Nepelometric Assay of Staphylococcal Coagulate

FRED J. STUTZENBERGER, CHARLES L. SAN CLEMENTE, AND DHARAM V. VADEHRA

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan

Received for publication 24 June 1966

ABSTRACT

STUTZENBERGER, FRED J. (Michigan State University, East Lansing), CHARLES L. SAN CLEMENTE, AND DHARAM V. VADEHRA. Nepelometric assay of staphylococcal coagulate. J. Bacteriol. 92:1005-1009. 1966.—Clotting of fibrinogen by staphylococcal coagulate was accompanied by an increase in light scattering; this property was used as a basis for a new nepelometric method. Reaction rates, which were now easily and precisely measured, were found to be directly proportional to coagulate concentration, when optimal conditions were maintained. These conditions included pH and concentrations of fibrinogen, coagulate-reacting factor, and sodium chloride in the reaction mixture. A standardized procedure for the assay is outlined, and a unit for the expression of activity is proposed.

Although staphylococcal coagulate and its ability to clot fibrinogen have been studied for over 60 years, no precise, quantitative method to determine the rate of clotting has been proposed. Current methods (7, 10, 14) employ serial dilution for the estimation of coagulate activity. Durhie and Haughton (3) used clotting time as the basis for the determination of the coagulate reaction rate. All of these methods, depending on visual determination of the clot, are subject to individual judgment and, thus, lack uniformity. However, the increase in light scattering which accompanies the clotting of fibrinogen seems to offer an accurate means of measuring coagulate reaction rates; light refraction properties have been used to study the shape and size of a range of macromolecules (1, 8). This report describes a precise, reproducible, and rapid nepelometric method for the assay of coagulate activity.

MATERIALS AND METHODS

 Cultures. Staphylococcus aureus strain 70 of the International-Blair Series (2) produced the coagulate used in this assay procedure.

Medium. The medium used for coagulate production contained the following: 0.3% Casamino Acids (Difco); 0.1% glucose; 0.001% each of thiamin, niacin, MgSO4·7H2O, MnSO4·H2O, and FeSO4·7H2O; and 0.5% each of K2HPO4 and KH2PO4. A 1-ml amount of the mineral solution of Tager and Hales (14) was added per liter. The Casamino Acids-salts solution was sterilized by autoclaving at pH 7.2 to 7.4; glucose and vitamins were sterilized by membrane filters (Millipore Filter Corp., Bedford, Mass.) and were added aseptically.

Growth conditions. After growth for 18 hr, 5% inocula was transferred to 190-ml volumes of the same medium contained in 500-ml Erlenmeyer flasks. Cultures were incubated on a rotary shaker at 37°C for about 18 hr, at which time an optical density of 0.5 to 0.6 at 625 mμ was attained.

Partial purification of coagulate. After removal of the cells by centrifugation, coagulate in the cell-free supernatant fluid was partially purified by repeated acid precipitation at pH 3.5. Diethylaminoethyl (DEAE) chromatography followed. The preparations used in these studies had a specific activity of 1.024 X 10⁶ reciprocal titer units per mg of protein.

Substrate system used in assay. A fresh stock solution of bovine Fraction I citrated fibrinogen (Sigma Chemical Co., St. Louis, Mo.), containing 300 mg of fibrinogen per 100 ml, was prepared daily in 0.01 M phosphate buffer having a final pH of 7.2. To determine optimal fibrinogen concentration for the assay in initial experiments, amounts were varied as follows: 12.5, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mg/ml. Higher concentrations of fibrinogen could be attained only by increasing the ionic strength of the solution. All solutions were clarified with membrane filters (Millipore Filter Corp.). Noninhibitory human plasma (2%), clarified by filtration or centrifugation, was added to each concentration of fibrinogen to provide coagulate-reacting factor (CRF). To determine optimal concentration of plasma-CRF, the amounts were varied as follows: 0.0, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0%. Effect of sodium chloride concentration in the final reaction mixture was studied over a range of 0.017 to 0.35 M; pH was also varied from 6.2 to 9.2. Spontaneous precipitation of the fibrinogen below pH 6.2 precluded any studies at lower values.

Assay procedure. The instrument used for the assay of coagulate activity was a Lumetron model 402E

1 Published with the permission of the Director of the Michigan Agricultural Experiment Station as Journal No. 3872.
colorimeter (Photovolt Corp., New York, N.Y.) with nephelometric attachment; the galvanometer was replaced by a Sargent model SR recorder (E. H. Sargent and Co., Detroit, Mich.). A Kahn antigen tube, cut to fit the instrument, served as the cuvette. The nephelometer was calibrated with BaSO₄ suspension as described by Kunkel et al. (9). A stock BaCl₂·2H₂O solution was prepared in a concentration of 1.15 g/100 ml of distilled water. A 3-ml amount of this solution was diluted to 100 ml with 0.2 N H₂SO₄. The resulting turbidity of the BaSO₄ suspension was assigned an arbitrary value of 20 nephelometric units. This suspension was serially diluted with 0.2 N H₂SO₄ to prepare standards for a calibration curve, where nephelometric units were plotted against recorder response in the 1.25-mv range.

Coagulase activity was quantitated in the following manner: 1 ml of the coagulase preparation to be assayed was thoroughly mixed with 2 ml of the fibrinogen-plasma substrate in the same cuvette used in calibration of the instrument. The increase in light scattering, which accompanied the clotting reaction, was automatically recorded, and the rate of the reaction was determined from the slope of the curve.

The clotting rates of similar reaction mixtures were determined in some exploratory experiments with a Brice-Phoenix light-scattering photometer (Phoenix Precision Instrument Co., Philadelphia, Pa.). The instrument employed in these studies was equipped with a laser as a light source, emitting wavelengths of 632.8 m. The increase in light scattering was measured at an angle of 90° from the incident light path. These increases were recorded on a Honeywell recorder (Honeywell Corp., Minneapolis, Minn.), and the rates were determined.

Unavailability of temperature control for either the Lumetron or the Brice-Phoenix instrument precluded any studies involving effects of temperature. All data reported in these studies were obtained at room temperature (22°C).

RESULTS

Preparation of the standard curve. After the BaSO₄ suspension was diluted to give nephelometric units equivalent to 2.5, 1.25, and 0.625, the recorder response to each value was determined. A linear relationship existed between nephelometric units and recorder response. This relationship was utilized in the conversion of recorder response to nephelometric units.

Effect of fibrinogen concentration upon rate of coagulase reaction. Fibrinogen concentrations of the stock substrate solutions were varied from 12.5 to 500 mg/100 ml, whereas 2% plasma-CRF was held constant. A 1-ml amount of the highly active coagulase preparation was mixed with 2 ml of each fibrinogen concentration and the reaction rate was determined (Fig. 1). Maximal activity was achieved with 300 mg of fibrinogen per 100 ml; higher concentrations showed no increase in clotting rate.

Effect of plasma-CRF concentration upon rate of coagulase reaction. The coagulase reaction rate was very dependent upon the percentage of plasma-CRF added to the substrate; maximal activity occurred in excess of 1.5% plasma (Fig. 2).

Dependence of coagulase reaction rate on pH. It was found that pH had a marked influence upon the rate of the coagulase reaction (Fig. 3). A change of one pH unit from the optimal value resulted in approximately 50% decrease in reaction rate. The optimal pH for this system was in the range of 7.1 to 7.3.

Sodium chloride dependence of coagulase reaction rates. Although fibrinogen solutions are
usually prepared in physiological saline (0.15 M NaCl), the optimal sodium chloride concentration for the coagulase reaction was 0.05 to 0.08 M. These data (Fig. 4), obtained with the Lumetron nephelometer, were verified in visually observed test-tube experiments, by plotting the reciprocal of the minimal clotting time (minutes required for the formation of a 4+ clot) against sodium chloride concentration. Precise instrumental methods and visual approximations both indicated that the salt concentration of the reaction mixture was highly critical.

Relationship of coagulase concentration to reaction rate. A linear relationship over a wide range of activities existed between coagulase concentration and clotting rate, when followed nephelemetrically. This proportional relationship between coagulase concentration and recorded increase in nephelometric units of the reaction mixture (Fig. 5) was the basis for the precise quantitation of coagulase activity. In comparison, it was found that the Brice-Pheonix light-scattering photometer could also be readily utilized in the assay procedure. The rate of increase in light scattering by the clotting mixtures, measured in preliminary tests simply as recorder units per minute, was plotted against coagulase concentration (Fig. 6). Because of the greater sensitivity of this instrument, the minimal amount of coagulase that could be accurately quantitated was about one-half that required with the Lumetron nephelometer. Also, with the more sensitive instrument, an inverse linear relationship (Fig. 7) existed between coagulase concentration and the lag period (time required for initiation of detectable increase in light scattering, after mixing of coagulase and substrate). With very active coagulase preparations, however, this lag period was quite brief and, therefore, was difficult to measure accurately.

FIG. 3. Effect of pH upon the rate of the clotting reaction, under conditions of optimal fibrinogen and plasma-CRF concentration, with a constant amount of coagulase (titer of 1:2,048 per milliliter). Spontaneous precipitation of fibrinogen occurred at pH values below 6.2.

FIG. 4. Effect of NaCl concentration upon the rate of clotting, under constant, optimal conditions of fibrinogen, plasma-CRF, and pH. Clotting rates nephelemetrically determined (●); rates determined as the reciprocal of the time in minutes required for the formation of a 4+ clot (○).

FIG. 5. Standard curve of the linear relationship between a wide range of coagulase concentrations and their corresponding reaction rates, determined by the Lumetron nephelometer.

DISCUSSION

The combination of the Lumetron nephelometer and Sargent recorder was quite suitable for a quantitative assay and required only the direct connection of nephelometer output to recorder input. The BaSO₄ standards were rapidly prepared, were easily reproducible, and were induc-
and concentration.

Brice-Phoenix light-scatterinig photometer.

If by amounts, uniformity of recording was found that such a salt concentration did not provide optimal conditions for maximal coagulase activity, at least in the present system. Many investigators have found that thrombin-induced clotting of fibrinogen is influenced by variations in ionic strength (11-13), but the mechanism of this influence has not been elucidated. Fitzgerald et al. (6) found that there was no change (as determined by light scattering and ultracentrifugation) in the characteristic shape or size of the fibrinogen molecule up to a pH of 10.8, regardless of protein concentration or ionic strength. Therefore, it is unlikely that a gross change in the tertiary structure of the fibrinogen molecule at a particular ionic strength could account for the influence of NaCl upon the coagulase reaction rate. Fibrinogen molecules are quite negatively charged (5), and it is possible that introduction of a cation, such as Na+, at a critical concentration could reduce the electrostatic repulsion between them and, therefore, could accelerate the polymerization process.

Based on the data presented, the following recommendations for the standardization of a nephelometric assay for coagulase are made: (i) substrate fibrinogen concentration, 0.3% (w/v); (ii) plasma-CRF concentration, in excess of 1.5% (v/v) depending upon source; (iii) NaCl concentration of reaction mixture, 0.05 to 0.08 M; and (iv) pH of the reaction mixture, 7.1 to 7.3 for this system.

Coagulase concentration was not critical; a linear correspondence was obtained with a variety of samples encompassing conventional reciprocal titers of 1:128 to 1:4,096. These appeared to be the lower and upper limits of sensitivity for the Lumetron instrumental system.

The availability of a quantitative method for the assay of coagulase activity makes it possible to define a standardized coagulase unit. It is suggested that a coagulase unit be defined as: that activity which causes a change of 0.01 nephelometric units per minute under the conditions employed in these studies. For purposes of comparison, a coagulase preparation having a conventional titer of 1:4,096 would have an activity of 1,250 coagulase units per ml. Specific activities for the evaluation of purification procedures could be computed as coagulase units per milligram of protein. Such standardization should

ing recorder response in direct proportion to nephelometric units.

In determining the parameters which affect the uniformity of the coagulase reaction rate as recorded by this system, it was found that both fibrinogen and plasma-CRF concentrations were critical. If either was present in rate-limiting amounts, the quantitation of coagulase activity was seriously impaired, particularly when dealing with highly active preparations. The reaction rate was also greatly influenced by pH, which must be considered important in the standardization of this nephelometric assay.

The marked influence that NaCl concentration has upon the coagulase reaction was unexpected, when one considered the routine practice of preparing fibrinogen solutions in 0.15 M saline. From the data obtained in these studies, we must conclude that such a salt concentration did not provide optimal conditions for maximal coagulase activity, at least in the present system. Many investigators have found that thrombin-induced clotting of fibrinogen is influenced by variations in ionic strength (11-13), but the mechanism of this influence has not been elucidated. Fitzgerald et al. (6) found that there was no change (as determined by light scattering and ultracentrifugation) in the characteristic shape or size of the fibrinogen molecule up to a pH of 10.8, regardless of protein concentration or ionic strength. Therefore, it is unlikely that a gross change in the tertiary structure of the fibrinogen molecule at a particular ionic strength could account for the influence of NaCl upon the coagulase reaction rate. Fibrinogen molecules are quite negatively charged (5), and it is possible that introduction of a cation, such as Na+, at a critical concentration could reduce the electrostatic repulsion between them and, therefore, could accelerate the polymerization process.

Based on the data presented, the following recommendations for the standardization of a nephelometric assay for coagulase are made: (i) substrate fibrinogen concentration, 0.3% (w/v); (ii) plasma-CRF concentration, in excess of 1.5% (v/v) depending upon source; (iii) NaCl concentration of reaction mixture, 0.05 to 0.08 M; and (iv) pH of the reaction mixture, 7.1 to 7.3 for this system.

Coagulase concentration was not critical; a linear correspondence was obtained with a variety of samples encompassing conventional reciprocal titers of 1:128 to 1:4,096. These appeared to be the lower and upper limits of sensitivity for the Lumetron instrumental system.

The availability of a quantitative method for the assay of coagulase activity makes it possible to define a standardized coagulase unit. It is suggested that a coagulase unit be defined as: that activity which causes a change of 0.01 nephelometric units per minute under the conditions employed in these studies. For purposes of comparison, a coagulase preparation having a conventional titer of 1:4,096 would have an activity of 1,250 coagulase units per ml. Specific activities for the evaluation of purification procedures could be computed as coagulase units per milligram of protein. Such standardization should

FIG. 6. Linear correspondence between coagulase concentration and recorder response, determined by the Brice-Phoenix light-scatterinig photometer.

FIG. 7. Inverse relationship between coagulase concentration and time required for initiation of recorder response, determined by the Brice-Phoenix light-scatterinig photometer.
Nephelemetric assay of coagulase

provide a common means of comparison in the purification and characterization of staphylococcal coagulase.

Acknowledgments

We thank H. L. Sadoff and J. B. Kinsinger for their cooperation and advice.

This investigation was supported by Public Health Service grant AI-05926 from the National Institutes of Allergy and Infectious Diseases.

Literature Cited


