Substances like starch and gelatin, acting as substrates, can obviously be prepared as films. Others, like DNA and RNA, must be included in a supporting medium. In the 3rd (1968) edition of this book an opinion was recorded that 'in the foreseeable future substrates will not simply be mixtures with their carrier. They will be attached by firm chemical linkages to an enzymatically inert part of the molecule. Specific peptides, for instance, will be attached by treatment with cross-linking reagents (e.g. carbodiimide, glutaraldehyde)'. There has been, it seems, little advance in this direction during the decade which has passed since the statement was made. It remains, nevertheless, as true as it was in 1968 but demand, the essential prerequisite for such new technology, has clearly been lacking.

The basic requirements for substrate film techniques are (1), the substrate must be insoluble (or immovable) in water, (2), it must be accessible to the enzyme, (3), its product, or at least the chromogenic portion thereof, must be water soluble and/or capable of removal by aqueous washing and, finally (4), it must be possible to show that any observed modification in the film is due solely to enzyme activity on the substrate.

In practical terms there are two main divisions of substrate film technology. After incubation the tissue section may be left in contact with the film or, alternatively, it may be separated therefrom and the two halves of the process then stained separately. The sequence of procedures involved in the second of these two options is shown in Figure 78.

This illustration refers to the technique for deoxyribonuclease, discussed on p. 258. With suitable adjustment of the captions it can be made to fit any of the separation techniques described in this chapter.

METHODS FOR PROTEASES

Gelatin-Silver techniques

A general technique for proteolytic activity, developed by Adams and Tuqan (1961a) from earlier work by Adams, Fernand and Schnieden (1958), was based on the incubation of fixed frozen sections on blackened photographic plates. Digestion of the gelatin layer by proteases left clear areas in the film which could be correlated easily with structures in the overlying tissue section (non-separation technique).

This method was used by Adams and Bayliss (1961) to demonstrate peripheral nerve cathepsins and by Adams and Tuqan (1961b) to show proteinase activity in Wallerian degeneration. Distinction of cathepsin from other proteolytic activity was made by showing that the enzyme concerned was pH-dependent and thermostable.

An essentially similar method was used by Bélanger and Migicovsky (1963) to demonstrate proteolysis in bone sections after parathyroid hormone administration. Activity was related especially to mature osteocytes.

There have been a number of modifications of the original gelatin techniques for proteases. Films coloured with an azo dye were used by Cunningham (1967) as the basis of a non-separation technique which gave better localization than that obtainable with blackened photographic films. Using a variety of inhibitors Cunningham showed that the enzyme in kidney sections which digested the cross-linked substrate closely resembled cathepsin D.

The technology was reviewed by Hallpike and Adams (1969) and an unfixed gelatin-silver substrate film technique was used by Shear (1969) for the demonstration of proteases in adipose tissues. Ilford Special Lantern Contrasty Glass Plates were exposed to light for 15 minutes, then developed in a Bromide Developer for 2 minutes, fixed in acid fixer for 4 minutes, washed and air-dried. Unfixed cryostat sections were applied to these plates and incubated for 3 hours. A similar substrate, prepared from Kodak AR10 autoradiographic film, was used by Penn *et al.* (1972) for the localisation of acrosomal proteases but Shear (1972) investigating the proteases of rodent salivary gland convoluted tubules, found that not only fixation of the gelatin film, but also the presence of silver

grains, inhibited enzyme activity. This prolonged the incubation period to 2–3 hours, allowing autolysis of the tissue section to occur with consequent deterioration in localisation of the enzyme. Shear essentially used Cunningham's (1967) method but with unstained gelatin post-stained with acid fuchsin.

Gelatin film technique

Unfixed and unmodified gelatin substrates had already been used by Denker (1969) in the first of a series of studies on reproductive system proteases. His original technique, proving not entirely satisfactory, was modified (Denker, 1974, 1977; Denker and Petzoldt, 1977) and details of the final version are given in Appendix 7. The kind of results which can be achieved are shown in Figures 80, 81 and 82. One of the best of a variety of stains used in post-staining variations of the original Adams and Tuqan (1961) technique is Mercuric Bromphenol blue, first used by Fried *et al.* (1976) for this purpose.

Colour film technique

In another elegant adaptation of the original principle Fratello (1968) placed his tissue sections on unexposed reversible colour film (Ferrania 3M, daylight type DIA 28). This type of film consisted of a nitrocellulose backing on which three layers of gelatin were superimposed. The first, furthest from the

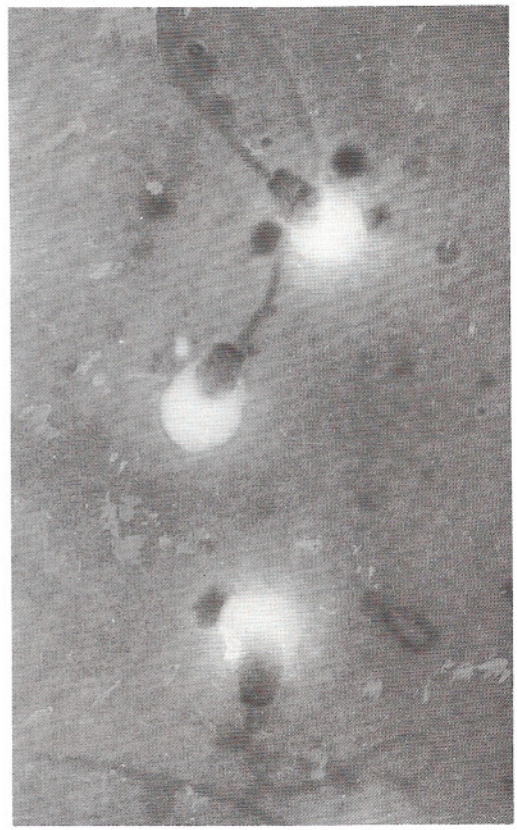

Fig. 80 Acrosomal protease (Acrosin). Rabbit semen, diluted 1 : 5 with TC 199 medium, spread on the substrate film using a blood smear technique. Gelatin substrate prepared according to Denker (1974) and soaked, prior to semen application, in 3×10^{-3} mol/litre buffered hyamine hydroxide. (Courtesy of Doz. Dr H. W. Denker.) × 1400.

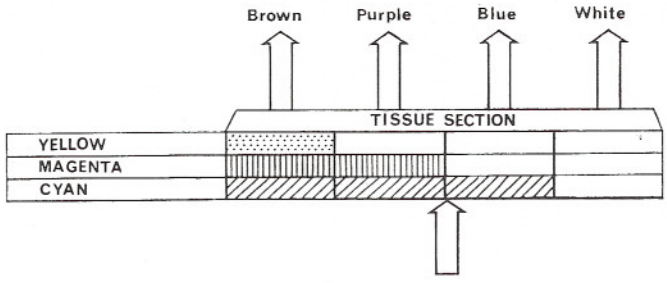

Fig. 79 Colour film technique. Effects of progressive digestion.

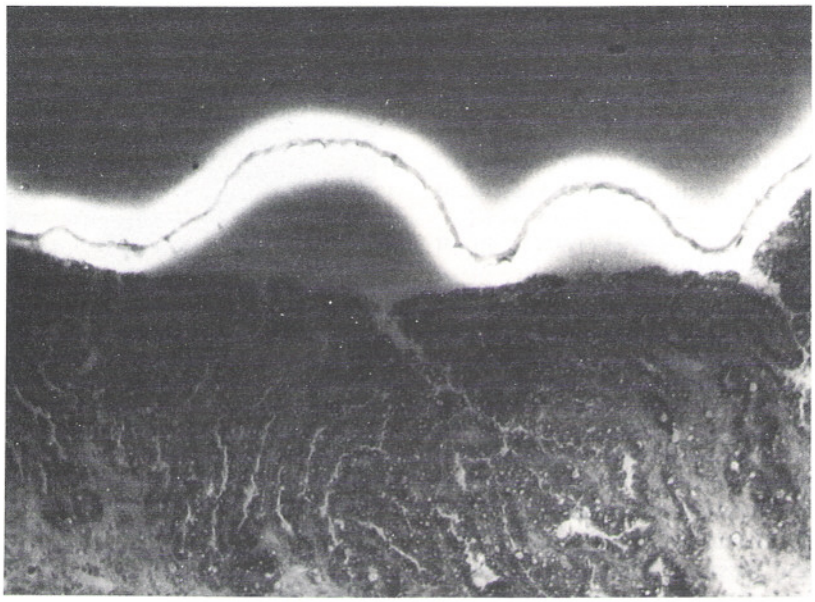

Fig. 81 Rabbit trophoblast-dependent blastocyst proteinase (blastolemmase). Fresh frozen cryostat section of blastocyst and uterus 7.3 d post coitum. Animal ovariectomised at 6 d post coitum which diminishes endometrical and blastocyst proteinase. Lysis confined to trophoblast and extracellular blastocyst linings. Endometrium shows little activity. (Courtesy of Doz. Dr H. W. Denker.) × 90.

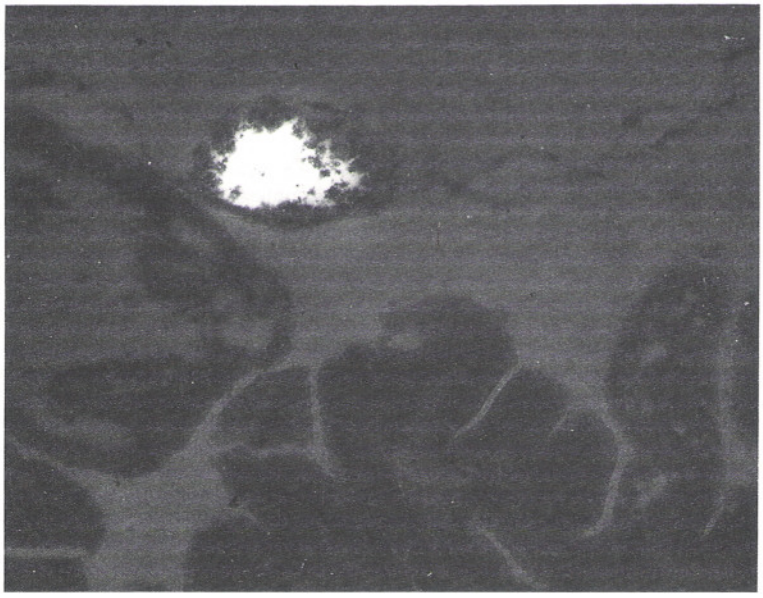

Fig. 82 Rabbit trophoblast proteinase. Fresh frozen (14 μm) cryostat section of blastocyst and uterus 8 d post coitum. Implantation prevented with antipain, shows the trophoblastic knob unattached to the endometrium. Enhanced proteinase activity is seen in its centre. (Courtesy of Doz. Dr H. W. Denker.) × 140.

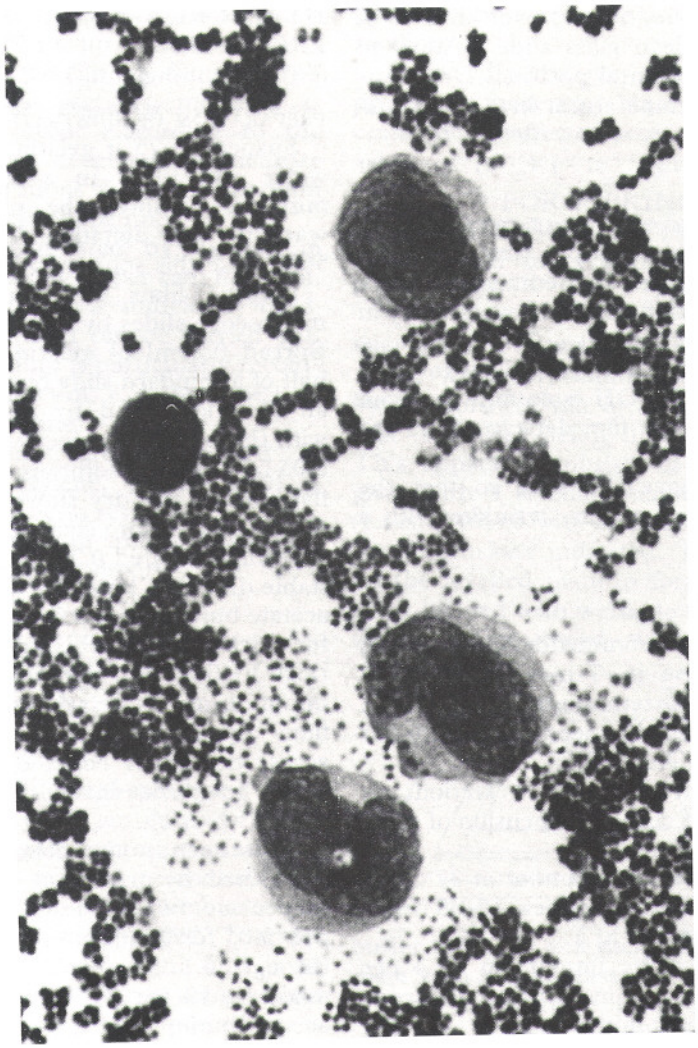

Fig. 83 Micrococcus lysodeikticus. Technique shows lysis of the walls of organisms surrounding two lysozyme-secreting blast cells. No activity shown by mature lymphocyte. May-Grünwald-Giemsa. (Courtesy of Dr D. Catovsky.) × 1400.

synthetic medium. Rapid dehydration and clearing are essential, in order to avoid crazing of the gelatin film.

Result. Protease activity is shown as a clear area where the gelatin film and its contained granules have disappeared. Cytological, but not intracellular, definition is obtained.

MODIFIED GELATIN METHOD FOR PROTEASES
(After Denker, 1977)

PREPARATION OF FILMS

(1) Spread 0.15 ml 5 per cent gelatin (Merck No. 4070 or Eastman Kodak No.

5247, w/v in distilled water) over an area of 2.5 × 5 cm of a clean glass slide.* Allow to solidify in the horizontal position. Dry completely at room temperature, in a horizontal position.

(2) Store overnight at 4°C in a moist chamber.

(3) Fix for 1 hr at room temperature in formal-alcohol (10 ml 40 per cent formaldehyde and 90 ml 50 per cent ethanol).

(4) Wash 20 min in deionised water. Dry in air. Store in plastic bags at −25°C.

(5) Immediately before use allow to swell in 7 per cent NH_4OH solution at room temperature until the film detaches from the slide.

(6) Gently remove the slide so that the film readheres. Smooth any wrinkles with a needle.

(7) Dry in air.

PROTEASE TEST

(1) (Optional) Recommended for sperm proteases. Immerse in 3 mM hyamine hydroxide (methyl benzethonium hydroxide) in 50 mM ethylene diamine-acetic acid buffer at pH 7.0 for 2 min. Air dry.

(2) Attach a fresh cryostat section, or spread one drop of chosen suspension of cells, and allow to dry.

(3) Incubate in a wet chamber at 37°C for up to 4 hr. (Sperm enzymes 15–45 min; blastocyst protease ½–4 hr.)

(4) Stop reaction by immersion in 4 per cent formaldehyde, or in saturated aqueous $HgCl_2$, at 4°C for 10 min.

(5) Wash in deionised water, or running tap water for 15 min.

(6) Stain in 0.5 per cent Toluidine blue in borate buffer (pH 10) at 4°C for 30 min.

(7) Wash briefly, dehydrate, clear and mount in synthetic resin.

Result

Sites of protease activity appear as clear areas or halos (Figs. 80, 81 and 82).

*Some slides are unsuitable for the purpose since they will not permit separation of the film to take place at stage 5. The gelatin solution should be kept at 50°C.

DYED GELATIN METHOD FOR PROTEASES

(After Cunningham, 1967)

PREPARATION OF SUBSTRATE FILMS

Suspend 1.75 g gelatin in 50 ml distilled water and place in a refrigerator for 30 minutes. Dissolve the swollen gelatin by warming and agitating for 3–5 minutes at 56°C. Use this solution within one hour.

Prepare films on standard (precleaned) microscope slides by the following procedure. Spread 0.2 ml of gelatin solution over one half of a standard slide by tipping the latter in all directions, and by stirring and tapping with the end of the pipette. For thin films use 0.05 ml of gelatin solution. Place the slide on a flat surface and dry slowly (12 hours). Load the dried films into staining racks and place in a freshly prepared 0.2 per cent solution of the stable diazotate Fast blue B in 40 mM veronal acetate buffer at pH 9.0. Leave for 15 minutes for thick FBB-15 films or for 45 minutes for thick FBB-45 and thin FBB-45 films. At 1–2 minute intervals detach gas bubbles from the films by drawing a metal spatula briskly across the upper edges of the slides. Wash slides in three changes of buffer, for 15 seconds in each.

Subsequently the colour of the films is intensified by treatment with a 2 per cent suspension of H-acid in veronal buffer at pH 9.0, for 5 minutes. Transfer the films through 0.1 N acetate buffer (pH 4.4), 5 minutes; 10 nM EDTA, 30 minutes; 0.1 N acetate buffer (pH 4.4), 5 minutes; distilled water, 5 minutes and, finally, a further bath of distilled water. Place slides, film surface upwards, on filter paper to dry. Remove excess, loosely bound, stain in 3 successive baths of 70 per cent ethanol, for 48, 3 and 2 hours, respectively. Dry slides once more.

INCUBATION OF TISSUE SECTIONS

For preference cold formalin or formol-calcium fixed tissues, post-treated in gum-sucrose, are cut on the cryostat at 6–10 μm. Sections are mounted directly on top of the film which is taken from 2.5 mM phosphate buffer (pH 6.8) and blotted with filter paper to

HISTOCHEMISTRY
Theoretical and Applied

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