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A Plate Method for Quantitation of Fibrinolytic Activity.* (32323)

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This report deals with a method for measuring fibrinolytic activity, based upon the principles used in the fibrin plate method of Astrup and Mullertz(1). Many investigators have modified the original Astrup and Mullertz procedure in order to overcome certain limitations of the method or to adapt it to a particular need. The method herein described facilitates the measurement of lysed areas, increases the accuracy of the measurement, minimizes the influence of a number of variables and requires a shorter incubation period. Although the techniques which this method employs are described in conjunction with the fibrin plate method they can also be applied to methods utilizing other substrates, such as plasma, and can be adapted to improve many of the modifications of the original Astrup and Mullertz procedure.

A new step introduced to the conventional fibrin plate method is the staining of the plate after incubation with the fibrinolytic agents(2). This innovation results in a striking contrast between stained unhydrolysed substrate and sharply demarcated transparent lysed areas (Fig. 1), thus facilitating the conventional manual measurement of the areas of lysis. More significantly, however, this procedure permits photometric quantitation of fibrinolytic activity. The latter is a rapid and more accurate technique than the conventional manual measurement of the lysed areas (*i.e.*, obtaining the product of two perpendicular diameters), particularly when the areas of lysis are irregular.

Materials and methods. Nine ml of 0.3% bovine fibrinogen (Armour) in phosphate buffer (pH 7.6) with an ionic strength of 0.15 are poured into a sterile flat bottomed petri dish (8.5 cm diameter) and clotted with 0.1 ml of thrombin solution (Topical Thrombin, Parke Davis, 100 NIH units per ml of 0.9% saline). Standardized movements of the dish are employed while thrombin is added to insure uniform mixing. Ten dishes placed on a level bench are used for each test. After clotting, the plates are allowed to stand for 15-30 minutes and then a 20-lambda drop of each test solution is placed on the surface of each plate. A plate can accommodate up to 8 drops without overlapping of the resulting areas of lysis. To minimize the influence of variables related to possible unevenness in the thickness of the fibrin plate or variations in its density, the dishes are rotated, each by a different arc, before application of the test solutions. Each sample drop is then placed on the surface of each plate on a site that has the same orientation in all dishes.

A practical method for placing the sample drops on the fibrin surface is to utilize a 1.0 ml capacity Hamilton syringe and a repeat-

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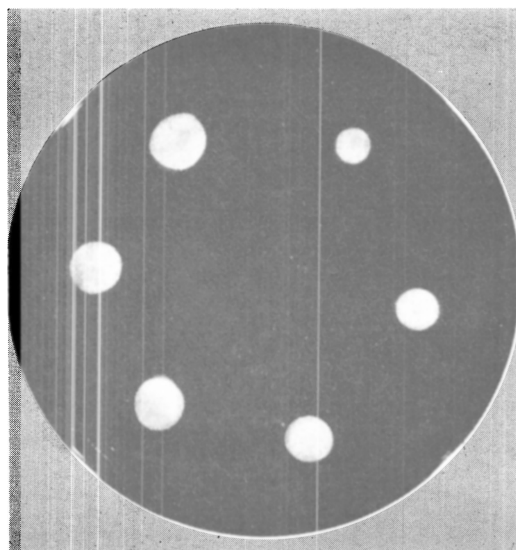

FIG. 1. Standard Fibrin plate stained with fast green after incubation with serial dilutions of fibrinolysin. Note the marked contrast between lysed areas and unhydrolysed substrate.

ing dispenser (Hamilton PB-600-1) which automatically delivers 20-lambda aliquots with each depression of a button. A trained technician requires less than 5 minutes to place drops of 4 different test solutions on each of 10 plates.

After 3 hours of incubation at 37°C, the plates are washed by careful immersion in tap water, stained by immersion for 15 minutes in a 0.2% aqueous solution of fast green, washed again by immersion in water and then allowed to dry in the air. While drying, staining solution which might have accumulated into the depressed lysed areas can be removed by gentle aspiration. Incorporation of the dye in the fibrin clot by adding it to the fibrinogen solution would be a simpler and more convenient staining procedure but it was found to interfere with fibrinolysis.

After the plates are dried, the areas of lysis can be measured either manually against a white background or photometrically. The photometric technique is based on the principle that the luminous flux transmitted through an area of lysis is proportional to the size of that area which in turn is proportional to the activity of the fibrinolytic enzyme. Transmission of light through the stained fibrin surrounding the areas of lysis is blocked by using a barrier filter of a color complementary to that of the stained fibrin. Fig. 2 illustrates the set up for this technique. A light source providing constant and uniform illumination is placed under a 2-3 cm diameter circular opening in a flat horizontal stage. A housing covers the light source permitting a beam of light to be directed to the stage opening only. The barrier filter is placed between the light source and the stage opening. A photoelectric cell connected to a meter is placed over the stage opening. It is preferable for the photoelectric cell to be recessed in order to minimize the effect of ambient light. The petri dish is placed on the stage so that an unlysed area covers the stage opening, and the light meter is adjusted to zero to compensate for light transmission through unlysed fibrin. The various lysed areas are then consecutively centered over the stage opening and corresponding readings are recorded. Light meter readings may be converted into square millimeters of lysed areas by reference to a curve (Fig. 3)

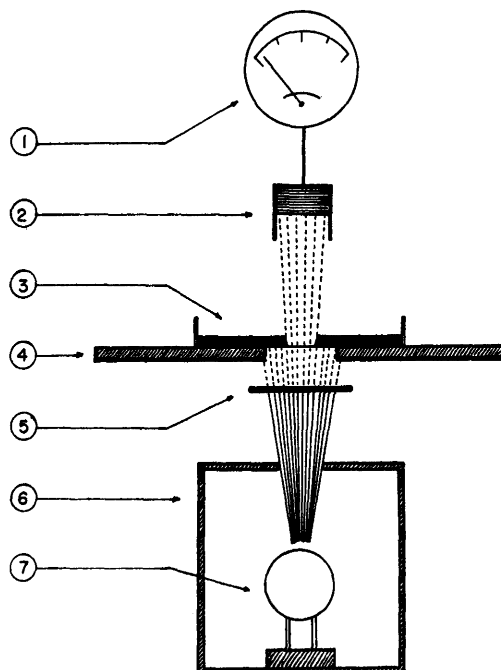

FIG. 2. Diagram illustrating the set up for photometric quantitation of fibrinolytic activity. 1. Meter. 2. Photoelectric cell. 3. Fibrin plate. 4. Stage with circular opening. 5. Barrier filter. 6. Light source housing. 7. Light source.

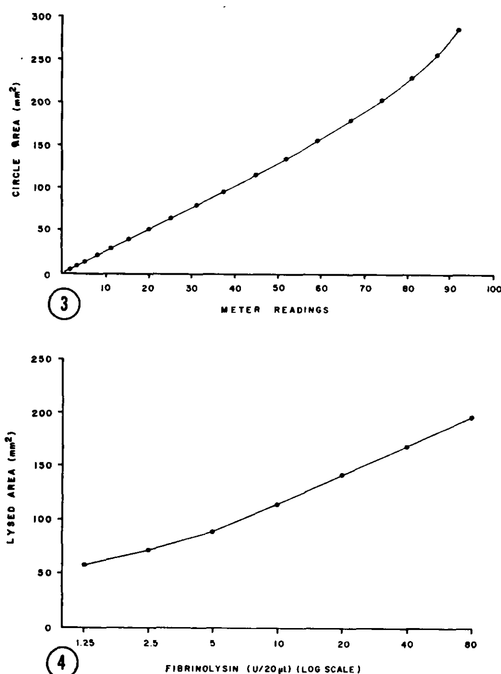

FIG. 3. Reference curve for converting light meter readings into surface area expressed in square millimeters.

FIG. 4. Linear relationship between serial concentrations of fibrinolysin and corresponding activities tested on standard fibrin plates and expressed in square millimeters.

obtained by plotting meter readings of the light transmitted through circles of known diameter against the surface of the circles expressed in square millimeters. Fibrinolytic activity can also be expressed as units of standardized enzyme preparations, such as plasmin or urokinase, by interpolation of the light meter readings on reference curves. These curves are obtained by plotting light meter readings of lysed areas produced by samples of a series of dilutions of the enzyme against the corresponding concentrations of the enzyme in the samples. The set up for photometric quantitation, described above, does not require specially made equipment. We have been using the built-in light source and stage of a microscope (with the condenser removed) and a "Photovolt" model 200M light meter.

Results. The following is a representative example of the results that can be obtained by utilizing the above described procedures: Table I presents the fibrinolytic activity (in mm^2) of 7 concentrations of fibrinolysin (Thrombolysin, Merck, Sharp and Dohme) all of which were tested on each of 10 standard fibrin plates. Fig. 4 demonstrates the linear relationship obtained when the mean values of activity are plotted against the respective concentrations of the enzyme on semi-logarithmic paper. Testing of the significance of the differences of the means provided a probability $P < 0.001$. According to our general experience, and as these findings indicate, a 3-hour incubation is sufficient to permit adequate quantitation of fibrinolytic activity with the material and procedures described above.

TABLE I. Activity of Serial Concentrations of Fibrinolysin (Expressed in mm^2 of Lysed Areas).

	Fibrinolysin units						
Plate No.	1.25	2.5	5	10	20	40	80
1	55	72	110	132	156	169	196
2	55	72	90	121	121	169	196
3	55	72	90	100	132	156	196
4	64	72	90	121	156	175	196
5	49	81	100	121	132	156	196
6	64	72	81	100	121	169	196
7	64	72	81	110	144	169	196
8	55	72	81	110	156	175	196
9	55	64	81	110	156	175	196
10	55	64	81	121	144	156	210
Mean	57	71.3	88.5	114.6	141.8	166.9	197.4
Standard deviation	4.8	4.5	9.4	9.8	13.7	7.5	4.2
Coefficient of variation	8.5%	6.3%	10.6%	8.5%	9.6%	4.5%	2.2%

Summary. A method is described for measuring fibrinolytic activity on fibrin plates. Staining of the plates facilitates the manual measurement of lysed areas and also permits a rapid and more accurate quantitation of fibrinolytic activity by a photometric technique. The procedures employed minimize the effect of a number of variables and require a relatively short incubation period.

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Toxicity of Cephalothin for McCoy Cell Cultures.* (32324)

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Sodium cephalothin was introduced for general clinical use in 1964 as a broad spectrum antibiotic with "the almost negligible toxicity of penicillin" for humans and experimental animals(1). Except for bone marrow depression with neutropenia in 4 patients (2,3) clinical reports so far have indicated a low toxicity for the drug. That it may possess considerable toxicity under some circumstances is suggested by reports from one laboratory which described marked morphologic changes in human amnion and mouse embryo cell cultures on exposure to cephalothin(4,5).

The present report describes the marked inhibitory action of cephalothin on the growth of a strain of cultured human cells at concentrations commonly in the sera of patients under treatment with the drug.

Materials and methods. Methods of propagating and counting the fibroblast-like McCoy strain(6) of human cells were identical with those previously described(7). Sterile vials of sodium cephalothin and sodium benzylpenicillin G were diluted and incorporated in the medium to give the final concentrations noted. Morphologic observations of the living cells were made directly and also by examination of Wright stained cover slips on which the cells were grown in a replicate series of culture tubes. Each experiment was repeated 3-4 times.

Results. The results shown in Fig. 1 indicate a progressive inhibition of cell growth by sodium cephalothin at concentrations of 10^{-5} M (4.18 μg/ml), 3×10^{-5} M (12.54 μg/ml), 6×10^{-5} M (25.08 μg/ml) and 10^{-4} M (41.8 μg/ml). Morphologic evidence of toxic effects was observed at the higher concentrations and consisted of rounding up of cells, increased granularity of cytoplasm, and at a concentration of 6×10^{-5} M a considerable number of large rounded cells. The same molar concentrations of sodium benzylpenicillin did not inhibit growth or produce morphologic changes indicating toxicity.

Discussion. Cephalothin and benzylpenicillin G are structurally related(8) and produce a similar biochemical injury to the staphylococcus by selectively inhibiting cell wall synthesis(9). Thus the finding of a significantly greater toxicity of cephalothin for the McCoy strain of human cells and other mammalian cultured cells(4,5) is surprising. Cephalothin possesses, however, a broad spectrum of antibacterial activity compared with penicillin. Broad spectrum antimicrobial activity implies injury to a biochemical process present in a wide variety of living cells. Since mammalian and bacterial cells have in common a number of similar metabolic pathways it would seem reasonable to anticipate that broad-spectrum anti-

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