THE CHARACTERIZATION OF STAPHYLOCOCCAL TOXINS

I. The Electrophoretic Migration of the Alpha Hemolytic, Dermonecrotic, Lethal, and Leucocidal Activities of Crude Toxin* ‡

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During the course of studies on the reaction of "immunologically unresponsive" rabbits to crude staphylococcal toxin (1) we became interested in the various reported activities of alpha hemolysin. It became important for us to know whether we were dealing with one, or more than one antigen, when we measured the alpha hemolytic, dermonecrotic, and lethal activities of crude toxin.

Several workers (2–10) have presented evidence which supports the "unitarian theory," namely, that the alpha hemolytic, dermonecrotic, and lethal properties of crude staphylococcal toxin represent different activities of one toxin. Contrariwise, an equally large group (11–23) have contended that these three activities do not reside in one toxin. It should be noted, however, that in most cases the evidence found pertaining to the "unitarian theory" was extracted from work which did not have as its principal objective the solution of this problem.

Another toxic effect of crude staphylococcal toxin, namely, its effect on rabbit leucocytes, has been described by Neisser and Wechsberg (24). Some workers (25–27) consider this effect to be a fourth activity of alpha hemolysin, while others (13, 19, 28) feel that this leucocidal effect is an expression of a toxin distinct from the alpha hemolysin.

This study deals with the migration of these four activities in an electrophoretic field under varying conditions.

Materials and Methods

Preparation of Staphylococcal Toxin.—The stability of the Wood 46 strain of *Staphylococcus aureus* used for the preparation of alpha hemolysin was maintained by lyophilization. The methods used by Leonard and Holm (29) for toxin production were modified in that the agar

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concentration of the medium was increased to 0.5 per cent, and the cultures were incubated without shaking in an atmosphere containing 50 per cent CO_2 and 50 per cent O_2. Following incubation the crude toxin was extracted from the medium by the method of Tasman and van der Slot (30). The cells were removed from the extracted toxin by centrifugation at 4°C, and the toxin was concentrated fourfold by perevaporation. The conditions used for the preparation of crude toxin were purposely selected to favor the production of alpha hemolysin.

Electrophoresis.—The Spinco (model CP) continuous flow paper electrophoresis apparatus was used at 4°C with a constant current of 98 milliamperes. Veronal buffer was used at pH levels of 9 and 7.6, while lactate buffer was used at a pH of 5.6 and 3.6. Both buffers were used at an ionic strength of 0.02. Fractions were collected for successive 6-hour periods. The 32 eluates obtained were tested for alpha hemolytic, dermonecrotic, lethal, and leucocidal activities, as well as for biuret-positive material and for carbohydrate concentration. In addition, all eluates were tested for the presence of beta and delta hemolysins.

Measurement of Alpha Hemolysin.—This activity was measured by incubating 0.5 ml of a 2 per cent suspension of washed rabbit red blood cells (washed and suspended in veronal buffer, pH 7.4, containing Mg++) with 0.5 ml of serial twofold dilutions of each eluate. The tests were incubated for 1 hour at 37°C followed by 1 hour at room temperature. The highest dilution of toxin showing complete hemolysis was considered to be the end point of the titration.

Measurement of Delta Hemolysin.—This activity was measured in the same manner as the alpha hemolysin, except that horse erythrocytes were used instead of rabbit erythrocytes.

Measurement of Beta Hemolysin.—The procedure used for the alpha hemolysin titration was used with two exceptions. Sheep red blood cells were substituted for rabbit red blood cells, and instead of incubating at room temperature during the 2nd hour, the tests were refrigerated for 1 hour at 4°C.

Measurement of Dermonecrotic Activity.—Adult New Zealand white rabbits were used for these measurements. The degree of dermonecrosis was determined by injecting 0.1 ml of each eluate intradermally into rabbits which were free of alpha antitoxin. Each eluate showing dermonecrotic activity, and one eluate on each side of the activity range, was then injected intradermally into each of four rabbits. Two days after injection, the diameter of the lesions was measured, and the average area of necrosis due to each eluate was calculated from these measurements. This method was used in preference to the titration of each eluate in an individual rabbit, because we had observed that individual rabbits varied widely in their response to toxin. The rabbits used for measuring dermonecrotic activity were pretested for the presence of anti-alpha toxin.

Measurement of Anti-Alpha Toxin.—An alpha hemolysin titration was carried out in the manner outlined above to establish the hemolytic unit. The hemolytic unit was taken as the smallest amount of alpha hemolysin causing complete hemolysis. Veronal buffer (pH 7.4, containing Mg++) was used to prepare serial twofold dilutions of anti-alpha toxin in 0.25 ml volumes. To each of these dilutions 2 hemolytic units of toxin were added in a volume of 0.25 ml. Following a 10 minute incubation period at 37°C, 0.5 ml of a 2 per cent suspension of washed rabbit erythrocytes were added to each tube. The tests were incubated for one hour at 37°C, followed by 1 hour at room temperature. Rabbits whose serum showed no inhibition of hemolysis were used in testing dermonecrotic activity.

Measurement of Lethal Activity.—"White Swiss" mice (6 weeks old) were used in these titrations. Two mice were injected intraperitoneally with 0.1 ml of each of the 32 eluates. In this way the region of lethal activity was located. Decimal dilutions were prepared in saline from each of the eluates showing lethal activity and from one eluate on each side of the lethal activity range. Three mice were inoculated intraperitoneally with 0.1 ml of each dilution of each eluate. Death within 12 hours was considered to be due to the toxin. The LD_{50} was calculated from these results by the method of Reed and Muench (31).

Measurement of Rabbit Cell Leucocidin.—Leucocytes were collected from rabbits according

to the following procedure. A rabbit was injected intraperitoneally with 500 ml of sterile saline. Eighteen hours later a second injection of 200 ml of saline was administered by the same route. Three hours after the second saline injection, fluid was aspirated from the peritoneal cavity. The leucocytes suspended in the fluid were sedimented by centrifugation at 500 RPM for 10 minutes. The sedimented leucocytes were resuspended in an equal volume of gelatin phosphate buffer. The leucocidin activity was assayed by the method described by Woodin (32).

TABLE I

Results of Continuous Flow Electrophoresis on Crude Staphylococcal Toxins Veronal Buffer, pH 9, Ionic Strength 0.02, Milliamperes 98

Tube No.	Optical density (biuret)	Alpha hemolysin titer*	Average dermonecrosis cm.²-rabbit	LD_{50} mice
1–9	0	0	0	0
10	0.01	0	0	0
11	0.20	64	3.3	$10^{-0.8}$
12	0.77	512	10.6	$10^{-1.5}$
13	0.52	128	4.3	$10^{-1.7}$
14	0.18	32	1.2	$10^{-0.8}$
15	0.17	32	0.1	0
16‡	0.16	0	0	0
17	0.16	0	0	0
18	0.14	0	0	0
19	0.14	0	0	0
20	0.13	0	0	0
21	0.11	0	0	0
22	0.11	0	0	0
23	0.10	0	0	0
24	0.06	0	0	0
25	0.11	0	0	0
26	0.06	0	0	0
27	0.04	0	0	0
28	0.01	0	0	0
29–32	0	0	0	0

* Reciprocal of titer.

‡ Point of application.

Measurement of Human Cell Leucocidin.—The microscopic method described by Gladstone and Van Heyningen (33) was employed.

Measurement of Biuret-Positive Activity.—To 1.0 ml of each of the eluates, 4.0 ml of standard biuret reagent was added. The optical density was read in a Bausch and Lomb spectronic 20 colorimeter at a wave length of 540 mu.

Measurement of Carbohydrate Concentration.—The colorimetric method described by Dubois *et al.* (34) was employed.

RESULTS

A. Electrophoretic Migration of Alpha Hemolytic, Dermonecrotic, and Lethal Activities.—Preliminary paper strip electrophoretic work using a variety of

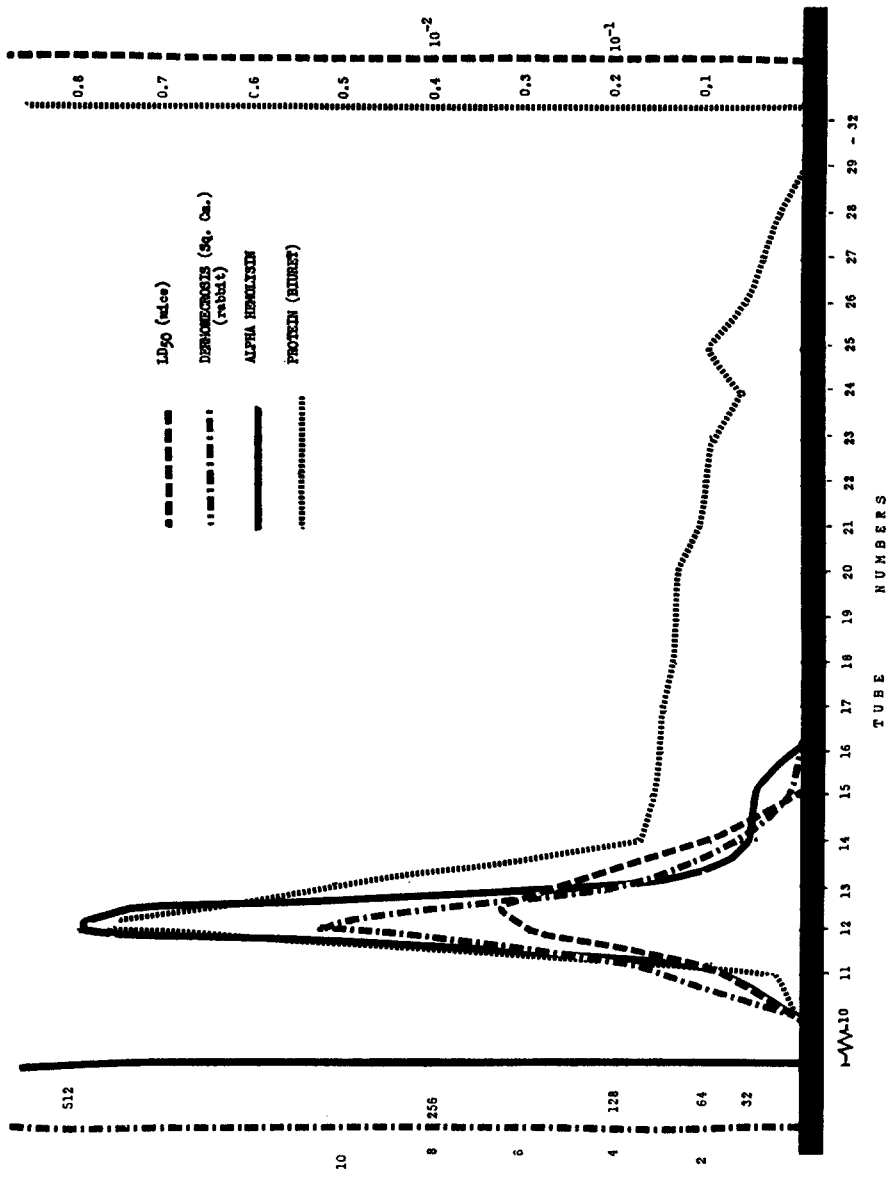
LD50 (mice)
DERMONECROSIS (Sq. Cm.) (rabbit)
ALPHA HEMOLYSIN
PROTEIN (BIURET)
TUBE NUMBERS

FIG. 1.

buffers under different conditions led to the conclusion that veronal and lactate buffers were the most satisfactory for the separation of alpha hemolysin from other components of crude toxin. Therefore, these buffers were used in migration studies employing continuous flow paper electrophoretic techniques.

TABLE II

Results of Continuous Flow Electrophoresis on Crude Staphylococcal Toxins Veronal Buffer, pH 7.6, Ionic Strength 0.02, Milliamperes 98

Tube No.	Optical density (biuret)	Alpha hemolysin titer*	Average dermonecrosis cm.²-rabbit	LD_{50} mice
1–10	0	0	0	0
11	0.05	0	0	0
12	0.81	64	3.0	10^{-1}
13	0.84	256	6.5	$10^{-1.8}$
14	0.85	512	16.0	$10^{-1.8}$
15	0.60	256	7.3	$10^{-1.8}$
16‡	0.32	64	3.7	$10^{-0.8}$
17	0.30	8	0	0
18	0.25	0	0	0
19	0.20	0	0	0
20	—	0	0	0
21	0.13	0	0	0
22	0.09	0	0	0
23	0.09	0	0	0
24	0.07	0	0	0
25	0.05	0	0	0
26	0.05	0	0	0
27	0.04	0	0	0
28	0.04	0	0	0
29	0.05	0	0	0
30	0.01	0	0	0
31–32	0	0	0	0

* Reciprocal of titer.

‡ Point of application.

Table I and Fig. 1 show the results of an electrophoretic experiment using veronal buffer at a pH of 9.

An examination of Table I and Fig. 1 shows that the biuret-positive material in crude toxin is found in 19 tubes (tubes 10 to 28). In addition, it may be seen that a sharp biuret-positive peak is found in the region of tube 12. Further, it will be noted that this biuret-positive peak covers the same region in which peak alpha hemolytic, dermonecrotic, and lethal activities are found. The probability that three distinct substances would migrate in this manner has

been calculated to be 1/406.[1] All eluates were tested for beta and delta hemolysins and were negative.

The results shown in Tables II, III, and IV were obtained using veronal buffer at pH 7.6, and lactate buffer at pH 5.6 and 3.6, respectively.

TABLE III

Results of Continuous Flow Electrophoresis on Crude Staphylococcal Toxins Lactate Buffer, pH 5.6, Ionic Strength 0.02, Milliamperes 98

Tube No.	Optical density (biuret)	Alpha hemolysin titer*	Average dermonecrosis cm.²-rabbit	LD_{50} mice
1	0	0	0	0
2	0.01	0	0	0
3	0.02	0	0	0
4	0.04	0	0	0
5	0.03	0	0	0
6	0.03	0	0	0
7	0.02	8	0.8	0
8	0.03	256	14.4	$10^{-1.5}$
9	0.14	1024	15.4	$10^{-2.5}$
10	0.17	256	13.8	$10^{-1.75}$
11	0.06	16	1.1	0
12	0.06	0	0	0
13	0.06	0	0	0
14	0.06	0	0	0
15	0.05	0	0	0
16‡	0.03	0	0	0
17	0.05	0	0	0
18	0.04	0	0	0
19	0.04	0	0	0
20	0.05	0	0	0
21	0.02	0	0	0
22	0.03	0	0	0
23	0.02	0	0	0
24	0.02	0	0	0
25	0.02	0	0	0
26	0.01	0	0	0
27–32	0	0	0	0

* Reciprocal of titer.

‡ Point of application.

It may be seen from an examination of Tables II, III, and IV, that the alpha hemolytic, dermonecrotic, and lethal activities migrated as a unit. Moreover, the three activities were consistently associated with a prominent peak of

[1] We are indebted to Dr. Charles E. Gates for the statistical analysis of these results (Statistician for Agricultural Experiment Station, University of Minnesota, St. Paul).

biuret-positive material. The probability that three different toxins would migrate in this manner by chance has been calculated to be 1/784, 1/280, and 1/378, respectively. All eluates in these four experiments were negative when tested for beta and delta hemolysins.

A close examination of Tables II and IV will reveal the fact that in these experiments the biuret-positive peak is relatively flat. However, it is also evident that the curves of the three activities are also relatively flat and con-

TABLE IV

Results of Continuous Flow Electrophoresis on Crude Staphylococcal Toxins Lactate Buffer, pH 3.6, Ionic Strength 0.02, Milliamperes 98

Tube No.	Optical density (biuret)	Alpha hemolysin titer*	Average dermonecrosis cm.²-rabbit	LD_{50} mice
1–15	0	0	0	0
16	0.01	0	0	0
17	0.04	0	0	0
18	0.02	0	0	0
19	0.06	0	3.2	0
20	0.27	64	8.4	$10^{-.8}$
21	0.71	256	17.1	$10^{-1.8}$
22	0.80	256	13.3	$10^{-1.8}$
23	0.83	512	12.9	$10^{-1.8}$
24‡	0.85	256	11.6	$10^{-1.6}$
25	0.43	0	0	0
26	0.04	0	0	0
27	0.04	0	0	0
28	0.03	0	0	0
29	0.04	0	0	0
30	0.03	0	0	0
31	0.02	0	0	0
32	0	0	0	0

* Reciprocal of titer.

‡ Point of application.

form nicely with the biuret curve. We feel that the "flattened" curve in these experiments resulted from a slight shift in the balance of the machine during operation.

B. The Electrophoretic Migration of Alpha Hemolytic and Leucocidal Activities.—Several workers have presented evidence for (25–27) and against (13, 19, 28) the contention that the leucocidin of Neisser and Wechsberg represents a fourth activity of alpha hemolysin. The results presented in Table V and Fig. 2 provide evidence in support of the view that this leucocidin and the alpha hemolysin are expressions of one toxin.

Table V and Fig. 2 present results which clearly show that eluate 9 possesses the highest

concentration of biuret-positive material as well as highest carbohydrate concentration. It will also be seen that eluate 9 has peak alpha hemolytic and leucocidal activity. When this eluate was injected intradermally into rabbits, large dermonecrotic lesions resulted. It was also lethal for mice.

An additional continuous flow electrophoretic experiment was conducted under the same conditions as above, except that veronal buffer, pH 9, was employed. In this experiment eluate 13 contained the highest concentration of biuret-positive material and of carbohydrate. This

TABLE V

Results of Continuous Flow Electrophoresis on Crude Staphylococcal Toxins Lactate Buffer, pH 5.6, Ionic Strength 0.02, Milliamperes 98

Tube No.	Optical density (biuret)	Optical density carbohydrate	Alpha hemolysin titer*	Reciprocal leucocidin titer
1–3	0	0	0	0
4	0.1	0	0	0
5	0.1	0	0	0
6	0	0	0	0
7	0.2	0	16	0
8	0.7	18	256	8
9	2.9	29	512	32
10	0.8	8	128	4
11	0.2	0	16	0
12	0.1	0	0	0
13	0.1	0	0	0
14	0.1	0	0	0
15	0.15	0	0	0
16‡	0.2	0	0	0
17	0.1	0	0	0
18	0.15	0	0	0
19	0.1	0	0	0
20	0.02	0	0	0
21–32	0	0	0	0

* Reciprocal of titer.

‡ Point of application of toxin.

eluate also showed peak alpha hemolytic, leucocidal, dermonecrotic, and lethal activities. Eluate 9 in the previous experiment and eluate 13 in this experiment were not toxic for human leucocytes, and were also negative when tested for beta and delta hemolysins.

DISCUSSION

The results herein reported strongly suggest that the alpha hemolytic, dermonecrotic, and lethal activities of crude staphylococcal toxin are but different expressions of one toxic component. The probability that three distinct toxins would have the same isoelectric point and the same charge density and therefore migrate in the same direction at the same rate is extremely remote.

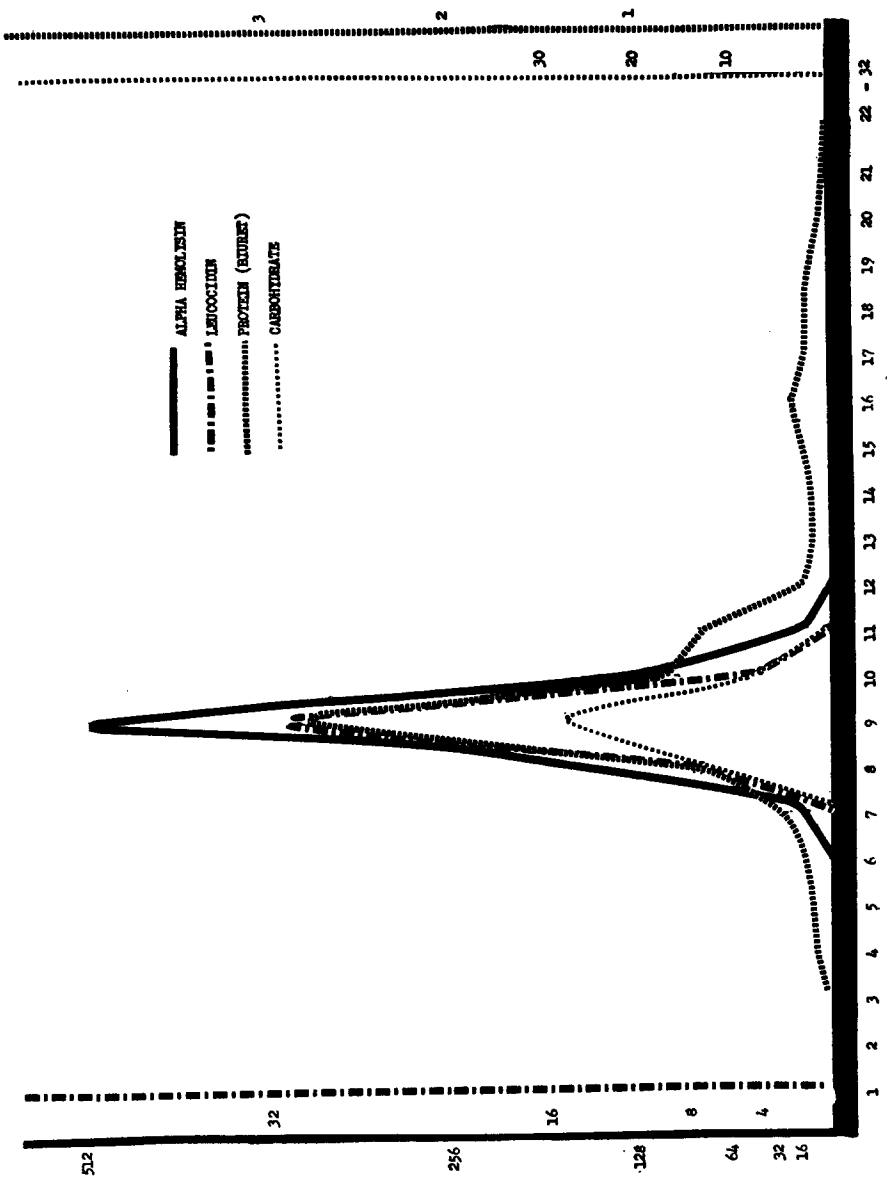
ALPHA HEMOLYSIN
LEUCOCIDIN
PROTEIN (MURES)
CARBOHYDRATE
TUBE NUMBERS
512
256
128
64
32
16
32
16
8
4
30
20
10
3
2
1

FIG. 2.

A calculation showed probabilities of 1/406, 1/784, 1/280, and 1/378, respectively, for each of the four experiments reported.

The limited studies which have been presented on the leucocidin of Neisser and Wechsberg support the view that this leucocidin represents a fourth activity of alpha hemolysin. This leucocidal activity migrated as a unit with the alpha hemolysin under two distinct sets of electrophoretic conditions. The fact that the eluates showing peak leucocidal activity against rabbit leucocytes were completely inactive against human leucocytes suggests that the leucocidin of Panton and Valentine (35) was not involved. Further, since the "leucolysin" of Gladstone and Van Heyningen (33) is believed to be an activity of the delta hemolysin, and since delta hemolytic activity was not detected in any of the eluates, it is very doubtful that this substance was involved. Additional evidence which supports the "unitarian theory" is presented in the article immediately following (36).

During preliminary paper strip electrophoretic studies, it became apparent that the alpha hemolysin consistently migrated toward the cathode at pH values ranging from 3.6 to 9.0. The continuous flow paper electrophoretic results recorded in Table I to V also show that within the same pH range the biuret-positive peak and the four associated activities consistently move to the cathode. This would indicate that under these conditions the alpha hemolysin carries a net positive charge. Butler (23) has also reported similar observations.

With this fact in mind, namely, that the alpha hemolysin is positively charged over a wide pH range, two explanations present themselves. If it is postulated that the alpha hemolysin is a conjugate containing protein and carbohydrate fractions, it would explain the fact that the peak biuret-positive material coincided with peak carbohydrate concentration (Table V and Fig. 2). Further, if it is assumed that the linkage between the protein and carbohydrate fractions is *via* the negatively charged carboxyl group of the protein fraction, one would expect a conjugate carrying a predominantly positive charge. A second explanation would, of course, be that the protein fraction of the conjugate contains a high concentration of basic amino acids. A contemplated amino acid and carbohydrate analysis of the purified preparation should provide information concerning the chemical composition of the alpha hemolysin.

SUMMARY

Continuous flow paper electrophoretic techniques have yielded results which strongly support the theory that the alpha hemolytic, dermonecrotic, and lethal activities of staphylococcal toxin are all expressions of one toxic unit.

Evidence is also presented which supports the contention that the leucocidin of Neisser and Wechsberg represents a fourth activity of alpha hemolysin.

Some of the peculiarities of the electrophoretic migration of alpha hemolysin are discussed.

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