

# Technical methods

## Estimation of plasminogen in biological fluids by agar/fibrinogen gel diffusion

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While studying antibody-antigen by an agar gel diffusion technique (Ouchterlony, 1948; Elek, 1948) using a rabbit anti-human plasminogen serum it was found that streptokinase could be used to identify plasminogen if fibrinogen was incorporated in the gel. The following method of plasminogen estimation was then devised.

### PRINCIPLE

The rate of diffusion of a protein in solution from a source in agar gel is, within limits, proportional to its original concentration (Ouchterlony, 1949).

When plasminogen in biological fluids is allowed to diffuse from a source in agar/fibrinogen gel for a given time, the distance diffused can be ascertained by activation of the plasminogen to plasmin by streptokinase after diffusion has taken place. The plasmin produces lysis of the fibrinogen in the gel which can easily be seen, thus the distance of the zone of lysis from the plasminogen source can be measured and the rate of diffusion calculated. This in theory would measure plasminogen concentration instead of plasmin activity.

The effect of inhibitors of plasminogen activation and plasmin activity should be greatly reduced or eliminated if only the distance the plasminogen diffused and not the amount of lysis could be measured.

Several methods can be devised on these principles. Unfortunately, however, it is extremely difficult to measure accurately the distance of the zone of lysis from the plasminogen source.

To measure the diffusion rate of plasminogen the method described below was devised in which the amount of fibrinogenolysis rather than its position is measured. It is not free from the effects of inhibitors but it has been found satisfactory for routine use.

Two gutters made in an agar/fibrinogen gel at an angle to each other are filled with dilute plasma and streptokinase respectively. From these sources the reactants diffuse and where they meet a line of lysis is formed. The length of this line of lysis is dependent on the diffusion rate of the plasminogen when all other reactants are kept constant.

### MATERIALS

All materials should be sterile.

- 1 Petri dishes 9 cm. in diameter.

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- 2 Filtered 3% agar in 0.9% NaCl.
  - 3 Filtered 0.2% bovine fibrinogen in Tris buffer pH 7.2  
NH<sub>2</sub>C (CH<sub>2</sub>OH)<sub>3</sub>..... 15 g.  
N/10 HCl. (make up in 1 litre distilled water) 106 ml.
  - 4 Plasma or serum diluted 1/64 with 0.85% NaCl.
  - 5 Streptokinase (Kabikinase) 5,000 u.ml. in NaCl.
  - 6 Brass mould to produce gutters 35 mm. × 5 mm.
- Reagents 2 and 3 are mixed in equal parts and heated to 94°C. for 30 minutes (the container with the mixture is placed in boiling water for 30 minutes).

### METHOD

A sterile technique was employed throughout.

The agar was first prepared as a 5% solution in boiling distilled water and after cooling and solidifying it was cut into cubes and washed in running tap water for two days. It was then remelted and diluted with a salt solution to give a 3% agar in 0.9% NaCl. It was stored in 50 ml. lots at 4°C. in sealed containers until used.

The plates were prepared on a perfectly flat surface and a layer of 8 ml. of 3% agar was poured into the Petri dishes to form a flat base. When set the mould was placed on the surface of the agar and 7 ml. of a mixture of equal parts of 0.2% fibrinogen and 3% agar (heated to 94°C. for 30 minutes to destroy the plasminogen contaminating the fibrinogen) was added. When the agar/fibrinogen mixture was set the mould was removed leaving three gutters. The mould was coated with sterile paraffin wax to prevent it sticking to the agar. The plates were now ready for use but could be stored in a moist atmosphere at 4°C. for a period of up to one week.

The gutters were filled with 0.3 ml. of the reactants. Reference plasma, diluted to give a lysis line length of 25 mm., was used in the left-hand gutter. It was stored diluted at -25°C. until used. The diluted test sample of

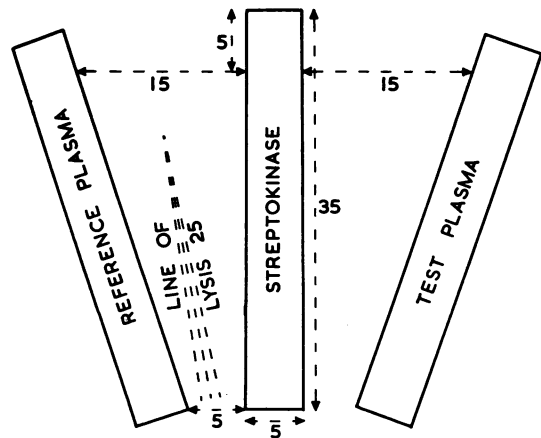


FIG. 1. Diagram of gutters in gel (enlarged).

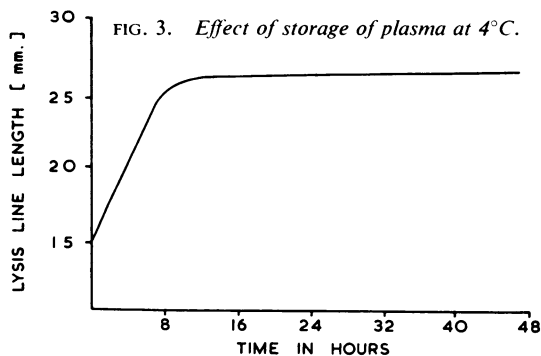
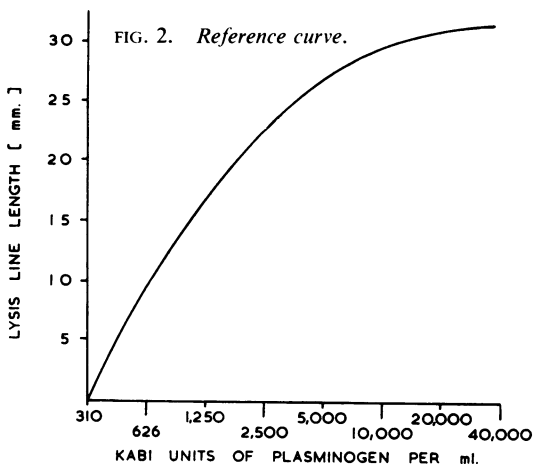
plasma was placed in the right-hand gutter and streptokinase in the centre gutter (Fig. 1).

The test was performed in duplicate. The plates were then incubated at 37°C. for exactly 16 hours and the length of the line of lysis appearing between the gutters measured with dividers. For convenience the measurement was made from the inside lower corner of the outside gutter to the tip of the line of lysis as the lower end of the lysis area was too diffuse. The results to date have simply been recorded as lysis line length in millimetres. The longer the line of lysis the greater the amount of plasminogen present.

#### OBSERVATIONS

Plates prepared with unheated fibrinogen, which therefore contained bovine plasminogen, were used to estimate the proactivator content of plasma. The length of the line of lysis obtained was almost identical to the length of line produced in plasminogen estimation. The zone of lysis between the gutters was much more diffuse and the unheated fibrinogen plates did not keep well. Therefore only heated fibrinogen/agar plates were used for plasminogen estimation.

Purified plasminogen which had a specific activity of 100,000 Kabi units per mg. was tested on the fibrinogen/agar plates in varying concentrations made up in saline or Tris buffer at pH 7.2. The length of the lines of lysis produced was recorded and a graph drawn which could



be used to convert line length into units per millilitre. During the 16 hours' incubation a zone of lysis usually appeared about the plasminogen gutter indicating plasmin activity. The results, however, were constant and in accordance with the pattern produced by varying protein concentrations in antibody antigen systems studied by the Elek-Ouchterlony agar/gel diffusion method.

When the plasminogen content of normal plasma is estimated by a method in which plasminogen is first converted to plasmin, a result of approximately 5,000 to 7,000 Kabi units per ml. of plasminogen is obtained. In the diffusion method described, normal plasma would appear to contain 50 times as much plasminogen, that is, approximately 300,000 Kabi units per millilitre. This difference in results is probably due to the instability in solution of purified plasminogen which was used to prepare the reference curve for the conversion of lysis line length to units of plasminogen. It is therefore better to express results as percentages of normal values rather than in units.

Storing plasma or serum at 4°C. before estimating plasminogen content resulted in an increase in the length of the line. This was probably due to the destruction of a labile inhibitor. The maximum length of line was obtained after eight hours. Therefore all plasma and sera in which plasminogen was estimated were first stored for eight hours.

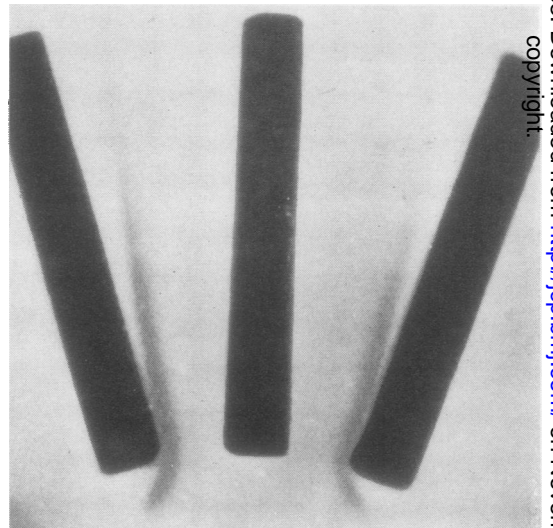


FIG. 4. Photograph showing lines of lysis. The line on the left is the line produced by the reference plasma and the line on the right is produced by plasma from a patient under streptokinase therapy in whom the plasminogen content has been reduced by approximately 50%.

The fibrinogen, plasminogen, and streptokinase used in this test were given by Kabi Ltd., Stockholm, Sweden.

#### REFERENCES

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