Technical methods

Anthocyanin BB: a nuclear stain substitute for haematoxylin

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Histopathologists daily look down microscopes at sections which are usually stained with haematoxylin and eosin. These sections are taken very much for granted. Our complacency in this matter was shattered in 1973 when a world-wide shortage of haematoxylin became apparent (Lamb, 1973; Lillie, 1974). Since then, in terms of relative commodity prices, haematoxylin has shown one of the biggest increases and now costs six times more than it did in 1973. These events have prompted considerable efforts to find an effective haematoxylin substitute (Lillie *et al.*, 1975). A fancied resemblance between haematoxylin stained fingers and blackberry stained fingers turned our attention to *Rubus fruticosus*—the common or garden blackberry.

Preliminary experiments showed that crude blackberry juice had some promise as a nuclear stain. Accordingly, a systematic study was undertaken in order to determine the optimum staining conditions and to characterise the stain.

Material and methods

PREPARATION

Garden fresh or supermarket frozen blackberries were shredded in a liquidiser and the resultant crude juice was clarified by filtration or centrifugation. It was established that ferric ions must be added for the stain to be effective, and the two best empirically devised formulae were:

- (a) 100 ml clear juice
1.0 g aluminium chloride
1.2 ml ferric chloride solution
- (b) 100 ml clear juice
5.0 g sodium chloride
1.2 ml 10% ferric chloride solution
3.0 ml glacial acetic acid

Unlike haematoxylin, the blackberry stain does not require 'ripening'. It is best stored at 4°C and

remains active for about two months. Its optimum pH range is 2.5-4.0.

STAINING PROCEDURE

- 1 Bring paraffin embedded sections to water.
- 2 Stain with formulae (a) or (b) for 10-12 minutes.
- 3 Wash in running tap water for at least two minutes.
- 4 Stain with eosin.
- 5 Dehydrate, clear, and mount.

CHROMATOGRAPHY AND SPECTROPHOTOMETRY

Concentrated blackberry juice extract was subjected to ascending chromatography on Whatman No. 1 paper in butanol, acetic acid, and water (4/1/5). Cyanidin chloride (K & K Laboratories Inc, Plainview, NY, USA) was used as the reference standard.

Spectrophotometry of the extract was performed in methanol/hydrochloric acid with the later addition of aluminium chloride.

Results

The nuclei are specifically stained a dark violet blue colour distinguishable from haematoxylin staining only by the faintest of green casts (Fig. 1). The stain is equally effective on formalin, Zenker, or Carnoy fixed tissue, on decalcified material, and on frozen sections. In the last case the staining time is only one to two minutes. It can also be used with special stains such as MSB, periodic acid-Schiff, and van Gieson (Fig. 2). Sections stained with blackberry juice have been exposed to daylight for over 18 months and showed very little fading.

On paper chromatography three spots were resolved with R_f values of 43, 29, and 24. The cyanidin chloride reference standard also produced three spots, the main one corresponding closely with the R_f 29 spot of the blackberry juice extract.

Spectrophotometry of the blackberry juice gave a peak in the visible spectrum at a wavelength of 530 nm. This compares with a peak of 528 nm obtained with cyanidin chloride. With the addition of aluminium ions a bathochromic shift was demonstrated and the visible peak moved 35 nm.

Discussion

The spectrophotometric and chromatographic evi-

Fig. 1 *Placenta stained with anthocyanin BB and E (BB and E $\times$ 220).*

Fig. 2 *Cervix stained with anthocyanin BB and van Gieson (BB and VG $\times$ 280).*

dence confirms that the natural dyes present in crude blackberry juice are of the anthocyanin type (Harbourne, 1967). For this reason the stain has been named anthocyanin BB—for blackberry. Extraction and purification of the stain from the crude juice was undertaken but the effort involved was not judged worthwhile as the purified extract was no better a nuclear stain than the crude juice.

Anthocyanins from a variety of sources such as red cabbage, elderberry, and dahlia were used fitfully as histological stains in the late 19th and early 20th centuries. These findings have been reviewed by Harms (1965). Blackberries were apparently first used by Claudius (1899) in Copenhagen and more recently by Gruber (1949). Reports in the literature of haematoxylin substitutes have recently been extensively surveyed (Lillie *et al.*, 1975). Anthocyanins featured prominently in that review but the authors, at that time, had not tested any of them.

The domestic availability, ease of preparation and use, resistance to fading, and, above all, its good nuclear staining properties strongly commend anthocyanin BB as an alternative to haematoxylin in routine histological procedures. The results are so comparable that an unsuspecting observer does not usually question whether the section under his microscope is stained with haematoxylin and eosin or anthocyanin BB and eosin. Nuclear contrast and clarity are particularly striking in the van Gieson

procedure and so it is probable that anthocyanin BB staining can be further improved by introducing a deliberate acid differentiation stage.

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Measurement of plasma volume using ^{59}Fe -labelled transferrin

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The measurement of plasma volume is usually based on the dilution of an intravenously injected labelled protein. The recommended method uses radioiodinated human serum albumin (ICSH, 1973) but there are a number of occasions when other labelled proteins are being used, in particular ^{59}Fe -labelled transferrin in the ferrokinetic investigation of blood disorders, which could also provide an estimate of the plasma volume. Intravascular proteins of similar

size and shape would be expected to have similar distributions, and there is no a priori reason why ^{59}Fe -labelled transferrin and ^{125}I -labelled albumin should not give equivalent plasma volumes. However, it has been suggested that there is a difference between the two measurements (Najean *et al.*, 1967) and that the ^{59}Fe plasma volume may be unreliable (Dacie and Lewis, 1975). These observations may have been due to non-specific labelling with ^{59}Fe ferric chloride (Cavill and Ricketts, 1974). We have therefore compared the dilution volumes of specifically labelled ^{59}Fe transferrin and ^{125}I albumin in patients with a variety of haematological disorders to see if the two are equivalent.

Subjects and methods

Fully informed consent was obtained from the 25 patients who took part in this study. One patient had haemochromatosis, one had a refractory anaemia, one had polycythaemia rubra vera, and two patients