Staining properties and stability of a standardised Romanowsky stain

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SUMMARY An evaluation of the standardised Romanowsky stain of Marshall *et al.* has been made in a routine haematology laboratory. It was noted that this stain had several advantages over the May-Grünwald Giemsa stain used in most British laboratories. These advantages include ease and speed of preparation, a shorter staining time, and reproducibility of results. These results are described in detail. The stability of the stock stain solution and of the 'working' stain (stock + buffer) has been studied by, respectively, thin-layer chromatography and visible spectroscopy. No change was detected in the composition of the stock solution at ambient temperature over a period of six months. Stability was unaffected by the composition of the container (polyethylene, PyrexTM, or soda-glass) or by daylight. The 'working' solution was stable for 3 hours. Thereafter a precipitate is formed, consisting of thiazine dyes and eosin in a molar ratio of $\sim 2:1$.

The major problem in the use of Romanowsky stains is batch-to-batch variation in their staining performance. This is a potential source of diagnostic error. Adoption of a reliable, standardised stain would not only eliminate such error but would also ensure comparability of stained films from different laboratories and facilitate reference to illustrative atlases. Moreover, such a stain is essential for use with automatic cell recognition systems. Variability in performance is usually due to variation in stain composition (Scott and French, 1924; Lillie and Roe, 1942; Lillie, 1943a, 1944a; Saal, 1964; Clemens and Toepfer, 1968; Toepfer, 1972; Marshall and Lewis, 1974b, 1975a; Löhr *et al.*, 1975; Marshall *et al.*, 1975a,b,c; Wittekind and Löhr, 1975; Wittekind *et al.*, 1976). We have therefore developed a stain prepared from pure dyes, which is of constant composition and performance (Marshall *et al.*, 1975c). Since our original report, in which the staining properties of different cell types were illustrated, we have continued to study the stain's properties. In this paper we describe its performance in routine use, list its staining properties, and provide data on its stability.

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Received for publication 25 July 1977

Material and methods

PREPARATION OF STAIN AND STAINING TECHNIQUE

Dyes were purified, as described previously (Marshall *et al.*, 1975c; Marshall and Lewis, 1975b). The stock solution of stain was prepared and used as described by Marshall *et al.* (1975c).

Stained preparations were made from most of the blood and bone-marrow samples arriving in our laboratory during one working week, approximately 1000 specimens in all. Duplicate preparations were made by our routine May-Grünwald Giemsa (MGG) technique (Dacie and Lewis, 1975).

STABILITY OF STAIN

The stock solution was stored at ambient temperature ($\sim 21^{\circ}\text{C}$) in polyethylene, PyrexTM, and soda-glass containers, in darkness and in daylight. All containers were tightly stoppered to prevent evaporation but were only partially filled to allow contact of the stain with atmospheric oxygen. Aliquots of stain were withdrawn at intervals over a period of six months, diluted with methanol, and analysed by thin-layer chromatography (Marshall and Lewis, 1974a). The stability of the 'working' stain (stock + buffer) was investigated as follows: samples were prepared and aliquots withdrawn at intervals of up to eight hours. Where necessary, these were centrifuged to remove precipitate, the supernatant was

diluted with excess methanol, and visible absorption spectra were made using a Unicam SP 800 Recording Spectrophotometer.

Results and discussion

During the test period, peripheral blood and bone-marrow films were examined from patients with a wide variety of disorders. The staining appearances of the vast majority of normal and pathological cell types were thus seen. With the standardised stain red cells stained a clear pink, in contrast to a brownish hue in our MGG preparations. The transition from basophilic through polychromatic to orthochromatic erythroblast was clearly demonstrated, as was the presence of red cell inclusions such as Howell-Jolly bodies and siderotic granules. Malarial parasites showed characteristic Romanowsky staining reactions.

In white cells, the specific granules of neutrophils, eosinophils, and basophils were well stained, as were the azurophil granules of lymphocytes and monocytes, the primary granules of promyelocytes, and platelet granules. Toxic granulation of neutrophils was shown clearly. The stain gave an excellent range of colouration in the mononuclear cells; cytoplasm was grey in monocytes, a clear pale blue in lymphocytes, a deeper blue in blast cells and atypical mononuclear cells, and a rich dark blue in plasma cells and proerythroblasts. Nuclear chromatin patterns were well stained and nucleoli were very clearly demonstrated.

The appearances observed were thus in all respects those of an excellent Romanowsky stain, and the results were comparable with the best MGG preparations. The latter will, of course, vary with the batches of stain used (see, for example, Marshall *et al.* (1975a) and Toepfer (1972)) whereas our stain is of constant composition and therefore gives consistent results.

Analysis of the stock solution of our stain showed that its composition remained unchanged in the six-month period under all conditions of storage. The

thiazine dyes underwent no oxidative demethylation even though solutions were in contact with atmospheric oxygen. There are few comparable data on other Romanowsky stains. Lillie (1944b) measured the changes in the visible absorption spectra of methylene blue-eosinate and Wright's stain with time. The stains were found to be highly stable in glycerol-methanol (1:1 v/v), and this solvent mixture was therefore selected for our stain. Stock solutions, which are totally methanol-based, are much less stable (Lillie, 1944b; Dean *et al.*, 1977). These include the stains of Jenner, Leishman, May-Grünwald, and the more recent stain of Wittekind *et al.* (1976).

Our 'working' solution proved stable in use for three hours. Thereafter, the formation of a precipitate made it necessary to prepare a fresh solution. Stain precipitation was investigated quantitatively by visible spectroscopy. Solutions of the 'working' stain diluted with methanol showed two absorption maxima, one at 642 nm due to the thiazine dyes (methylene blue and azure B), and another at 525 nm due to eosin. Thus, by measuring the reduction in the absorbances at these wavelengths, it was possible to follow the amount and composition of the precipitate formed. It was found that the solution was stable for three hours (Figure). After this, significant amounts of precipitate were formed, the rate of formation being greatest three to four hours after preparation. Precipitation ceased at eight hours, when the concentration of eosin was effectively zero. The composition of this precipitate was deduced as follows: the 'working' solution contains thiazine dyes and eosin in the ratio of 2:1 by weight. Taking the molecular weight of the thiazine dyes as 350 (the molecular weight of the predominant dye, azure B hydrobromide—that of methylene blue hydrochloride is not significantly different) and that of eosin (colour acid) as 648 (ignoring the small proportion of 2',4',5'-tribromofluorescein), we see that in the freshly prepared 'working' solution the cationic and anionic dyes are present in the molar ratio of about 3.7:1. By eight hours the concentration of the cationic dyes in solution has been halved, whereas the

Figure Stability of 'working' solution of the stain: (left) change of absorbance at 642 nm (thiazine dye absorption maximum) with time; (right) change of absorbance at 525 nm (eosin absorption maximum) with time.

concentration of eosin has fallen to virtually zero. Therefore, the molar ratio of thiazine dyes:eosin in the precipitate is about 2:1. Similar studies on the 'working' solutions of commercially available stains indicate the formation of similar precipitates, the rate of formation again being greatest three to four hours after dilution of the stock.

In practical terms, the standardised stain has several advantages in the routine laboratory. In addition to its stability, no lengthy process of dissolution of dyes and ageing of solutions is necessary. Filtration of the stock solution is not required, and the staining time is considerably shorter than in the routine MGG technique. This can produce a significant saving of time when slides are stained intermittently during the day, while providing for the emergency service a simple staining procedure that gives results equal to those obtained with optimal technical facilities. It is probable that the stain will be commercially available in the near future.

This work was assisted by a grant from the Department of Health and Social Security. Mr W. Johns provided skilful technical assistance.

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