The Application of the Prussian Blue Stain to Previously Stained Films of Blood and Bone Marrow

By R. Dorothy Sundberg and Harriette Broman

A SIMPLE METHOD FOR STAINING non-hemoglobin iron within erythrocytes and their precursors and within macrophages and other cells containing particulate iron has been in use in our laboratories since 1951. This method has made possible rapid estimates of the amount of non-hemoglobin iron in the marrow and in the blood in both new and old films of marrow or blood as well as in both new and old section material.

LITERATURE

Grüneberg described “siderocytes” as erythrocytes containing granules which were positive for iron when subjected to the prussian blue reaction. After fixation with absolute methyl alcohol, blood films were treated with a freshly mixed solution of 1 per cent potassium ferrocyanide and 1 per cent hydrochloric acid for 3 to 5 minutes and then counterstained with biebrich scarlet.

Pappenheimer, Thompson, Parker and Smith (1945) used methyl alcohol fixation followed by a freshly mixed solution of 1 per cent hydrochloric acid (3 parts) and 2 per cent potassium ferrocyanide (1 part). They counterstained with basic fuchsin. Of particular interest in their study of siderocytes was the fact that they photographed granules and rod-like structures demonstrable in erythrocytes with the Giemsa stain, decolorized with acid alcohol, and then applied the prussian blue reagent. They showed that the stippled erythrocytes of the blood stained with Giemsa were actually erythrocytes containing some type of iron granules. Pappenheimer et al. (1945) also showed that the granules which were prussian positive did not take the Gram stain and were Feulgen negative.

When heparinized blood from their cases with high percentages of siderocytes was sedimented, large numbers of the iron-containing bodies were recovered. When these were treated with sulphuretted hydrogen (0.9 per cent salt solution, most took on a brown color; only occasional black bodies were found. Since hemosiderin is blackened by this reaction, Pappenheimer et al. felt that the iron-containing bodies were not hemosiderin. Here one might comment that it is of some interest that occasional iron-containing bodies had been blackened. Further proof that siderocytes actually contained iron was obtained by demonstrating their migration toward the magnetized side of a tube while the unaffected erythrocytes settled to the bottom. During the course of their study, Pappenheimer et al. sent their slides to R. M. Case. Case applied the “iron stain” over Giemsa and found many siderocytes present but for some reason felt that the granules were not identical with siderotic granules.

Rath and Finch (1948) have made extensive use of the prussian blue reaction in estimating the total amount of stainable iron in fixed or free reticuloendothelial cells in unstained films of fragments of bone marrow.

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Mills, Huff, Krupp and Garcia (1950) illustrated stippling in rubricytes and erythrocytes with the Wright-Giemsa stain and also showed similar stippling in different rubricytes and erythrocytes subjected to the prussian blue reaction and counter-stained with safranine.

Despite the early report of Pappenheimer et al. (1945) no real emphasis was placed on the practice of using a reaction designed to demonstrate iron as a type of counterstain until Koszewski (1952) showed that one could decolorize marrow films which had been stained with May-Grünwald Giemsa years before and could then apply the Turnbull blue reaction. He illustrated plasma cells containing granules which stained first with the May-Grünwald Giemsa and then again, after decolorization, with Turnbull blue.

Recently Kaplan, Zuelzer, and Mouriquand (1954) have described a method which they used on fresh films of marrow. Their method involved fixation in formalin vapor for 30 minutes prior to staining for one hour in equal parts of a solution of 2 per cent aqueous potassium ferrocyanide and 2 per cent hydrochloric acid. After a brief wash in tap water, their slides were counterstained for 30 to 60 seconds with dilute basic fuchsin.

TECHNIC

Dry films of a large variety of cellular fluids including blood, bone marrow, lymph, sputum and ascitic fluid or imprint or touch preparations of various tissues or organs can be stained for nonhemoglobin iron in the following manner.

1. The film or imprint is dried rapidly.
2. The slide is flooded with Wright's stain and the stain should remain in this undiluted state for 4 minutes.
3. The stain is diluted with buffer and allowed to remain on the slide for a period of time suitable to the strength of the stain and the cellularity of the film.
4. The slides are immersed in the prussian blue reagent which is made up of freshly mixed 1 per cent hydrochloric acid (3 parts) and 2 per cent potassium ferrocyanide (1 part). The slides are left in the prussian blue reagent for 10 minutes, washed briefly in distilled water until a pink color appears and then allowed to drain dry.

Slides stained in this way are comparable to the original films stained with Wright's stain, but particulate iron is colored a vivid blue or blue green, and there has been a minimal amount of decolorization. Individual cells are still easily recognizable and the original staining reaction is altered very little. The nongranular cytoplasm of many of the hemosiderin-filled macrophages is often also a vivid blue or blue green, and fragments of this cytoplasm are a similar color.

Insofar as particulate iron is concerned, that which once appeared deep blue or even purple with Wright's stain is changed to an even more vivid blue or blue green color than

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* This paper was brought to our attention by Dr. Rudi Schmid at a time when we were attempting to distinguish between various types of stippling. The illustrations showed cells resembling many we had seen. Because many of the cases studied had previous biopsies of the marrow, it was desirable to have a method for proving the presence of particulate iron in old specimens.

† 0.5 per cent and 4 per cent potassium ferrocyanide have also been used with apparently equal success. The proportions and strengths used were those used by Pappenheimer et al. They were recommended by Dr. Roy Holly who had consulted Dr. Clement Finch regarding the best stain.

‡ This reaction is reasonably clear at 2 minutes, but the granules seem less discrete than at 5 or 10 minutes. At 30 minutes (the time recommended for tissues), decolorization is still not too great, but nuclei show some fading, and Jolly bodies may be very difficult to see.
that obtained with the prussian blue reaction alone, and that which appeared blue or blue-green with Wright’s stain takes on a more intense color than it showed originally. No intentional separate phase of decolorization is necessary. The prussian blue reagent decolorizes and changes the color of the particulate iron sufficiently to make the iron easily identifiable. This method, using the prussian blue reagent as a type of counterstain, has been used with equal success on films originally stained with Wright’s or the Giemsa stains as long as 30 years ago* and on films stained with Wright’s stain immediately prior to using the prussian blue reagent.

Controls of this method have included various studies. Comparable films of the same marrow having many macrophages laden with phagocytosed erythrocytes and particulate iron (hemosiderin?) as well as many siderotic normoblasts and siderocytes have been subjected to:

1. Methyl alcohol fixation (4 minutes) and the prussian blue reagent (10 minutes).
2. The outlined prussian blue reaction followed by Wright’s or the Giemsa stains or by safranine or basic fuchsin.
3. Wright’s stain followed by the Prussian blue reaction.

In these three types of preparations the numbers of iron-laden macrophages are similar and the blue to blue-green color of the prussian positive granules and cytoplasm of macrophages is similar in each of the types of specimens. The siderotic normoblasts and siderocytes are similar in percentages in the latter two types of specimens. In the first type of specimen in which no counterstain is used, the siderotic granules are visible in large numbers of cells believed to be erythrocytes and precursors, but the cell types are not clearly recognizable. The Wright’s stain followed by the prussian blue reagent affords the best cytology.

Efforts to predict which of the granules in macrophages or which type of stippling in erythrocytes and normoblasts will be prussian blue positive have not been uniformly successful. Much of the deep blue granulation in either macrophages or normoblasts and erythrocytes will become a brilliant blue or blue-green with the prussian blue reagent. Numerous macrophages containing blue blue-green, and olive green pigments and numerous stippled erythrocytes and normoblasts have been circled, sketched, subjected to the prussian blue reagent and reidentified. Some, but not all of the blue or blue-green pigment in macrophages or normoblasts and erythrocytes proves prussian positive. Some of the olive-green pigment remains olive green or disappears. Some of the stippling disappears. Although most often it is the fine blue-green basophilic stippling which disappears, occasionally the cell which contained that stippling will show a somewhat coarser, brilliant blue-green stippling after the prussian reaction.

Particulate iron has been found in neutrophils, lymphocytes, monocytes, and plasma cells as well as in macrophages and in erythrocytes and precursors. The blue-green Döhle bodies of neutrophils are prussian negative.

Because it seemed that it was the precipitated basophilic spongiosplasm (ribonucleic acid bearing cytoplasm) or the ordinary basophilic stippling of erythrocytes and normoblasts which disappeared with the prussian reagent, films of reticulocytes were also studied. The reticulation of reticulocytes stained with brilliant cresyl blue or stained with brilliant cresyl blue plus a Wright’s counterstain also disappeared when subjected to the prussian reagent for 10 minutes.

* Blood film from pernicious anemia from files of Dr. Hal Downey.
In the course of this investigation, films of bone marrow and imprints of liver from a patient with malignant melanoma were also studied. In both of these types of preparations golden yellow* to brown pigment (not distinguishable from unstained hemosiderin) was seen in many of the macrophages and melanoma cells. When stained with Wright's stain, the pigment still was not distinguishable from hemosiderin. With the prussian reagent used as a counterstain, only very occasional bright blue-green granules could be found. With the simple prussian reaction alone, only a very few hemosiderin-containing macrophages were found in the films of marrow or imprints of liver, although numerous cells containing the golden brown pigment had been identified prior to application of the prussian reagent. These studies afforded further evidence that all pigment in the marrow which resembles some hemoglobin breakdown product does not contain iron.

The prussian blue reagent has also been used on sections of particles of bone marrow and, of course, other tissues. Here the technic is as follows:

1. Take the tissue through xylol and decreasing strengths of alcohol to distilled water.
2. Stain for 30 minutes with the prussian reagent.
3. Wash in tap water for 20 minutes.
4. Counterstain with hematoxylin for 2 minutes and eosin for a few seconds.
5. Do not decolorize the hematoxylin.

With this technic bright blue-green granules of varying sizes are visible in macrophages and phagocytic cells of all types and also in normoblasts and erythrocytes. Some of the masses of prussian positive material in the macrophages are extremely large; their color is, however, similar to that seen in the erythrocytes (siderocytes) and normoblasts.

If new section material is not available, the coverslips can be removed from old slides, the tissue passed through decreasing strengths of alcohol to water, and the prussian reagent applied. Restaining with hematoxylin is generally not necessary, but the original eosin usually fades, and an additional passage through eosin is desirable.

Because Kaplan, Zuelzer, and Mouriquand’s method used on unstained films of marrow allowed estimation of normoblasts and reasonable identification of various cell types, and because their results seem similar to those obtained with the method described here, we decided to use their method on previously stained films of marrow. It was found that the method described by Kaplan, Zuelzer, and Mouriquand can be applied to previously fixed and stained blood and marrow films. The original stain is generally fairly well maintained, and the particulate iron is the expected vivid blue-green color. The siderocyte percentage, based on 1000 cell counts, was estimated on blood films from two patients with remarkable siderocytoses (exogenous hemochromatosis and gastrectomized splenectomized megaloblastic anemia). By using these counts as an objective means of comparison, it was determined that a higher percentage of

* The pigment seemed very similar in color to that described as hemosiderin by Rath and Finch and to that seen in hemolytic anemias, hemochromatosis, megaloblastic anemias and refractory anemias.
PRUSSIAN BLUE STAIN

Table 1.—Patient with Exogenous Hemochromatosis—Siderocytes in Blood

<table>
<thead>
<tr>
<th></th>
<th>F10 P10 B</th>
<th>F30 P60 B</th>
<th>KZM</th>
<th>WP10</th>
<th>WP60</th>
<th>W F10 P60</th>
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<tr>
<td>RDS</td>
<td>9.6</td>
<td>13.5</td>
<td>10.1</td>
<td>7.0</td>
<td>6.4</td>
<td>9.6</td>
</tr>
<tr>
<td>HB</td>
<td>7.2</td>
<td>9.2</td>
<td>9.5</td>
<td>7.1</td>
<td>6.4</td>
<td>7.2</td>
</tr>
<tr>
<td>LMG</td>
<td>10.3</td>
<td>11.0</td>
<td>11.0</td>
<td>8.2</td>
<td>8.3</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Gastrectomized Splenectomized Patient with Megaloblastic Anemia—Siderocytes in Blood

<table>
<thead>
<tr>
<th></th>
<th>F10 P10 B</th>
<th>F30 P60 B</th>
<th>KZM</th>
<th>WP10</th>
<th>W F10 P10</th>
<th>W F30 P60</th>
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<tbody>
<tr>
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<td>39.7</td>
<td>37.3</td>
<td>19.6</td>
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<td>37.1</td>
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<tr>
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<td>37.5</td>
<td>35.5</td>
<td>34.9</td>
<td>22.7</td>
<td>39.9</td>
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</tr>
<tr>
<td>LMG</td>
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<td>18.8</td>
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</table>

Formalin vapor—30 minutes. F10—Formol vapor—10 minutes. P10—Prussian reagent—concentration as described in present report—10 minutes. P60—Prussian reagent—same as above—60 minutes. B—Basic fuchsin. KZM—Method of Kaplan et al.7 as outlined. W—Wright’s stain. K—Prussian reagent—concentration as described by Kaplan, et al.7—60 minutes.

Siderocytes was found when formalin fixation was used either originally or after the methyl alcohol fixation employed with Wright’s stain than when the only fixative was methyl alcohol. Formalin seemed to be the only variable responsible for a higher percentage of siderocytes and fixation with formalin for 10 minutes followed by immersion in the prussian reagent (concentration as described in present report or as recommended by Kaplan et al.7) for 10 minutes provided a stain with as great a degree of “positivity” as that obtained with 30 minutes of fixation and 60 minutes of exposure to the prussian reagent. The increased positivity provided by formalin fixation seems to be based upon the blue-green color reaction of minute particles and, sometimes, delicate fuzzy aggregates of stainable iron.

The siderocyte counts in table 1 give an idea of the variability of even 1000 cell counts as well as the variability apparently induced by formalin fixation.

Counts were done by three of us (R. D. S., H. B., and L. M. G.*).

Discussion

The technic described has proved valuable in the problem of differentiating the siderotic granules of siderocytes and normoblasts from basophilic stippling (ribonucleic acid) and Jolly bodies. It has also shown that all pigment in macrophages which resembles hemosiderin does not contain iron which stains with the prussian blue reagent. Because, with an added 10 minute staining period, the prussian blue reagent can be applied after films, imprints, or sections (30 minutes) have already been stained and still provide a clear-cut method of dis-

* Lorraine M. Gonyea, M.S., Instructor in Medical Technology, University of Minnesota Hospitals.
Distinguishing particulate iron, the method is invaluable in the comparison of old and new specimens which may contain excesses of particulate iron. It is also useful in the study of iron deficiency anemia, but in the latter, the staining of smears made from marrow particles as recommended by Rath and Finch is desirable since often virtually no particulate iron can be found in ordinary films made from the myeloid-erythroid or buffy coat layer of centrifuged marrow.

Bone marrow and blood from a variety of conditions has been studied, and the results are the subject of a separate report.

The term “particulate iron” is used here to refer to the granules and masses of prussian positive material which show colors varying from blue-green to blue, to purple with Wright’s stain, but which, with the addition of the prussian blue reagent, all assume a reasonably similar vivid blue-green color. The present studies suggest that if the material in macrophages is to be regarded as hemosiderin, probably the siderotic granules in normoblasts and siderocytes are also hemosiderin. The diffuse blue-green color in the cytoplasm of macrophages could be due to the more soluble form of iron, ferritin.

The method described has been compared with that recently presented by Kaplan, Zuelzer and Mouriquand. Their method shows a variably higher degree of positivity which appears to be based upon the ability of formalin vapor to allow staining of minute granules or fuzzy particles of iron with the prussian blue reagent. The method described by Kaplan et al. minus the basic fuchsin counterstain, can be used on previously stained dry films of blood, marrow, etc., a feature not pointed out by these investigators.

**Summary**

A simple method for staining non-hemoglobin iron in erythrocytes, normoblasts, macrophages, and other cells containing particulate iron in new or old films of cellular fluids or imprints of tissues and organs is presented. This method consists in using the prussian blue reagent as a type of counterstain; no separate decolorization is necessary. The preparations obtained resemble the original preparations except that iron stands out as a vivid blue-green material.

The method is particularly useful in studying conditions accompanied by varying degrees of iron excess or hemosiderosis of the marrow.

The stainable iron is virtually the same color. The diffuse blue-green color of the cytoplasm of macrophages might be attributed to the more soluble form of iron, ferritin.

Stainable iron is visible in normoblasts and erythrocytes as well as in macrophages in sections subjected to the prussian blue reaction.

A prussian blue method using formalin fixation on fresh films of marrow has also been shown to be useful in the demonstration of particulate iron in previously stained films of marrow and blood. The formalin fixation appears to bring about a higher percentage of siderocytes.

**Summario in Interlingua**

Es presentate un simple methodo pro colorar ferro non-hemoglobinie in erythrocytos, normoblastos, macrophagos, e altere cellulas a particulias continente ferro—si ten in nove e preservate frotis de fluido cellular como etiam in impres-
Prussian blue stain

Siones de textos e organismos. Iste método consiste in usar le reagente blu de Prussia como un specie de contra-colorante. Nulle processo specificamente de-colorante es requisit. Le preparatos obtenite es simile al preparatos original excepte que ferro se distingue como materia de color vivemente blu-verde.

Le método es specialmente utile in studiar conditiones accompaniate de varie grades de excesso de ferro o de hemosiderosis del medulla.

Omne le ferro colorate per le método exhibi practicamente le mesme nuance. Le diffuse blu-verde que appare in le cytoplasma de macrophagos es possibilemente attribuibile a ferritina que es un forma plus solubile de ferro.

Ferro colorabile es visible in normoblastos e erythrocytos e etiam in macrophagos in sectiones subjicite al reaction a blu de Prussia.

Le método a blu de Prussia con fixation a formalina que Kaplan, Zuelzer, e Mouriquand ha usate pro nove frotis de medulla es etiam utile in le demonstratio de ferro intraparticular in previemente colorate frotis de medulla e sanguine. Le fixation a formalina pare distinctificar un plus alte procentage de siderocytos.

REFERENCES

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The Application of the Prussian Blue Stain to Previously Stained Films of Blood and Bone Marrow

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