An evaluation of some commercial Romanowsky stains

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SYNOPSIS The staining properties of 43 commercial Romanowsky-type stains have been studied. Considerable differences in the appearance of stained blood films were observed with different batches of these stains, the staining of red cells being particularly variable. Attempts have been made to correlate staining patterns with stain composition as revealed by thin-layer chromatography and sulphated ash analyses. In this way it has been possible to define some essential requirements for satisfactory staining.

Romanowsky-type stain variants such as those of Giemsa, Jenner, Leishman, and Wright are routinely employed for the coloration of blood and bone-marrow films. All these stains contain a mixture of methylene blue and other closely related thiazine dyes and eosin. The variations in the staining properties of these different stain variants are well documented (Baker, 1970; Baker *et al*, 1966; Dacie and Lewis, 1968; Lillie, 1969) as are the variations between different batches of stain which are nominally of the same type (Cramer *et al*, 1973; Lillie, 1944; Lillie and Roe, 1942; Price, 1968; Scott and French, 1924). Staining variability is particularly troublesome when the needs of a routine department and the use of automatic staining equipment call for a reliable, standardized procedure.

In this study we describe the variation observed in the coloration of blood films which have been stained with a selection of commercially available Romanowsky-type stains. By correlating staining performance with the chemical composition of the stains, it has proved possible to define the characteristics of some successful commercial stains.

Materials and Methods

INVESTIGATION OF STAIN COMPOSITION

The dye components of Romanowsky-type stains were separated by thin-layer chromatography. The method of Marshall and Lewis (1974a) was employed. Sulphated ash determinations on certain of the stains which are commercially available in powder form

were made by the National Physical Laboratory, Teddington, Middlesex.

STAINING METHODS

Specimens of venous blood from three subjects were collected into EDTA-K₂ anticoagulant (1.5 mg/ml of blood). One of the subjects was haematologically normal, one had iron deficiency anaemia, and the third chronic granulocytic leukaemia. Thin films were made shortly after blood collection. They were dried in air and were fixed in methanol in accordance with standard practice (Dacie and Lewis, 1968). Throughout this work only microscope slides from a single manufacturer (Chance Proper Ltd, Smethwick, Warley) were employed since the staining of films may be affected by variations in the quality of the glass on which they are spread (Scott and French, 1924). Films were stained with the batches of Diff-Quik, Giemsa, Jenner, Leishman, May-Grünwald, Romanowsky, and Wright stains indicated in table I. Jenner and May-Grünwald stains were used in combination with Giemsa stain. In these combination stains, a single batch of Giemsa stain (R. A. Lamb, 3025) was used. All staining was performed 'on slide' by the techniques described below. In all cases the aqueous buffer employed was 0.066 M Sørensen's buffer, pH 6.8 diluted before use 1:20 with water (Dacie and Lewis, 1968).

Diff-Quik Stain

Films were fixed in methanol, stained for 15 sec in solution I, followed by 15 sec in solution II.

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Giemsa Stain

The commercial solution or a stock solution of 5 g/l stain powder in a mixture of glycerol (2 volumes) and methanol (3 volumes) was diluted 10 times with buffer. Methanol-fixed films were stained in this solution for 15 min.

Jenner-Giemsa Stain

Methanol-fixed films were stained for 5 min in a solution comprising the commercial solution or a 3 g/l stock methanolic solution of Jenner stain powder (1 volume) and buffer (1 volume). Films were then stained with Giemsa stain as already described.

Leishman Stain

Films were stained for 2 min with the commercial solution or a 1.5 g/l methanolic solution of the commercial powder. This solution was then diluted with twice its volume of buffer and allowed to act for a further 10 min.

May-Grünwald-Giemsa Stain

Methanol-fixed films were stained for 5 min in the commercial solution or in a 3 g/l methanolic solution of the May-Grünwald stain powder. Films were then stained with Giemsa stain as already described.

Romanowsky Stain

Methanol-fixed films were stained for 5 min in the commercial solution diluted with an equal volume of buffer.

Wright Stain

Methanol-fixed films were stained for 5 min in a solution comprising a 3 g/l methanolic solution of the stain powder and the commercial solution (1 volume), and buffer (2 volumes).

After staining, slides were differentiated for 4 min in buffer, drained, air-dried, and mounted in Diatex (R. A. Lamb Ltd).

Results

The staining properties of the commercial stains, together with their dye components, are presented in table I. Sulphated ash analyses are given in table II.

Discussion

A standardized Romanowsky-type stain is highly desirable not only to ensure consistently good staining, which is the essence of morphological diagnosis, but also to facilitate the exchange of material between laboratories. The advent of automatic cell recognition systems makes such a stain essential.

One of the objectives of this study was to assess the suitability of the Romanowsky-type stains which are commercially available at the present time for the routine staining of blood films. In this assessment, the generally adopted staining procedures were carefully standardized, but no attempt was made to adjust staining conditions to yield optimum results, since this is not practicable in busy, routine laboratories. There is a generally accepted scheme of staining which is expected of Romanowsky stained preparations, viz purple chromatin, blue leucocyte cytoplasm, purple-black basophil granules, red-pink eosinophil granules, purple neutrophil granules, purple platelet granules, and pink red-cells. Based on this scheme, stained films have been examined and the stains assessed as useless, very poor, poor, fair, good or excellent (table I). These subjective ratings indicate the suitability of the stains for routine diagnostic purposes. Lowly rated stains are those which stain some cell components adequately but leave others unstained or stained colours differing considerably from the accepted ones. It can be seen that staining properties varied considerably in stains obtained from different suppliers. Indeed, this variability cannot be reduced by dealing with a single supplier. It is unfortunate that supply houses have done little to improve the situation which has long been a cause of concern (Lillie, 1944; Lillie and Roe, 1942; Scott and French, 1924).

An attempt has been made to correlate staining properties with stain composition. In this section only the single stain procedures are discussed since the results obtained with combination stains are considered to be too complex for interpretation. Using those staining procedures described above, it was noted that the simplest stain of those yielding good or excellent results (Giemsa, BDH 685704/560113) contained dyes of R_f 0.059 (methylene blue), 0.11 (azure B) and 0.60 (eosin), all as major components. Other relatively simple stains of this group (Giemsa, G. T. Gurr 06707; Leishman, E. Gurr, no batch No.; Romanowsky, R. A. Lamb 2627) contained, in addition, a dye of R_f 0.72 (tribromofluorescein) and in two instances one of R_f 0.79 (fluorescein). However, the majority of successful stains contained all the following dyes, at least some of which were in quantities greater than traces: R_f 0.059 (methylene blue), 0.11 (azure B), 0.15 (a dye chromatographically indistinguishable from toluidine blue but whose identity is unknown), 0.19 (azure A), 0.24 (sym.—dimethylthionine), 0.60 (eosin), and 0.72 (tribromofluorescein). It thus appears that the components of R_f 0.15, 0.19, 0.24, and 0.72 are unessential for, although not detrimental to, successful staining. In this study, we

Stain and Batch No.	Components Present ¹											
	0-059	0-11	0-15	0-19	0-24	0-25	0-36	0-47	0-59	0-60	0-72	0-79
'Diff-Quik'												
1 Harleco 3058 P Solution I										●	○	○
2 Solution II	○	○	○	○	○							
Giemsa												
1 BDH 685704/560113	●	●								●	○	○
2 Difco 1169	●	●	○	○	○		○	○		●	○	○
3 Gurr 1324	●	●	○	○	○		○	○	○	●	○	○
4 E. Gurr—no batch No.	●	●	○	○	○		○	○		●	○	○
5 G. T. Gurr 06707	●	●	○	○	○		○	○		●	○	○
6 G. T. Gurr 17869	●	●	○	○	○		○	○		●	○	○
7 Hopkin and Williams 013954	●	●	○	○	○		○	○		●	○	○
8 R. A. Lamb 2670	●	●	○	○	○		○	○		●	○	○
9 R. A. Lamb 3025	●	●	○	○	○		○	○		●	○	○
10 R. A. Lamb 3328	●	●	○	○	○		○	○		●	○	○
11 Merck 416168	●	●	○	○	○		○	○	○	●	○	○
Jenner²												
1 Difco 0402	○	○	○	○	○					○	○	○
2 E. Gurr—no batch No.	●	●								●	○	○
3 G. T. Gurr 05993	●	●	○	○	○					●	○	○
4 G. T. Gurr 10886	●	●	○	○	○					●	○	○
5 G. T. Gurr 16406	●	●	○	○	○					●	○	○
6 G. T. Gurr 17372	●	●	○	○	○					●	○	○
7 Hopkins and Williams 93797	●	●	○	○	○					●	○	○
8 Hopkin and Williams 10064B4803/1	●	●	○	○	○					●	○	○
9 R. A. Lamb 2656	●	●	○	○	○					●	○	○
Leishman												
1 BDH D 874630	●	○	○	○	○					●	○	○
2 E. Gurr—no batch No.	●	●								●	○	○
3 E. Gurr Feb. 1965	●	●	○	○	○					●	○	○
4 G. T. Gurr 646	●	●	○	○	○		○	○		●	○	○
5 G. T. Gurr 1246	●	●	○	○	○		○	○		●	○	○
6 Hopkin and Williams 013595	●	●	○	○	○		○	○		●	○	○
7 Hopkin and Williams 13121 7824/1	●	●	○	○	○		○	○		●	○	○
8 R. A. Lamb 0506	●	●	○	○	○		○	○		●	○	○
9 R. A. Lamb 2414	●	●	○	○	○		○	○		●	○	○
May-Grünwald³												
1 Difco 0272	○	○	○	○	○					○	○	○
2 E. Gurr—no batch No.	●	●								●	○	○
3 Hopkin and Williams 93797	●	●	○	○	○					●	○	○
4 Hopkin and Williams 90239B 011356	●	●	○	○	○					●	○	○
5 R. A. Lamb 2668	●	●	○	○	○					●	○	○
6 Searle 17300/57699	●	●	○	○	○					●	○	○
Romanowsky												
1 G. T. Gurr 0557		○	○	○	○	○	○			○	○	○
2 G. T. Gurr 1155		○	○	○	○	○	○			○	○	○
3 R. A. Lamb 2259	○	○	○	○	○	○	○			○	○	○
4 R. A. Lamb 2541	○	○	○	○	○	○	○			○	○	○
5 R. A. Lamb 2627	○	○	○	○	○	○	○			○	○	○
Wright												
1 E. Gurr—no batch No.	○	○	○	○	○			○	○	○	○	○
2 R. A. Lamb 0074		○	○	○	○			○	○	○	○	○

Table I Dye composition and staining properties of Romanowsky-type stains

¹Components are designated by their R_f values obtained using the thin-layer chromatographic method of Marshall and Lewis (1974a). The tentative identities of these components are: 0-059 methylene blue, 0-11 azure B, 0-15 identity unknown but chromatographically indistinguishable from toluidine blue, 0-19 azure A, 0-24 *sym.*-dimethylthionine, 0-25 azure C, 0-36 thionine, 0-47 methylene violet Bernthsen, 0-59 methyl thionoline or thionoline, 0-60 eosin, 0-72 tribromofluorescein, 0-79 fluorescein (Marshall and Lewis, 1974b).

Major components are indicated by ●, trace ones by ○, and those present in intermediate amounts by ○.

²See text for details.

³These stains were used only in combination with Giemsa stain.

Stain and Batch No.	Staining Properties						Red Cells	Rating of Stain's Suitability for Diagnostic Purposes ¹
	Chromatin	Leucocyte Cytoplasm	Granules					
			Basophil	Eosinophil	Neutrophil	Platelet		
'Diff-Quik'								
1	} Purple	Blue	Purple	Dull red	Purple	Purple	Blue-pink	Fair
2								
Giemsa								
1	Purple	Blue	Purple-black	Dull red	Red-purple	Purple	Grey-pink	Good
2	Purple	Blue	Purple-black	Dull red	Purple	Purple	Pink	Excellent
3	Purple	Blue	Purple-black	Orange	Purple	Red	Green	Fair
4	Pale purple	Pale blue	Pale purple	Pink	Red	Pink	Red	Poor
5	Purple	Blue	Purple-black	Orange-red	Red-purple	Purple	Pink	Excellent
6	Purple	Blue	Purple-black	Orange-red	Purple	Purple	Pink-brown	Good
7	Purple	Blue	Purple-black	Red	Purple	Purple	Grey-pink	Good
8	Purple	Blue	Purple-black	Orange-red	Purple	Purple	Pink	Excellent
9	Purple	Blue	Purple-black	Red	Purple	Purple	Pink	Excellent
10	Purple	Blue	Purple-black	Dull red	Purple	Purple	Blue-green	Fair
11	Purple	Blue	Purple-black	Dull red	Purple	Purple	Blue-pink	Fair
Jenner ³								
1	Purple	Grey-blue	Purple-black	Orange-red	Red-purple	Red	Pink	Fair
2	Purple	Blue	Purple-black	Red	Purple	Purple	Pink	Excellent
3	Purple	Blue	Purple-black	Red	Purple	Purple	Pink	Excellent
4	Purple	Blue	Purple-black	Red	Purple	Unstained	Blue-pink	Poor
5	Red-purple	Violet	Unstained	Dull red	Purple	Unstained	Blue-pink	Very poor
6	Purple	Blue	Purple-black	Red	Red	Purple	Pink	Poor
7	Purple	Blue	Purple-black	Dull red	Purple	Purple	Blue-pink	Fair
8	Purple	Blue	Purple-black	Red	Purple	Purple	Blue-pink	Fair
9	Purple	Blue	Purple-black	Red	Purple	Purple	Blue-pink	Fair
Leishman								
1	Purple	Blue	Purple-black	Red	Purple	Purple	Pink	Excellent
2	Purple	Blue	Purple-black	Red	Purple	Purple	Pink-purple	Good
3	Purple	Blue	Purple-black	Red	Purple	Purple	Brown-pink	Good
4	Purple	Blue	Purple-black	Dull red	Blue-purple	Purple	Grey	Fair/poor
5	Purple	Blue	Purple-black	Red	Blue-purple	Purple	Grey-pink	Fair/poor
6	Purple-blue	Blue	Purple-black	Red	Red-purple	Purple	Pink	Fair/poor
7	Purple	Blue	Pale purple	Red	Red-purple	Purple	Dull pink	Poor
8	Purple	Blue	Purple-black	Dull red	Red-purple	Purple	Blue-pink	Poor
9	Purple	Blue	Purple-black	Dull red	Purple	Purple	Grey-pink	Good
May-Grünwald ³								
1	Purple	Blue	Purple-black	Red	Purple	Purple	Pink	Excellent
2	Purple	Blue	Purple-black	Red	Purple	Purple	Dull pink	Good
3	Purple	Blue	Purple-black	Red	Purple	Purple	Grey-pink	Good
4	Purple	Blue	Purple-black	Red	Purple	Purple	Blue	Fair
5	Purple	Blue	Purple-black	Red	Purple	Purple	Blue-pink	Good
6	Purple	Blue	Purple-black	Red	Purple	Purple	Pink	Excellent
Romanowsky								
1	Pale blue	Pale blue	Red-purple	Red-purple	Red	Pale red	Red	Useless
2	Unstained	Unstained	Unstained	Red-violet	Pale red	Purple	Pink	Useless
3	Purple	Blue	Purple-black	Orange-red	Purple	Purple	Pink	Excellent
4	Purple	Blue	Purple-black	Orange-red	Purple	Red	Pink	Excellent
5	Purple	Blue	Purple-black	Orange-red	Purple	Red	Pink	Excellent
Wright								
1	Blue	Blue	Unstained	Unstained	Unstained	Blue	Green	Useless
2	Blue	Blue	Unstained	Red	Red	Unstained	Pink	Useless

Table 1—continued

have been unable to correlate the staining of specific substrates with specific dye components as has been done by other workers. For example, Lillie (1944) found that Romanowsky-type stains in which azure A, azure B, and methylene blue predominated gave respectively red-purple, violet-purple, and blue chromatin. A knowledge of the predominant thiazine dye component of our commercial stains did not enable such a prediction to be made of their staining of chromatin. Contrary to such predictions,

in two of the three stains yielding blue chromatin (Romanowsky, G. T. Gurr 0557; Wright, R. A. Lamb 0074) methylene blue was absent.

It will be seen from table I that certain stains gave unsatisfactory results (that is, those other than excellent or good) even though they contained the components characteristic of the satisfactory stains. In a proportion of these (Giemsa: Gurr 1324, R. A. Lamb 3328, Merck 416168; Leishman: Hopkin and Williams 013595 and 13121 7824/1; Wright: E.

<i>Stain and Batch No.</i>	<i>Sulphated Ash (%)</i>
Giemsa	
BDH 685704/560113	1.87
E. Gurr—no batch No.	14.31
Hopkin and Williams 013954	3.30
R. A. Lamb 2670	5.23
Merck 416168	1.23
Leishman	
BDH D 874630	4.45
E. Gurr—no batch No.	9.08
G. T. Gurr 646	10.60
G. T. Gurr 1246	11.51
Hopkin and Williams 013595	2.59
R. A. Lamb 0506	14.90
R. A. Lamb 2414	7.07

Table II *Sulphated ash analyses of Romanowsky-type stain powders*

Gurr, no batch No.) it was noted that dyes of R_s 0.47 (methylene violet Bernthsen) and/or 0.59 (methyl thionoline or thionoline) were present in greater than trace amounts. The detrimental effects on staining of high concentrations of methylene violet Bernthsen under certain conditions was observed by Lillie (1944). He believed that such effects were observed only in Coplin jar staining, or 'on slide' staining using solutions with relatively low methanol content. We have observed it with several of our 'on slide' procedures irrespective of the methanol concentration of the stains. The detrimental effects of the dye of R_s 0.59 have, to our knowledge, not been previously reported. The mechanisms of action of these effects are obscure.

The failure of the rest of this group (Giemsa: E. Gurr, no batch No.; Leishman: G. T. Gurr 646 and 1246; R. A. Lamb 0506) may be explained in terms of excessive metal salt contamination. Such contaminants are present in the thiazine (Clemens and Toepfer, 1968) and xanthene (Marshall *et al.*, in press) dyes used to produce Romanowsky-type stains. Additional contaminants may also be introduced during 'polychroming' procedures. Estimates of the total amount of metal salt contamination of these stains may be obtained by sulphated ash determinations. Table II shows that the aforementioned stain powders are all exceptional in that they yield greater than 10% sulphated ash (this is equivalent to > 0.05 g/l in the Leishman and Giemsa staining solutions). Most batches of stain yield considerably less ash. The importance of these salts in histological staining has received only scanty attention (but see Bennion and Horobin, 1974; Horobin and Goldstein, 1974; Singer, 1952) and their importance in Romanowsky staining has not hitherto been reported. Numerous explanations are available for the effects of salts upon staining, including salt-induced dye activity changes,

(Mukerjee and Ghosh, 1970) and, more specifically, salt-induced dye aggregation (Coates, 1969) and Donnan equilibrium effects (Bennion and Horobin, 1974). The applicability of any of these explanations to Romanowsky-type staining is at present unknown.

Conclusions

The staining properties of commercially available Romanowsky-type stains are extremely variable. It is not possible to correlate, with any precision, these variations with stain composition as determined in this study. It is possible to identify three properties of these stains which, when used alone (that is, not in combination procedures), will produce results closely approximating to the generally recognized Romanowsky scheme of colouring:

(1) Dye components of R_s 0.059 (methylene blue), 0.11 (azure B), and 0.60 (eosin) are revealed by thin-layer chromatography probably in amounts greater than traces. Any of the components of R_s , 0.15, 0.19, 0.24, 0.25, 0.36, 0.72, and 0.79 (these have tentatively been identified as, respectively, a dye of unknown identity, azure A, *sym.*—dimethylthionine, azure C, thionine, tribromofluorescein, and fluorescein) may also be present.

(2) Dye components of R_s 0.47 and 0.59 (these have tentatively been identified as, respectively, methylene violet Bernthsen and methyl thionoline/thionoline) must be absent or present only in trace amounts.

(3) The stain must not be excessively contaminated with metal salts. Giemsa and Leishman stains in powder form should give $< 10\%$ sulphated ash. This is equivalent in both cases to $< 0.005\%$ sulphated ash in the final staining solutions.

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