Technical methods

A standardized Romanowsky stain prepared from purified dyes

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Stains consisting of combinations of various thiazine
dyes with eosin, for example, those of Giemsa,
Leishman, and Wright, are used routinely to study
the cellular morphology of blood and bone marrow.
The major problem in the use of these Romanowsky-type
stains is batch-to-batch variations in their
staining properties as a result of variations in their
dye composition and the presence of contaminating
metal salts (Lillie, 1943 and 1944a; Lillie and Roe,
1942; Marshall et al, 1975a; Saal, 1964; Scott and
French, 1924).

The extent of these variations in a large number of
commercially available Romanowsky-type stains
was demonstrated in a previous study (Marshall
et al, 1975a). In this study it was found that the
simplest successful stain examined contained methy-
lene blue, azure B, and eosin and was only mini-
mally contaminated with metal salts. Accordingly,
we have produced a stain from purified samples of
these dyes.

Materials and Methods

PURIFICATION OF DYES
Methylene blue and azure B were purified as de-
scribed previously (Marshall and Lewis, 1975).
Commercial samples of eosin (table) were freed of
contaminating metal salts and volatile material
and converted to the free acid by the following
scheme:

(a) The commercial dye was dissolved at 10 g/l
in 0-1 m aqueous ammonia. Any insoluble material
was removed by filtration.

(b) 0-2 m aqueous hydrochloric acid was added
to this solution until precipitation ceased.

(c) The precipitate was removed by filtration
and washed several times with dilute aqueous
hydrochloric acid (ca 0-0005 m).

(d) Finally, the precipitate was dried at 135-140°C
for approximately 4 hours.

The purity of this free acid was confirmed by
sulphated ash analyses.

<table>
<thead>
<tr>
<th>Dye Supplier and Batch No.</th>
<th>Sulphated Ash yielded %</th>
</tr>
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<tbody>
<tr>
<td>or Purchase Date</td>
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<tr>
<td></td>
<td>Product</td>
</tr>
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<td>21-59</td>
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</tr>
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<td>R. A. Lamb 0715</td>
<td>19-53</td>
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<tr>
<td>R. A. Lamb 2621</td>
<td>18-18</td>
</tr>
</tbody>
</table>

Table Sulphated ash analyses of eosin samples

FORMULATION OF STAIN

Purified methylene blue, azure B, and eosin were
mixed in varying proportions. In some mixtures,
either methylene blue or azure B was omitted.
The mixtures were dissolved in 1:1 v/v glycercol-
methanol, in which the dyes are readily soluble and
highly stable (Lillie, 1944b). These solutions were
diluted with varying proportions of Sorensen’s
phosphate buffer, pH 6-8 or 7-2, 0-00132 m (available
from Mercia, Watford, Herts). Films prepared on
good quality microscope slides (Chance Propper
Ltd, Smethwick, Warley) were fixed in methanol,
stained for varying times in these solutions, and
differentiated in buffer pH 6-8 or 7-2. They were then
air-dried and mounted in a synthetic resin (Diatex,
R. A. Lamb Ltd).

The stained films were examined by an observer
who was unaware of the composition of the stains
used and they were compared with duplicate films
stained with a traditional May–Grunwald-Giemsa
sequence as routinely used in this laboratory (Dacie
and Lewis, 1968).

OPTIMAL STAINING PROCEDURE

After testing a number of staining solutions and
staining times, the procedure presented below was
found to give excellent results:

(a) The following quantities of the purified dyes
were mixed: methylene blue, 9-0 mg; azure B,
17-0 mg; eosin, 13-0 mg. The mixture was dissolved
in 20 ml of 1:1 v/v glycercol-methanol by gentle
stirring for a few minutes. (This stock solution

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Figs 1 to 6  Material stained using the procedure described in the text.

Figs 1 and 2  Photomicrographs of a normal blood film.

Fig 3  Photomicrographs of a blood film from a case of β-thalassaemia major showing anisochromasia, polychromasia, and punctate basophil stippling.

Fig 4  Photomicrograph of a blood film from a case of acute myeloblastic leukaemia. The blast cell has prominent nucleoli and contains an Auer rod.

Figs 5 and 6  Photomicrographs of a bone marrow film from a case of megaloblastic anaemia showing erythroid and granular precursors.
may be stored for many months without alteration in its composition.)

(b) The solution was diluted with 100 ml of Sörensen's phosphate buffer, pH 6·8, 0·00132 M.

(c) Films of blood (collected into EDTA-K₂ anticoagulant, 1·5 mg/ml of blood) and aspirated bone marrow were fixed for 5-10 minutes in methanol. They were stained by immersion in the diluted solution for 10 minutes.

(d) Stained films were differentiated for 1 minute in the pH 6·8 buffer, air-dried, and mounted.

Results and Discussion

Typical examples of the appearances of films stained by the recommended technique are shown in figs 1 to 6. These show the typical scheme of colouration produced by successful Romanowsky-type stains. It should be noted, however, that the colour balance and some of the more subtle features seen in the films, especially fine granulation, have been lost in the reproduction. Although the number of coloured components in this stain is small (see figs 7 and 8) compared with that typically seen in commercial stains (Marshall et al, 1975a) the characteristic subtle variation in colour was present.

Stains not containing methylene blue gave results approaching a Romanowsky effect but lacking this subtle colour variation. The presence of methylene blue enhanced nucleolar staining and red cell polychromasia. In addition, it resulted in monocytic cytoplasm being stained grey in contrast to the blue-grey colouration seen in its absence. Those stains not containing azure B stained chromatin blue instead of purple.

Replacement of the pH 6·8 buffer by one of pH 7·2 resulted in an orange-grey colouration of red cells. The staining of white cells was unaffected. Stains prepared from different batches of purified dyes gave identical results. To date, about 200 films of normal and pathological blood and bone marrow have been stained. On no occasion was any staining variation seen. This reproducibility obtained from a stain prepared from purified dyes represents a distinct advantage over that of currently available commercial stains (Marshall et al, 1975a). Another advantage which makes the stain particularly suitable for routine use is that the stock solution in glycerol-methanol is rapidly prepared and may be used immediately. It may be noted that the preparation of stock solutions of Giemsa, Jenner, and Wright stains (Baker et al, 1966; Lillie, 1969) involves a lengthy solution and ageing process. The stock solution of our new stain is extremely stable. Once diluted with buffer, the stain must be used within a period of about 3 hours; diluted solutions of the conventional Romanowsky-type stains show similar stability.

The effectiveness of the procedure for desalting eosin is illustrated in the table. Commercial samples of eosin contain a small proportion of tribromo-
fluorescein (Marshall et al., 1975b). This is not removed by the desalting procedure, although it may be removed chromatographically (Graichen and Molitor, 1959). However, we have found that this is unnecessary for the present staining procedure. Tribromofluorescein-free eosin and eosin deliberately contaminated with a much larger proportion of tribromofluorescein than is present in commercial samples gave indistinguishable results.

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References


Modifications to improve the reliability of the Coulter ‘S’

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The Coulter Counter Model ‘S’ System (Coulter Electronics Ltd) measures automatically the haematological parameters haemoglobin concentration, white cell count, red cell count, and mean cell volume and also computes from them the haematocrit, mean cell haemoglobin, and mean cell haemoglobin concentration. The instrument can handle one sample every 20 seconds and can be both precise and reliable (Barnard et al., 1969). However, in our laboratory the model ‘S’ is used to process up to 800 samples per day, and we have found that these conditions of virtually continuous operation expose limitations in the design of the instrument with a concomitant fall off in its reliability. We now describe some of these limitations and suggest ways of modifying the machine to overcome them.

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The Rinse and Drain Mechanisms

The most troublesome limitation exposed when the instrument was run continuously was the failure of the aperture baths to drain completely. (This happened several times within a period of three months.) In the original version of the instrument, during each cycle waste fluid was drawn under vacuum from the aperture baths into the waste chamber and then discharged to waste under 0-35 kgf/cm² positive pressure. Owing to the low surface tension of the effluent, caused by the lying agent, and the turbulence created by the vacuum, there was a tendency for excess foam to accumulate in the waste chamber which then overflowed into the trap bottle and from there into the switching valve (SV) No. 6, which controls the vacuum in the pneumatic card (L2). This caused the switching valve to become progressively less efficient with the result that the aperture baths were sometimes only partially drained, and carry-over between successive samples was observed. The valve eventually failed completely. The manufacturers then modified the instrument by fitting an anti-foam device (Coulter Alteration Number ECO-0367) which gave only partial improvement; however the excess foam still over-