TECHNICAL METHODS

Photographic Recording of Precipitin Bands in Agar Gel

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The method for producing antigen-antibody precipitin bands in agar gel, due to Ouchterlony, is becoming increasingly useful in diagnostic immunology as well as in research into antibody-antigen reactions. For both these purposes a dimensionally accurate record of all bands, including faint lines, crossovers, and spurs, is necessary for correct interpretation of the plate. The dimensional accuracy is obligatory if an investigation is to be carried out on the relative molecular weights of the proteins involved (Korngold and Van Leeuwen, 1957).

The method of drying the agar plate on to a lantern slide is not one of choice, as it is both laborious and the record is liable to deteriorate (Gell, 1955; Hayward and Augustin, 1957). Freehand drawings are both inaccurate and liable to bias. Most methods of photography used until now have employed the capacity of the precipitin band to reflect the incident light, making it necessary for the camera to be slightly inclined from the perpendicular to the plate to give an image of a well-lit band. This leads to dimensional inaccuracy, halation, and loss of definition, and grey bands and spurs are not recorded. Other disadvantages are that it is necessary to employ a very powerful light source a small distance from the plate, generating considerable heat, and a specially constructed apparatus and good recording camera, both expensive, are necessary.

This paper describes a method of photography giving high contrast, and definition, and dimensional accuracy which is both cheap and capable of being carried out without an advanced knowledge of photographic technique.

Materials and Methods

Petri Dishes.—Pyrex petri dishes, 7–12 cm. in diameter, are used. Any which show bubbles in the glass, scratches, or excessive convexity of the inside surface of the base are discarded, but it has been found that strain marks do not affect the photography. Each preparation must be inspected, as it has been found that bubbles form as the plates age. Those passing the inspection are washed, dried, rendered completely clean and grease-free in an acid cleaning mixture, and, after rinsing thoroughly with filtered distilled water, are dried inverted, in a completely dust-free atmosphere. From this point until the plates are poured great care must be taken that there is no contact between the fingers and the inside surface of the plate. The clean plates are stored in a dust-free container until used.

Preparation of the Agar.—Davis New Zealand agar at double concentration (2%) is prepared. This allows for the incorporation of a suitable buffer or salt solution at double the required strength. A phosphate-citrate buffer at a final ionic strength of 0.1, pH 7.6, was suitable for use with blood coagulation factors. A stock of agar is prepared by making 2 litres of 2% agar in distilled water, the agar being brought into solution by placing the container in a steam chest for one hour with occasional mixing by swirling. The solution is then filtered *twice* through a 20 cm. Büchner funnel containing one sheet of Green's "504½" filter paper covered by a 3 cm. thick layer of paper pulp with a thin layer of medium sand on it. The funnel and its contents are preheated by passing hot tap water through it for 20 minutes before filtration. A very gentle suction is applied (5–10 cm. Hg) to bring the agar through the funnel, at the same time avoiding boiling and subsequent froth. The first 20 ml. of filtrate is discarded. After the second filtration into the same flask the agar is distributed in 50 ml. amounts into clean, sterile bottles using a clean preheated measuring cylinder. The bottles are immediately stoppered and stored at 2–4° C. Neither bacterial nor fungal contamination or deterioration of the agar has been found over a six-month period of storage.

Agar Plates.—A flat, levelled board is used for the plates, and should be of such a size that a large relatively air-tight cover such as a glass tank or the lower half of a desiccator can be inverted over it to provide a dust cover and prevent undue evaporation of water from the agar. It has been found that it is not advisable to use another, larger, petri dish to cover each individual plate, as there is a tendency for condensed water to drop on to the agar and affect the development of the lines. The volume required to fill the plates to a depth of 3 mm. is calculated, and as the plates were of four standard diameters, lines of those lengths were drawn on the pouring board with the volumes of agar required inscribed on them so that the size of the plates could be rapidly checked without unnecessary handling. The plates are placed inverted on the board together with a clean 10 ml. "blowout" pipette with a wide jet (wider than B.S.S.).

A bottle of stock agar is placed in the steam chest and left for one hour to melt. During the time that the agar is melting an equal volume of the required buffer or salt solution is made up at double strength, containing 0.04% sodium azide as a preservative. This is then filtered twice through the same Whatman No. 1 filter paper into a 250 ml. Erlenmeyer flask standing in a boiling water-bath. The agar is removed from the steam chest and slowly poured through a funnel into the filtered buffer or salt solution, and the mixture swirled for a few minutes to ensure complete mixing. The flask is then removed

FIG. 1.—Ouchterlony plate demonstrating clarity obtainable by this method of photography. The plate was photographed after two days' development. The centre well contained the antiserum and wells 1 to 6 the antigenic solution. The black spot beside well 3 is dust and the circle in well 2 shows the appearance of an air bubble in the glass of the dish.

FIG. 2.—Ouchterlony plate showing the resolution obtainable. The background of this plate is dirty owing to bacterial growth, as this plate had to be developed for 10 days to show maximum development of lines. The experiment was concerned with the comparison of the antigens in two stages (A) and (B) of the preparation of a bovine clotting factor (antihaemophilic globulin). The antiserum had been prepared in a rabbit by intravenous injection of a fraction of bovine plasma rich in this factor. The centre well contained the antiserum. Outer wells 1, 3, and 5 contained the preparation A at increasing dilution and wells 2, 4, and 6 contained preparation B, likewise at increasing dilution. This plate shows the following patterns used in the interpretation of the results described by Wilson and Pringle (1955, 1956). Surrounding the centre well there is a "folded ribbon." Fusion of lines occurs between wells 1 and 2, and a "spur" from the third from centre line of well 2 joins two components of well 1, the line demonstrating a "crossover" as it passes to the outermost line at well 1.

from the boiling water-bath and the agar distributed into the plates, care being taken to apply very gentle suction to the pipette to avoid the formation of bubbles. The glass cover is then placed on the board and the plates left for about one hour to cool and gel. It is worth noting that a pH of below 6.5 or above 8 will probably interfere with the setting of the plates, as will autoclaving the agar at any stage or clearing with albumin. It has been found that the plates usually remain uncontaminated by bacteria or fungi for about a week after pouring.

The "Camera."—An ex-R.A.F. continuous processing unit enlarger (Fig. 3) was used, but the addition of a lamphouse to the back of a disused half- or whole-plate camera or projector will serve equally well. The lamp, a 75 watt "flashed opal" enlarger bulb, was adjusted to give a parallel beam of light incident on the petri dish. A large (15–20 cm.) diameter petri dish containing glycerol is supported on a screen having a circular aperture slightly larger than the agar plate and about 2 cm. above the condenser lens. Initially immersion oil was used to cancel out the refraction errors in the base of the plate, but as it had to be changed frequently owing to dust contamination this would have led to great expense, and glycerol was found to have a refractive index near enough to the glass to serve. The photographic plate was supported in a quarter-plate printing frame, having a mask with a circular aperture on the camera side (chiefly for aesthetic appeal in the finished prints) and a black backing plate to eliminate stray light reflected from the walls of the darkroom. The black sheet of metal at the top of the camera was to prevent reflection from the ceiling and was found to give a great improvement in contrast in the negative.

Photographic Technique.—The following materials are required for the preparation of the agar plates: Clean, dry, lint-free rag, filtered distilled water, lead shot, fine forceps, small spatula, filter paper, and a large petri or crystallizing dish as a receptacle for the agar. Photographic materials required are: Ilford N.40 $3\frac{1}{2} \times 4\frac{1}{2}$ process plates, Kodak white smooth glossy hard single weight paper (WSG 3S). Johnson's "M-Q" and "contrast" developers, and an acid hardening and fixing solution.

Method.—Lead shot is pushed into the agar above a predetermined point for identification on the final print. The dish is then inverted over the receptacle and the edge of the agar disk gently lifted with the spatula until the agar drops out of the petri dish, which is then carefully cleaned and placed on a clean filter paper. The disk of agar is carefully picked up and held between thumb and forefinger and washed on both sides with a stream of filtered distilled water from a plastic wash bottle. It is then returned to its dish, care being taken to avoid minute air bubbles under the agar, which is then covered with a 2–3 mm. layer of filtered distilled water. The prepared dishes are stood on filter paper, and covered with damp filter paper until photographed.

FIG. 3.—The camera is supported between two vertical strips of laboratory girder. The upper inset shows, in exploded view, the plate holding apparatus, and the lower inset shows an exploded view of the arrangement of the agar plate on the dish of glycerol supported over the condenser lens.

The dish is carefully lowered into the glycerol container in the camera, avoiding the formation of bubbles in the glycerol under the dish. The front of the camera is replaced and the image focused on a screen placed in the plate holder (a fogged, undeveloped photographic plate was found to provide the best screen). A photographic plate is then marked in one corner on the emulsion side with diamond with its serial number, and then substituted for the focusing screen, and the exposure made by switching on the "camera" light. Care must be taken to avoid vibration. A small aperture of $f8-22$ is recommended together with a neutral grey filter placed on the lens to give a controllably long exposure time of 5 to 10 sec. Shortening the exposure time for plates with only very faint bands does not increase the definition but only leads to printing difficulties. The exposure should be the same for every plate, regardless of the appearance of the bands.

The plates are developed for three minutes at 20°C. , in Johnson's "M-Q" developer containing 0.5% wetting agent, constantly agitating the developing dish, fixed, and washed. It was found that a good criterion of correct exposure was that,

if the plate was held 3 in. away from the red safe-light after fixing, the difference between the edge of the lamphouse and the red screen was just sharply definable through the densest part of the negative. For most purposes the negatives were printed contact, and developed for about one minute in the "contrast" developer diluted one part with three parts of water, the development being stopped when the agar background just began to show. Any print developed in under 45 or over 75 seconds was discarded because faint lines and spurs were lost. The prints were fixed, washed, dried in a glazer, and mounted on paper for examination and filing. Lantern slides can be made by contact printing the plates.

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