MECHANISM OF ACTION OF STAPHYLOCOCCAL ALPHA-HEMOLYSIN

I. SOME FACTORS INFLUENCING THE MEASUREMENT OF ALPHA-HEMOLYSIN

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ABSTRACT

Marucci, Americo A. (Upstate Medical Center, Syracuse, N.Y.). Mechanism of action of staphylococcal alpha-hemolysin. I. Some factors influencing the measurement of alpha-hemolysin. J. Bacteriol. 86:1182–1188. 1963.—A kinetic method for the accurate and reproducible measurement of the action of staphylococcal alpha-hemolysin on rabbit erythrocytes is described. The activity of the alpha-hemolysin depends upon the temperature used for measurement. At 37 C the hemolysin lyzes cells faster, but it in turn is rather quickly inactivated. At 0 C there is no inactivation, but the rate of lysis is greatly decreased. There is no change in the activity with change in the total reaction volume, providing that the concentrations of cells and hemolysin are kept constant. The fraction of rabbit red cells lysed by a given amount of hemolysin in a given time is constant and independent of the total number of cells in the reaction mixture.

During the past 10 years, investigations on all phases of Staphylococcus aureus have been renewed and expanded. The literature up to 1959 has been comprehensively reviewed by Elek (1959). Most of the recent work relating to the alpha-hemolysin has been concerned with purification, characterization, and methods of measurement (Jackson and Little, 1957; Mangold and Raynaud, 1959; Robinson, Thatcher, and Montford, 1960; Butler, 1959; Kumar and Lindorfer, 1962; Madoff and Weinstein, 1962; Lominski and Arbuthnot, 1962; Bernheimer and Schwartz, 1963).

There have been no studies devoted exclusively to the mechanism of action of the staphylococcal alpha-hemolysin. Since this was our objective, it was imperative that an accurate and reproducible method of measurement be employed. The system of measurement should also be flexible enough to permit variation of reagents and conditions which might affect the alpha-hemolysin activity. The present paper is concerned with the description of the kinetic method of analysis developed for the measurement of staphylococcal alpha-hemolysin and of some factors which influence its measurement.

MATERIALS AND METHODS

Hemolysin. Batches of 200 ml of Brain Heart Infusion broth (Difco) in 500-ml Erlenmeyer flasks were inoculated with 25 ml of an 18-hr culture of Staphylococcus aureus (Wood 46 strain), grown under 10 to 20% CO2 in the same medium. The 500-ml flasks were placed in anaerobic jars and CO2 was added. The sealed jars were fixed on a shaker which imparted rotary motion at 150 complete 1-in. strokes per min. After overnight growth at 37 C, the cells were removed by centrifugation at 2000 × g at 3 C for 30 min; the supernatant fluid was then Seitz-filtered with the receiving flask kept in an ice-bath. To this filtrate, at −10 C, 95% ethanol was added slowly with continuous stirring until the final ethanol concentration was 45% (v/v). This mixture was left overnight at −10 C. The precipitate was collected by centrifugation at −10 C at 2000 × g for 30 min, and was suspended as quickly as possible in the minimal amount of ice-cold distilled water sufficient for quantitative transfer to dialysis bags. The dialysis was carried on with continuous shaking for 18 to 20 hr at 2 to 5 C against 20 liters of distilled water. During this time the precipitate dissolved. The solution of hemolysin was removed from the dialysis bags and distributed into Pyrex tubes in 1.1- or 0.6-ml samples. This hemolysin solution contained 0.640 mg of N/ml. The tubes were capped with rubber-apron stoppers and were stored at −20 C. For use in an experiment, one of the tubes was removed from the deep-freeze, thawed, and diluted in ice-cold buffer.

Agar diffusion in petri plates against the anti-
serum described below showed this fraction to consist of at least eight antigens reactive with this serum.

**Erythrocytes.** Rabbit blood was collected by cardiac puncture and added to an equal volume of Alsever's solution. To this mixture (usually 60 ml), Merthiolate was added to a final concentration of 1:10,000, and the cells were stored at 2 to 5 C for at least 4 days before being used.

For use in an experiment, a portion of these cells was washed three times with the staph-buffer described below; 1 ml of the packed, washed erythrocytes was then suspended in 19 ml of buffer, and 1.0 ml of this 5% suspension was added to a 15-ml volumetric flask. The cells were lysed by bringing the volume up to the 15-ml mark with 0.1% NaHCO₃. The optical density (OD) of this hemoglobin solution was determined in a Beckman DU spectrophotometer in 10-mm cuvettes at a wavelength of 541 nm with distilled water as the blank. We had determined that an OD of 0.600 under these conditions represents 5 x 10⁶ cells per ml in the original suspension. We adjusted the cell suspension to contain 5 x 10⁶ cells per ml by use of the following relationship:

$$\text{OD}_0 \times 19 = 19 + x$$

where OD₁ = optical density of the 5% suspension when 1 ml is lysed in 15 ml, and x = number of ml of buffer to be added to 19 ml of 5% suspension to adjust the OD to 0.600, i.e., 5 x 10⁶ cells per ml.

**Staph-buffer.** Buffer was prepared by dissolving 4.0 g of 5,5-diethylbarbituric acid, 0.78 g of Na, 5,5-diethylbarbiturate, 83.6 g of NaCl, and 2.52 g of NaHCO₃ in a 2-liter volumetric flask and bringing the volume up to the mark with distilled water. This 5 x isotonic buffer was stored at 2 to 5 C. For use each day, a portion was accurately diluted to isotonicity, and sufficient gelatin was added so that the final gelatin concentration would be 0.5%. The pH of the diluted buffer should be 7.0 to 7.2.

**Antiserum.** The antiserum used throughout the present study was Staphylococcus Antitoxin, lot 96-1, purchased from Connaught Medical Research Laboratories, Toronto, Canada. This antiserum was made up by pooling serum from horses inoculated with filtrates of various strains of staphylococci. It was modified by treatment with pepsin and was labeled to contain 20,000 antitoxin units per vial, which represents approximately 2220 antitoxin units per ml.

**Volumetric glassware.** Ostwald-Folin volumetric pipettes, calibrated in this laboratory to an accuracy of better than 2 parts in 1,000, were used throughout. All volumetric glassware was cleaned in sulfuric acid-dichromate cleaning solution, and was thoroughly rinsed with tap water and then distilled water before being dried in an oven set at 40 to 45 C.

**Water baths.** Two 10-gallon aquaria were used. Temperature was maintained in each water bath by use of a mercury thermoregulator connected through a relay to a low-lag immersion heater. Each bath was equipped with a mixer. For temperatures below ambient, ethylene glycol at 0 C was circulated through a copper cooling coil which was immersed in the water bath. The flow of ethylene glycol was regulated so that the heating element was not overtaxed. The temperature variation of the baths did not exceed ±0.2 C. For experiments done at 0 C, the baths were filled with shaved ice and water.

A shaking arrangement was constructed over the water baths. By use of this, Erlenmeyer flasks which contained the reaction mixtures could be clamped so that they remained partially submerged in the water while wrist-action motion was imparted through an angle of 60 to 70° at about 38 complete strokes per min. In this way, the cells were kept in suspension throughout the experiment.

**Kinetic analysis.** The kinetic analysis was patterned after the method developed by Mayer (1961) for study of immune lysis. Buffer and hemolysin, kept ice-cold, were pipetted into the reaction flasks, which were kept in an ice-bath. Upon completion of the addition of the reagents, the flasks were placed in the kinetic water bath set at 20 C and, after sufficient time had elapsed for temperature equilibration, the standardized red blood cells, also at 20 C, were added while the flasks were shaking. Experiments were done to show that the order of mixing, i.e., adding the dilutions of hemolysin to the erythrocytes in the flasks, did not affect the kinetic results. Addition of the last reagent initiated the reaction. Samples (3 ml) were removed at appropriate intervals and centrifuged immediately in table model centrifuges (Clay-Adams, Inc., New York, N.Y.; CT 1002/D). The total time in the centrifuge was 3 to 3.5 min; acceleration time, 0.3 min; decelera-
TABLE 1. Protocol* and results of a typical kinetic experiment

<table>
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<tr>
<th>Start time</th>
<th>Sample no.</th>
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<th>T'</th>
<th>OD</th>
<th>OD'</th>
<th>No. of cells lysed</th>
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<td>2.26</td>
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<td>0.002</td>
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<td>0.224</td>
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<td>0.342</td>
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<tr>
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<td>0.003</td>
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<td>37.84</td>
<td>37.84</td>
<td>0.003</td>
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</tbody>
</table>

* Flasks A, B, C, D, E, and F contained, respectively, 3.0, 2.5, 2.0, 1.5, 1.0, and 0 ml of hemolysin (1:50) in 25.0, 25.5, 26.0, 26.5, 27.0, and 28.0 ml of buffer. To each flask were added 2.0 ml of cells (optical density = 0.000).

a Time of addition of the erythrocytes. Start time 1.65 was with flask A; 1.07 was with flask B; 0 was with flask F.

b T = time centrifuge started with sample.

c T' = T minus start time, i.e., net time of reaction.

OD = optical density of the supernatant fluid.

OD' = optical density of the supernatant fluid minus the optical density of the supernatant fluid from the cell control taken at the same time (flask F).

* Number of cells lysed X 10^8.

poured into appropriately labeled tubes. These tubes were kept in an ice-bath until the completion of the experiment, at which time the optical densities were determined and converted either into number of cells lysed or into percentage of lysis.

The time for lysis of 50% of the cell suspension (T50) was determined directly from the plot of the curves relating lysis to time of reaction.

RESULTS

Representative kinetic analysis. To demonstrate the method of computation of the results, the protocol for a representative set of data in which varying amounts of hemolysin were used in a kinetic experiment is given in Table 1, and a plot of the complete data for this experiment is shown in Fig. 1.

The kinetic curves are characterized by three general regions: (i) a lag period, the length of which varies inversely with the amount of hemolysin used; (ii) a period of rapid, though not constant, lytic rate; and (iii) a period of continual creeping lysis approaching complete lysis.

Stability of alpha-hemolysin at 0 C. Since different time factors are involved in the preparation of

FIG. 1. Kinetic analyses with varying amounts of staphylococcal alpha-hemolysin.
the various reaction flasks for the kinetic analyses, it is of paramount importance to ascertain that there is no loss of activity of the diluted hemolysin kept at 0 C. To study this, an experiment was set up in which the same amount of hemolysin was added to four flasks (A, B, C, and D). These were held in a kinetic water bath at 0 C. The standardized rabbit erythrocytes, also at 0 C, were added to start the reaction. At 15 min, flask B was placed in the 20 C kinetic bath, and samples were removed at appropriate time intervals. Flasks C and D were removed at 30 and 60 min, respectively, and were treated as was flask B. Flask A was left at 0 C, and samples were removed at prolonged intervals. The results of this experiment are shown in Fig. 2.

There is no loss of activity up to 1 hr at 0 C. This is shown by the parallel time-lysis curves for flasks B, C, and D. The times for 50% lysis after these flasks were placed at 20 C were 14.6, 13.8, and 12.5 min, respectively. The probable explanation for this slight difference in the T50 values is that the hemolysin is already acting on the cells at 0 C, as shown in flask A, and this then gives a slight advantage to the cells left at 0 C for the longer periods. In other experiments, hemolysin activity remained constant over a period of 4 to 5 hr at 0 C.

Optimal temperature for kinetic analyses. The approach to this type of experiment was to add the standardized rabbit red cell suspension to identical amounts of hemolysin which had been held at a given temperature for specified lengths of time. Samples were taken in the usual fashion, and, from the plot of the data, the time for 50% hemolysis was taken (Table 2).

At 0 C there is no loss of activity, even after 4 hr. The lag period is greatly prolonged at this temperature, resulting in no observable lysis for 1 to 1.5 hr (Fig. 2). From an operational standpoint, it would be most inconvenient and wasteful of time to use this temperature for routine kinetic analyses. As the temperature for carrying out the analyses is increased, the reactions proceed more

**TABLE 2. Time for 50% lysis of standardized rabbit erythrocyte suspension by identical hemolysin dilutions held at a given temperature for definite periods of time**

<table>
<thead>
<tr>
<th>Temp</th>
<th>Time (min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>20.6</td>
</tr>
<tr>
<td>20</td>
<td>14.6</td>
</tr>
<tr>
<td>25</td>
<td>8.0</td>
</tr>
<tr>
<td>37</td>
<td>4.8</td>
</tr>
</tbody>
</table>

**FIG. 2. Stability of the alpha-hemolysin at 0 C.**
TABLE 3. Time for 50% lysis of erythrocytes from different rabbits by the same alpha-hemolysin dilution

<table>
<thead>
<tr>
<th>Donor rabbit no.</th>
<th>T50 min</th>
<th>Avg. T50 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.5</td>
<td>14.8</td>
</tr>
<tr>
<td>2</td>
<td>14.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>19.0</td>
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<tr>
<td>8</td>
<td>14.9</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>15.9</td>
<td></td>
</tr>
<tr>
<td>Pool*</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>Average†</td>
<td>15.1</td>
<td></td>
</tr>
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</table>

* Prepared by mixing 1.0 ml of standardized erythrocytes from each rabbit.
† Average for cells from all rabbits.

quickly. However, there is appreciable diminution of activity when the diluted hemolysin is kept at the higher temperatures for varying periods.

Since experiments were planned in which the reactions would be followed for 1 to 2 hr, it was essential that the deleterious effect due to temperature be minimized. With this end in view, we chose 20 C as the temperature for our kinetic analyses.

Effect of different rabbit erythrocytes. Erythrocytes from nine adult rabbits were standardized, and a pool was prepared by mixing 1.0 ml from each standardized suspension. Kinetic analyses were run with the same hemolysin dilution and each of the cell suspensions.

There is a pronounced difference in the susceptibility of rabbit erythrocytes from different animals (Table 3). In this experiment the greatest variation in T50 was 42% (13.4 min vs. 19.0 min). The T50 of the pooled cells was between the extremes of the most and least susceptible cells, but it did not correspond to the average of the nine cell suspensions from which it was made. These results mean that direct quantitative comparison of hemolysin activity is not possible when different batches of erythrocytes are used.

Effect of total reaction volume. Since experiments were planned involving various volume relationships, it was deemed advisable at the outset to establish whether the course of hemolysis was affected in any way by a change in total volume. To study this, an experiment was set up in which the total volume of the reaction mixtures was varied from 15 to 60 ml, but the relative proportions of hemolysin, red blood cells, and total volume were kept constant. The results of this experiment are shown in Fig. 3.

The fourfold difference in total volume causes no detectable change in the kinetic course of hemolysis when the proportions of alpha-hemolysin, cells, and total volume are kept constant. The absolute number of cells lysed with respect to time is the same under these changes in conditions.

Effect of red cell concentration. Varying amounts of hemolysin were assayed against varying numbers of the same batch of standardized rabbit erythrocytes. The experimental protocol is given in Table 4 and the results are shown in Fig. 4.

Under the conditions of this experiment, i.e., constant total volume, constant hemolysin concentration, and sixfold variation in red cell concentration, the fraction of cells lysed by a given amount of hemolysin is constant and independent of the number of erythrocytes originally present in the reaction mixture.

**Discussion**

The most frequently employed method of measurement of alpha-hemolysin activity involves re-
acting twofold dilutions of the hemolysin with a constant volume of red cells for a stated period of time (Thal and Egner, 1954; Butler, 1959; Johnson et al., 1961; Madoff and Weinstein, 1962; Bernheimer and Schwartz, 1963). The hemolysis is estimated visually or with a photometer, and the titer is taken as the dilution of hemolysin in the tube showing 50% lysis. The inherent error in this dilution procedure is 100%. In addition, in view of the continued creeping lysis shown in the kinetic curves, the end points will vary with the time selected for observation. One group of workers used 100% lysis as the end point in this type of analysis (Kumar and Lindorfer, 1962). Since the kinetic curves are sigmoidal, this end point selection introduces an additional uncertainty into these estimates.

Mangalo and Raynaud (1959) and Lominski and Arbuthnott (1962) carried out the reactions in spectrophotometer cuvettes. They then followed the course of hemolysis by density measurements. Jackson and Little (1957) employed a kinetic method in which the total volume was 6.0 ml. A standardized cell suspension was used, but supernatant fluids had to be diluted in order to be in a range for suitable optical density measurements.

For purposes of determining the mechanism of action of alpha-hemolysin, the published methods suffer from inherent inaccuracy or are restrictive in terms of number of reactants and conditions which can be varied. A flexible, yet accurate and reproducible, kinetic method for measurement is given in the present paper.

The shape of the kinetic curve describing staphylococcal alpha-hemolysin activity is sigmoidal with three general regions: a lag period, a period of accelerated lysis, and a terminal period of continued creeping lysis.

The apparent activity of the hemolysin depends on the temperature used for measurement. At 0 C, the lag period is greatly prolonged, but there is little or no loss in activity. At 37 C, the lag period is abbreviated, but the hemolysin is rapidly inactivated. In view of these wide temperature differences, it is essential that the temperature used for assay of the hemolysin be kept constant and within close limits. As the temperature for performing our analyses, we have selected 20 C, since neither the lag nor thermal inactivation are maximal at this temperature.

Variation in susceptibility of cells from different rabbit donors is well known (Jackson and Little,

1957; Bernheimer and Schwartz, 1963). The reason for this effect, however, has not been elucidated. Age of the donor animal does not seem to play a role, for the rabbits used in these studies were healthy young adults in the same general age and weight groups. The variability may relate to the differing availability of reactive sites on the red cells from different rabbits. The answer to this problem will have to await further detailed experimentation on the rabbit erythrocyte receptor sites.
The fraction of cells lysed by a given amount of alpha-hemolysin is independent of the number of cells in the reaction mixture. This is analogous to the “percentage law” of Andrewes and Elford (1933) with relation to the inhibition of phage by specific antisera. The significance of this finding and its relation to the mechanism of action of alpha-hemolysin are discussed in the accompanying paper (Marucci, 1963).

ACKNOWLEDGMENT

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LITERATURE CITED


