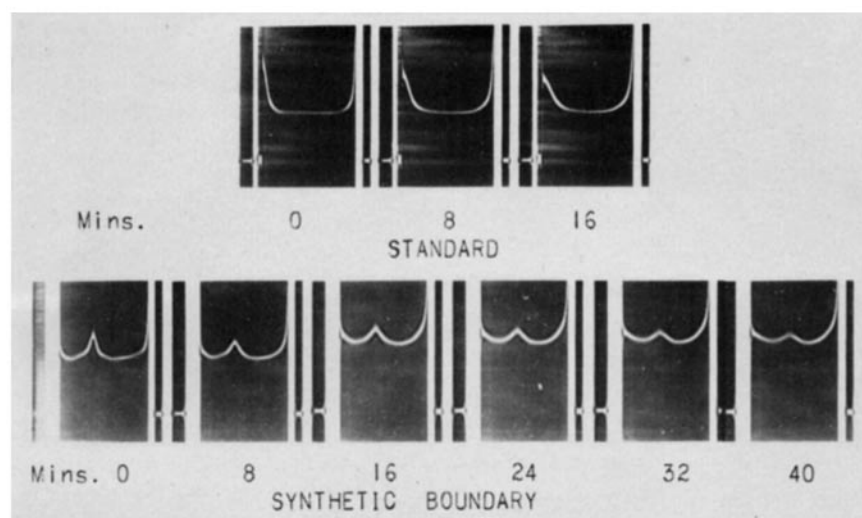


FIG. 3. The first two photographs of migration in the synthetic boundary cell were taken at a bar angle of 35° .

accurately measure the rate of sedimentation under these conditions. To circumvent this difficulty a synthetic boundary cell was employed. Fig. 3 (lower photographs) shows the results of subjecting the purified toxin to approximately $240,000 \times g$ for a period of 40 minutes.

Note that only one peak remained throughout the centrifugation. These results further point to the homogeneity of the product. Calculations made from

the rate of sedimentation in the synthetic boundary cell indicate that the alpha hemolysin has an observed S value of 1.4. This would suggest a molecular weight of the order of 10,000 to 15,000.

The three tests for purity which were applied directly to the purified preparation all point to homogeneity. However, since it has been demonstrated that extremely small amounts of antigen may call forth the production of relatively large amounts of antibody, it was decided to attempt to stimulate the production of antibodies to possible trace antigenic impurities in the preparation. To this end the sera from fifteen rabbits were tested for antibodies to staphylococcal products by use of microimmunoelectrophoretic techniques. Each



FIG. 4. Both wells contained crude staphylococcal toxin obtained from an alpha hemolytic strain. The moat contained serum from a rabbit hyperimmunized with the purified alpha hemolysin (eluate 14).

rabbit serum was also tested against crude toxin from an alpha hemolytic staphylococcus and against crude toxin from a beta hemolytic strain. Of the fifteen sera tested only three were negative to all of these tests. These three rabbits were immunized by a series of injections with purified alpha hemolysin. Following immunization, the sera of these rabbits was tested by microimmunoelectrophoretic methods against crude toxin produced by an alpha hemolytic staphylococcus. Each of the three sera gave only one line of precipitate when tested in this manner. The results of one of these tests is shown in Fig. 4.

The results presented in Fig. 4 show that only one line of precipitate occurs when immunoelectrophoretic techniques using crude toxin and specific anti-alpha hemolysin are applied. This demonstrates that detectable antibodies to possible trace contaminants did not appear in the serum of animals hyperimmunized with purified alpha hemolysin.

DISCUSSION

Agar double diffusion, immunoelectrophoretic, and ultracentrifugation techniques, when applied to purified alpha hemolysin, all point to homogeneity. Further, antibodies to possible impurities in this preparation could not be detected in the serum of animals which were hyperimmunized with this preparation. The fact that this homogeneous preparation possessed alpha hemolytic, dermonecrotic, leucocidal, and lethal activity provides strong evidence that these four activities are but different expressions of one toxic component of crude staphylococcal toxin. This evidence is substantiated by the previously reported observation (1) that these four activities migrate as a unit which is closely associated with a prominent biuret-positive peak and with peak carbohydrate concentration under a variety of electrophoretic conditions. It is felt that this evidence gives direct and rather conclusive support to the unitarian theory.

The importance of having available alpha hemolysin of demonstrated purity becomes evident when one considers the fact that to date the activities of alpha hemolysin have been studied using crude or partially purified preparations. Under these conditions there is no real assurance that the effects observed are truly the result of alpha hemolysin activity, and, therefore, its role in the pathogenesis of staphylococcal infections was difficult to assess. In addition, the availability of purified alpha hemolysin makes possible quantitative studies concerning the immunizing potential of this toxin. This aspect, namely, a knowledge of the immunizing potential of the various individual staphylococcal products, is of fundamental importance, if the present immunization methods are to be improved. Concerning this problem Dubos (2) has emphasized that the present studies on acquired immunity to staphylococcal infections are hampered by the fact that the constituents or products of the staphylococcus responsible for establishing infection have not been properly identified. The proper identification of the factors responsible for infection and lethal damage may well lead to a replacement of the present empirical immunization methods by a rational well oriented approach to the problem.

Studies are contemplated or are already in progress on the immunological, pathological, physiological, and chemical properties of purified alpha hemolysin.

SUMMARY

The continuous flow paper electrophoretic methods used to isolate alpha hemolysin are presented.

The purity of the alpha hemolysin preparation is demonstrated by double agar diffusion, immunoelectrophoretic, and ultracentrifugation techniques.

Indirect evidence of purity is provided by the fact that rabbits immunized with purified alpha hemolysin produced detectable antibody only to alpha hemolysin.

Direct support is given to the "unitarian theory" by the demonstration that purified alpha hemolysin also possessed dermonecrotic, lethal, and leucocidal activities.

Further studies dependent on the availability of purified alpha hemolysin are outlined.

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