MICROTECHNICAL DEMONSTRATION OF IRON *

A CRITICISM OF ITS METHODS

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Iron contained in the tissues occurs in two forms. In one group of compounds, generally included under the denomination "hemosiderin," the iron reacts similarly to any inorganic, insoluble iron compound, readily demonstrable by the well known iron reactions of analytic chemistry. In the second group of compounds iron can be demonstrated only after thorough chemical destruction, *e.g.* incineration. In the latter group belong, among others, hemoglobin, methemoglobin and hematin. In this paper I wish to deal with the demonstration of hemosiderin-iron only.

There are three methods and their modifications, respectively, known for the demonstration of iron in microscopic preparations: (1) the Berlin blue method of Perls; (2) the iron sulphide reaction of Quincke (described by Mayer as early as 1850); and (3) the Turnbull blue method of Tirmann and Schmelzer. This last method is based on Quincke's reaction. According to the Berlin blue method of Perls the microscopic section is treated with hydrochloric acid and potassium ferrocyanide. Iron dissolved from hemosiderin by the hydrochloric acid reacts with the potassium ferrocyanide, producing a precipitate of Berlin blue. According to the iron sulphide reaction of Quincke, iron compounds are converted by yellow ammonium sulphide into iron sulphide. On account of the energetic reducing properties of this reagent, ferrous sulphide is formed from both ferrous and ferric compounds. Ferrous sulphide is highly sensitive to oxygen and acids, and preparations will deteriorate within a few days in spite of all possible care. Therefore, the Turnbull blue method of Tirmann and Schmelzer transforms ferrous sulphide by means of an acidulated solution of potassium ferricyanide into Turnbull's blue.

The question as to which of the methods mentioned is the best to

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demonstrate the greatest amount of iron present and to give the truest picture has been rather extensively debated. Generally speaking there are but a few adherents of the Berlin blue method (Zaleski, Arnold and Sumita). According to Sumita no method is superior to that of the Berlin blue. He reproduces a table of Neumann's showing that colorimetric demonstration of ferri-iron as Berlin blue is about 250 times more sensitive than its demonstration as iron sulphide. On the contrary, the great majority of workers on this topic are of the opinion that the iron sulphide reaction and Turnbull's blue method are more sensitive, demonstrating a greater amount of iron than the Berlin blue method. According to Quincke, the details, as revealed by the iron sulphide reaction, are much finer than those by the Berlin blue method. In his opinion this is due to the fact that iron is present in the tissues partly in the form of ferrous compounds which do not react with potassium ferrocyanide. Moreover, he supports the greater sensitivity of the iron sulphide reaction with the observation that egg yolk is stained dark green by ammonium sulphide, whereas if treated with hydrochloric acid and potassium ferrocyanide it becomes but pale blue. He also contends that the Berlin blue method will occasionally produce false positive reactions in the absence of iron compounds. According to Hueck, Turnbull's blue method is more reliable, especially if small amounts of iron are to be demonstrated. He is unable to explain this, as he never could demonstrate ferrous compounds in the tissues with the direct Turnbull's blue reaction. He observed that potassium sulphocyanate demonstrates a conspicuously smaller number of granules than ammonium sulphide. Nishimura's modification of the Tirmann-Schmelzer method consists of employing potassium ferrocyanide instead of ferricyanide after previous treatment with ammonium sulphide by reason of quick oxidation of the white ferro-ferrocyanide formed at first into ferri-ferrocyanide (Berlin blue) by the oxygen of the air. He declares that he was often able to prove the presence of ferrous in addition to ferric compounds in tissue sections. In his opinion the alleged higher sensitivity of Turnbull's blue method is due partly to the fact that it demonstrates both ferric and ferrous compounds. Mallory, too, prefers modifications of Turnbull's blue to the Berlin blue method, partly on the basis of the theory just mentioned, partly because Berlin blue is soluble in an excess of potassium ferrocyanide, causing blurring of the picture. Lubarsch also recommends Turn-

bull's blue method as the most sensitive of all. In short, according to the generally accepted opinion in the medical literature on this question, Turnbull's blue is the most dependable, demonstrating the greatest amount of iron: this tenet is accepted by all textbooks of microtechnique.

In the course of the year I performed the iron reaction on a great number of microscopic preparations and made observations contrary to the general view mentioned. Therefore I set out to reinvestigate the entire question of the microtechnical demonstration of iron. My material was rather miscellaneous: formalin and formalin-alcohol fixed organs from cases of pernicious anemia, organized hematomas of various ages, a case of pigmented giant celled xanthoma and the organs of a rabbit dying of phenylhydrazine intoxication. Many microscopic sections were prepared from all tissue blocks and all types of iron demonstration, as found in the literature, were tried on them, together with some methods devised by me. My results were as follows:

1. I was unable to demonstrate even traces of ferrous compounds in any material with the direct Turnbull's blue reaction. Therefore I see no reason why the Stoeltzner method, using a mixture of potassium ferro- and ferricyanide in order to ensure demonstration of both ferric and ferrous compounds, should ever be employed.

2. Different modifications of the Berlin blue method are by no means of the same value. Those modifications using hydrochloric acid and potassium ferrocyanide separately will invariably show a smaller amount of iron than those using them simultaneously. Those using acid first (method of Stieda) are fundamentally wrong, since the iron dissolved by the acid before the application of potassium ferrocyanide is irretrievably lost. But even those using the reagents in reverse order are objectionable. This is but natural. Ferric chloride is but slowly split from hemosiderin by hydrochloric acid; in the meantime almost all the potassium ferrocyanide taken up by the section during previous treatment with this substance will diffuse away and become so highly diluted in the excess of hydrochloric acid that its concentration will become entirely insufficient. This applies even to the method of Sumita (incubating the sections with a concentrated solution of potassium ferrocyanide). In this way both theoretically and practically only those methods are justifiable that use both reagents simultaneously. But even in the

latter case too short a time of exposure may constitute a serious source of error. Even when using a 10 per cent solution of hydrochloric acid (commercial pure concentrated hydrochloric acid, conchloric about 40 per cent of hydrochloric acid gas by weight, being taken here for 100 per cent) it can be easily observed that for about 20 to 25 minutes the number of blue granules will steadily increase, whereas after 25 minutes no more new granules are noticed. If less concentrated solutions are used the completion of the reaction, of course, will take an even longer time. This has been observed also by Nishimura. The concentration of the potassium ferrocyanide seems to be less important: a 1 to 2 per cent solution is quite sufficient. However, in order to prevent the Berlin blue precipitate from dissolving it is necessary to use a more concentrated solution (this point will be dealt with once more under Step 3). On the basis of experience I suggest the use of a mixture made up of equal parts of a 10 per cent potassium ferrocyanide and a 20 per cent hydrochloric acid solution, prepared freshly with distilled water and filtered. The time of exposure should be 30 minutes. This mixture has a satisfactory stability; it begins to show a greenish tinge after about 45 minutes, but no precipitate is formed even after several hours. Even so, it is advisable to place the slide with the section side down in order to avoid the deposition of any possible precipitate. Berlin blue will not be damaged by the acid as it is almost insoluble in dilute acids; by alkalis, however, it will be bleached. Therefore, counterstaining with lithium carmine is not advisable. For nuclear staining I used Kernechtrot (a red nuclear stain obtained from the German firm, Hollborn), with invariably satisfactory results. The method outlined yields an extraordinarily sharp and complete iron reaction, equal to the iron sulphide but without its drawbacks and decidedly superior to the Tirmann-Schmelzer method and any of its modifications. This was especially conspicuous in cases where minute amounts of iron had to be demonstrated. I did not observe a single case in which Turnbull's blue method gave a critically acceptable evidence of a greater amount of iron than the method described.

3. That in spite of the facts mentioned Turnbull's blue is considered the most reliable method by the majority of workers on this problem is due to several reasons, no one of which, however, can stand objective criticism. First, there are theoretical assumptions

which cannot be substantiated in practice, *e.g.* the presence of ferrous compounds. Hueck's statement that a conspicuously small number of granules react with potassium sulphocyanate is by no means a proof since iron sulphocyanate is easily soluble and its rapid diffusion renders its exact observation impossible. It is interesting that Nishimura, who also uses potassium ferrocyanide when performing the Tirmann-Schmelzer reaction on the basis of the rapid transformation of white ferro-ferrocyanide into Berlin blue, accepts as a possible cause of the alleged lower sensitivity of the Berlin blue method the fact that it fails to demonstrate both ferric and ferrous compounds; the obvious contradiction between his two statements seems to have escaped his attention. Moreover, as has been shown, hemosiderin does not contain ferrous compounds. In the egg yolk test of Quincke the darker color produced with the iron sulphide reaction is not a proof of higher sensitivity but is due simply to the fact that the staining power of ferrous sulphide is greater than that of Berlin blue. This can easily be shown by the following simple test: put one drop each of ammonium sulphide and of a mixture of sodium ferrocyanide and hydrochloric acid on a blotting paper moistened with a highly diluted (0.1 per cent) solution of ferrous sulphate: the spot caused by the first reagent will be dark green, almost black, whereas that caused by the second reagent will be much lighter blue, although both reactions may be considered quantitative. The objection of Mallory to the Berlin blue method is that Berlin blue is soluble in an excess of potassium ferrocyanide, which causes a diffuseness of the staining. I at once state that I never observed blurring attributable to Berlin blue itself; moreover, it can be easily shown that the assertion of Mallory does not hold. Prepare an aqueous suspension of Berlin blue, divide it into four equal parts, dilute these portions to equal volumes with (1) distilled water, (2) 10 per cent hydrochloric acid, (3) a 5 per cent solution of potassium ferrocyanide, and (4) a mixture of the two last solutions. Centrifuge for several minutes and observe the supernatant fluids. In (1) it will be dark blue, in (2) pale blue, and in (3) and (4) pale greenish yellow. This shows clearly that potassium ferrocyanide strongly reduces the solubility of Berlin blue. If the Berlin blue and Turnbull's blue methods are performed on microscopic preparations of the same material, the preparations treated according to Turnbull's blue method often will look without question more blue. If the sections

are compared under the microscope it will be noticed that with the Berlin blue the blue granules are tiny, dense, homogeneous and sharply outlined, similar to the granules of eosinophilic leukocytes. No other morphological element but round granules can be seen. On the contrary, in sections treated according to Turnbull's blue method the blue granules are of a much larger size (this has been noticed by Tirmann, who erroneously attributed this phenomenon to the greater sensitivity of his method); very often they have darker outlines and a paler center; not rarely they are surrounded by a more or less extensive pale blue halo. In addition, from larger granules very often bizarre, whorly or twig-like processes (also with darker outlines) take their origin. These peculiarities observed with Turnbull's blue method are the more marked the more acid is used. If the mixture suggested by the originators (equal parts of 1 per cent hydrochloric acid and a 20 per cent potassium ferricyanide solution) is used, they are quite conspicuous, but are much less so if only one drop of hydrochloric or, even better, acetic acid is added to each 50 cc. of the ferricyanide solution. The section, however, does not become blue as rapidly. If both sections are compared with a third one, made according to the method of Quincke (in which a 1 hour exposure to ammonium sulphide has proved amply sufficient), it will be noticed that the morphology of the iron, according to the Berlin blue method and the iron sulphide reaction, is absolutely identical as regards the number as well as size and shape of the granules; whereas the formations observed with Turnbull's blue are not duplicated by any other method. The cause of this interesting fact is the following: with the Berlin blue method ferric chloride is but slowly split from hemosiderin and bound at once, locally, by potassium ferrocyanide. On the contrary, in the case of Turnbull's blue method, hydrochloric acid dissolves iron sulphide almost with the vehemence of an explosion, but anyhow at a rate greatly surpassing the reaction speed of the formation of Turnbull's blue, the latter being retarded also by a semipermeable membrane of its own substance formed on the surface of the granules. In addition, particles are torn and carried off by gas bubbles. The curious artifacts described are formed in this way, as can easily be observed if the conversion in sections of ferrous sulphide into Turnbull's blue is examined under the microscope. Of course, if, following the suggestion of Mallory, no acid is used these faults will not occur, but the transformation of

ferrous sulphide into Turnbull's blue will be an incomplete one, and in addition many granules will acquire a dingy greenish color, rapidly fading out under the coverslip. Another method devised by Mallory, using 5 per cent acetic acid, is in no respect superior to the original one. Should someone, convinced of the superiority of the iron sulphide reaction, prefer the same, I would advise him to render his preparations durable, not by the Turnbull blue method, but rather by converting ferrous sulphide into either copper or lead sulphide. This can easily be accomplished by washing the section (treated previously with ammonium sulphide) first with pure diluted ammonia, then with distilled water and placing it for 1 hour in either a 5 per cent copper sulphate or lead nitrate solution. The deep black granules of copper or lead sulphide will give in sections counterstained with methylene blue or any red nuclear stain a beautiful picture. Hematoxylin is not recommended for counterstaining. The granules will be but slightly larger than those of the original ferrous sulphide, the difference being insignificant.

While working on this subject I was much annoyed by the fact that both the Berlin blue and Turnbull's blue preparations soon began to lose their color and within a few months, or even weeks, became entirely unsuitable for comparison. This phenomenon is well known and was described by Gans in 1923. Although the preparations can be restored by removing the coverslip, I tried to find a method by which the sections could be made permanent. My experiments were based on the following idea: fading out of Berlin blue or Turnbull's blue sections is quite different in nature from that of hematoxylin or methylene blue. In a section stained with hematoxylin, fading starts from the margins and is progressive toward the center; the process is irreversible. It is probably caused by oxidation. Decolorization of the methylene blue stain can be almost completely prevented by the use of acid-free balsam; therefore it is to be attributed to an acid reaction. Fading out of an iron reaction invariably starts at the center and if the section is not much smaller than the coverslip, its peripheral parts will not lose their color. The decolorization cannot be retarded by using neutral balsam. These facts, together with the quick restoration of the preparations if exposed to air or, even better, to a diluted hydrogen peroxide solution, point to the decolorization being due to reduction. Canada balsam, too, like resins in general, probably takes up oxygen while

drying, depriving the section of its oxygen in this way and reducing both Berlin blue and Turnbull's blue to colorless ferro-ferrocyanide. On this basis it seemed that a mounting medium rich in oxygen would prevent or at least retard the fading out of sections. This idea proved to be sound. I diluted inspissated, almost dry Canada balsam with old, resinified oil of turpentine (known to contain peroxides) until it became of syrup-like consistence and tried to mount sections with it. It turned out that all sections mounted with this medium were much more durable than those mounted with simple Canada balsam. On account of the shortness of time that had elapsed since my experiments (5 months) I am unable to state the exact duration of sections preserved in this way. At any rate, all my preparations mounted with Canada balsam almost completely faded out in this time, whereas those mounted with the new medium remain unchanged. Canada balsam preparations all faded out within less than 2 days if kept at 56° C., whereas those preserved with oil of turpentine completely withstood this temperature for more than 14 days. Oil of turpentine does not destroy either carmine or Kernechtrot stain. I tried also to dilute Canada balsam with a solution of benzoyl peroxide in xylene; this method, however, proved to be much inferior to the first one. Therefore, I recommend the mounting of iron reaction preparations with Canada balsam diluted with old, resinified oil of turpentine.

Summary and Conclusions

1. Hemosiderin does not contain ferrous compounds demonstrable with the direct Turnbull's blue reaction.

2. The alleged superiority of the Tirmann-Schmelzer modification of Turnbull's blue method is based partly on erroneous theoretical conceptions and partly on the misinterpretation of artifacts.

3. The best microtechnical reagent for iron is a mixture of equal parts of 20 per cent hydrochloric acid and a 10 per cent solution of potassium ferrocyanide. The exposure should be 30 minutes. Results of equal quality are produced by converting ferrous sulphide obtained in Quincke's reaction into copper or lead sulphide.

4. Both Berlin blue and Turnbull's blue preparations can be made durable by diluting the Canada balsam to be used in mounting sections with old, oxidized oil of turpentine.

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DESCRIPTION OF PLATE

PLATE 120

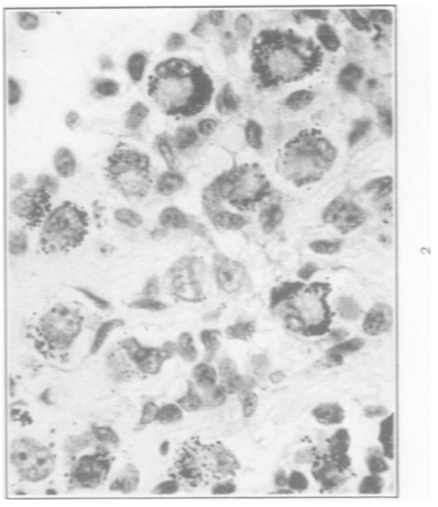
All photomicrographs were made of the same material (pigmented giant celled xanthoma of a tendon sheath) under identical optical conditions. Hematoxylin nuclear stain.

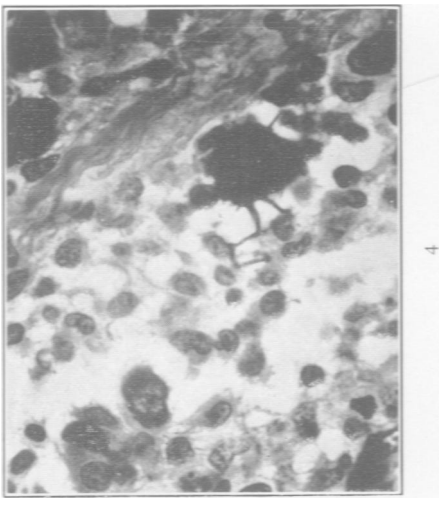
FIG. 1. Author's modification of the Berlin blue method. × 300.

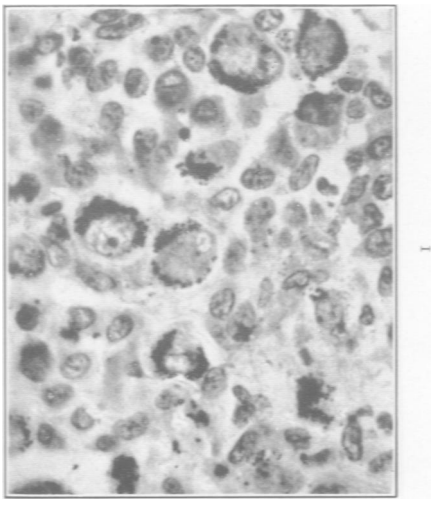
FIG. 2. Author's modification of the Tirmann-Schmelzer method (conversion of ferrous sulphide into lead sulphide). × 300.

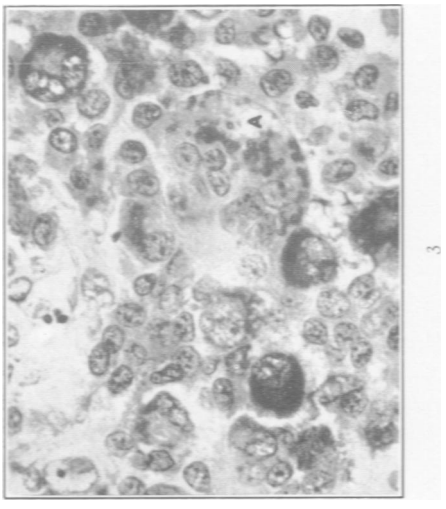
FIG. 3. Turnbull's blue method. At "A" granules appear as tiny ringlets (diffusion artifact). × 300.

FIG. 4. Typical artifact (twig-like processes) as produced with the Turnbull's blue method. × 300.









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