Blood and Malaria Parasite Staining with Eosin Azure Methylene Blue Methods

R. D. LILLIE, M.D.
Senior Surgeon, U. S. Public Health Service, Division of Pathology,
National Institute of Health, Bethesda, Md.

In 1939 it became apparent to those interested that many field workers in malaria were using Grübler's Giesma stain for thick film work, and were unable to obtain results satisfactory to them with the Giemsa stains then manufactured in the United States. The difficulty was that when staining at pH 7.0, which seems best for thick film work, the German stain gave a clear, light blue coloration to plasmodial and lymphocyte cytoplasm and a sharp reddish-purple chromatin stain, whereas American samples then available gave a grayish-lilac cytoplasmic coloration and a denser red-purple chromatin stain. This gray-lilac was difficult to distinguish in the similarly but less intensely stained background of detritus in the thick film, and from the gray-pink cytoplasm of red corpuscles at pH 7.0.

In attempting to solve the difficulties the available pure dyes of the methylene blue thionine series were tried in combination with eosin and with each other. To afford greater ease in manipulation, we decided to use the Nocht method, which consists in using 1:1,000 solutions of eosin and thiazin diluted in buffered water, on account of its extreme flexibility.

Azure B turned out to be the best single dye, although MacNeal and others had thought this dye of relatively low value. It was also found that the desired depth of cytoplasmic and background staining required a slight excess of basic dye over the amount equivalent to the eosin. This accords with Giemsa's original work.

Spectroscopic studies done at this time revealed that the imported Giemsa stain contained chiefly azure B and methylene blue, agreeing perfectly with our experimental findings.

Our first formulae were derived directly from the Nocht method. One consisted of methylene blue 270, azure B 200, azure A 50 and eosin 500 dissolved as such in 100 ml. of a mixture of equal parts of glycerin and methyl alcohol. This stain was highly satisfactory.

However, as soon as an attempt was made to translate this into commercial production, variation in dye content of the four dyes led to difficulties. So, believing that the precipitated eosinates would be of more constant dye content, further formulae were evolved on that basis, and the first few commercial samples were eminently satisfactory. Such samples consist basically of 50 mg. azure A eosinate, 250 mg. azure B eosinate, 200 mg. methylene blue eosinate and 100 mg. methylene blue.

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in 100 ml. of the glycerin and methyl alcohol mixture.

After a time this method in turn broke down and the spectroscope showed that the difficulty lay in qualitative variation in composition of the azure B. One of the methods of making azure B is to oxidize methylene blue to crude azure A with hydrochloric acid and potassium bichromate and then again to reduce this with formaldehyde by heating under a reflux condenser. This was supposed to yield only azure B. However, a trial of this reduction process in my hands has yielded on at least one trial almost pure methylene blue, as well as products spectroscopically intermediate between azure B and methylene blue.

It would appear that the commercial method of manufacture of azure B will have to be revised by introducing spectrographic control, by further study of the variation factors in the formaldehyde synthesis, or by going over to the partial-oxidation process which appears to yield quite constant products spectroscopically when the same proportions of chromate and methylene blue are used. This has been done and has been found to yield a more uniform product.9

However, with the cooperation of the Stain Commission and the dye companies, after excluding some samples which do not turn out well, we are now able to obtain on the market quite satisfactory Giemsa stain for blood and parasite work.

Turning to Wright's stain we find that commercial samples of this on the market, found in various laboratories and furnished for certification to the Stain Commission, vary considerably in staining capacity. Some samples yield pale blue nuclear staining with satisfactory light blue lymphocyte cytoplasm, good neutrophil and eosinophil granules and orange-pink-red corpuscles staining at pH 6.5 with a 1:2 dilution. Such samples, though offered as Wright's stain, are essentially eosinates of unaltered methylene blue, or Jenner's stain. Spectroscopically these give absorption maxima between 660 and 665 for the thiazin, 515–518 for the eosin. I consider these unsatisfactory. Other samples give blue-violet to reddish-purple leucocyte and parasite chromatin, clear, light blue parasite and lymphocyte cytoplasm, good granules and orange-pink-red cells. Here absorption maxima range from 660 for the bluer samples to 645 or 650 for the redder. These are quite good for blood and parasite work. Still other samples give denser reddish-purple chromatin in white cells and parasites, more gray-lilac cytoplasm in lymphocytes and parasites, perhaps less satisfactory eosinophils, good azure granules and orange-pink-red corpuscles. Here absorption maxima range from 620 to about 640. Some workers prefer such samples for blood work, but they seem less satisfactory for malaria parasites, trypanosomes, etc.

Such variation occurs even in successive samples from the same manufacturer and appears to be inherent in the Wright method of polychroming. This calls for heating at about 98°C for an hour, in relatively shallow layer, of 1 per cent methylene blue in 0.5 per cent sodium bicarbonate. Extended experimental work has shown that marked variations in such products are produced with variations in the amount of evaporation occurring, in exposure to air, in pH of the initial mixture, and apparently by taking the sodium bicarbonate out of different bottles.

However, by prevention of evaporation, by rigidly controlling pH by using standard buffer mixtures in place of the apparently variable sodium bicarbonate, and by close control of time, temperature, dye content of methylene blue and its concentration in solution, it is possible consistently to produce polychrome methylene blues in
the azure A or azure B range which will vary by less than 5 milli-microns in the position of the absorption band on repeated trial. So it would appear that, when the preference of workers as to color effect can be learned, it should now be possible to duplicate closely Romanowsky stains of Wright type in successive batches.

We have been greatly aided in these attempts at standardization of Romanowsky stains by study of their absorption spectra and the correlation of these with the staining capacity of the samples. It was my belief that a similar method of study would be valuable in determining the causes of deterioration of solutions in methyl alcohol or glycerin and methyl alcohol of these stains. Accordingly, studies were initiated last August using a number of samples of Wright's stain solution which had been on the laboratory shelves for intervals varying from 1 week or so up to over 3 years, as well as freshly prepared solutions of two samples, one with an original thiazin absorption maximum at 656, the other at 621 μ. To these latter various contaminants were deliberately added at 2 per cent concentration and spectra were run at intervals from 2 days up to 2 months at the present writing. The samples have also been tested as blood and parasite stains at the start and at the end of this period.

Summing up this work to date, old samples which no longer give satisfactory stains show absorption peaks generally below 615 μ, which represents a fairly pronounced shift from the original in many on which spectra were made when they were fresh. Crude chemical separations of some of these samples indicated formation of considerable amounts of methylene violets, apparently more than the remaining azares, though thoroughly satisfactory separations have not yet been worked out.

The deliberately contaminated samples all showed progressive shift of the absorption maxima toward 600 μ. This was fastest when repurified methyl alcohol was shaken with Na₂CO₃ and then filtered and used as a solvent. Acetone and formaldehyde both accelerated the shift, and methyl alcohol redistilled over silver oxide to destroy aldehydes showed a slower shift than commercial C.P. grade. Two per cent water had little appreciable effect. Formic acid seemed to act as a stabilizer for the thiazin, but this ruins the eosin component for staining purposes. However, after 2 months the repurified methyl alcohol solution had reached the same point as those contaminated with acetone or formaldehyde. Hence it would seem that the repurification of methyl alcohol was of little value.

However, the most obvious and striking finding was that solutions from the sample with an initial absorption peak at 656 were still good stains after 2 months in solution in pure, commercial, or acetone- or formaldehyde-contaminated methyl alcohol, while the solutions made from the sample with an initial maximum at 621 were all worthless in less than a month in spite of originally brilliant staining.

In the first instance absorption maxima were still above 630 after 2 months, while in the second they were below 615 in less than 2 months.

Hence it would seem that we should require initial absorption maxima for Wright's stains between 650 and 660 μ.

REFERENCES
4. Lillie, R. D. Romanowsky Staining with Buffered Solutions. III. Extension of the Method to


