THE VALUE OF HYDROGEN PEROXID IN THE MICROCHEMICAL DETERMINATION OF IRON.*

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PLATE LXII.

The use of hydrogen peroxid in the microchemical determination of certain forms of iron has proved of such great value to the author that he feels that the method deserves a wider application and should fill a definite need in our present technique.

Without considering in detail all the methods and their minor modifications which have been recommended for the microchemical determination of organic iron in tissues, it may be said that two general forms of technique are now in use.[1] One method is applicable to iron-containing substances, such as hemosiderin, which react directly when treated with ammonium sulphid or with potassium ferrocyanid, or ferricyanid, and hydrochloric acid. The other method is employed to convert the masked iron of such elements as cell nuclei into a form capable of manifesting a reaction with the above reagents. By the first method, all so-called albuminate iron will react, while by the second, the albuminate iron is usually removed from the section coincident with the liberation of the masked iron. By neither of these methods is a perfect composite picture obtainable. Although the ammonium sulphid glycerin method of Macallum[2] may possibly be employed to obtain an iron reaction from hemoglobin, certainly no method has been devised, that is at once reliable and easy of application, for the demonstration of iron in hemoglobin and its various modifications and such iron-containing derivatives as hemin and hematin.

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[1] For a detailed consideration of methods and literature, see Tracy, *Jour. Med. Research*, 1905–6, xiv, 1.

[2] *Proc. Roy. Soc. of London*, 1891, l, 277.

There is thus a large amount of tissue iron which by the present technique is with difficulty, or not at all, demonstrable, and there are possibly other forms than those that have been indicated. It was especially for the purpose of rendering demonstrable, in tissues, the iron of hemoglobin and its various modifications and iron-containing derivatives that the investigation of the action of oxidizing agents was undertaken.

It is a well known fact that when a pure solution of hemoglobin is treated with hydrogen peroxid, it is rapidly decolorized and decomposed with the production of a flocculent precipitate and the liberation of the iron in such a form that it can be detected by any of the usual tests for iron. It is equally well established that hematin and hemin are amenable to destructive oxidation by hydrogen peroxid, as well as by other methods of oxidation, with the liberation of the iron in a demonstrable form. On the other hand, nothing definite is reported of the changes produced in fixed red blood corpuscles by treatment with hydrogen peroxid, nor of intermediate stages in the oxidation of hemoglobin, hematin, or hemin. Neither has it been shown that these substances will yield an iron reaction short of their complete disintegration. These facts formed the basis upon which this investigation began.

The hydrogen peroxid employed was the perhydrol of Merck and Company (30 per cent. strength), and the dioxygen of the Oakland Chemical Company (3 per cent. strength), most of the work being done with the latter preparation. The stronger preparation seems to possess only two advantages, which are, rapidity of action and ability to oxidize some tissues, occasionally encountered, which show unusual resistance to oxidation. These advantages may be offset by the difficulty in controlling the action so as to obtain the degree of oxidation desired. Various dilutions of the strong peroxid can be used to good advantage.

Although hydrogen peroxid is unable to effect any appreciable degree of oxidation in unfixed red blood corpuscles, even after prolonged action, the change produced in fixed cells is quite rapid and pronounced. If a blood clot, fixed with alcohol, with formalin, or by boiling, be treated with 30 per cent. hydrogen peroxid, it rapidly changes color, becoming lighter brown and ultimately bleaching

to a perfectly colorless condition. The entire change requires but a few minutes, the actual time depending upon the size of the clot thus treated. The same changes are produced by weaker solutions of hydrogen peroxid, but require a much longer time. When such bleached clots are thoroughly washed with distilled water to remove the excess of the hydrogen peroxid and teased upon a slide, the fragments will give an instantaneous and intense Prussian blue reaction for iron, or the greenish black color with ammonium sulphid. If sections are prepared from oxidized clots—preferably fixed with formalin, which produces the best preservation of the red blood corpuscles—and treated with potassium ferrocyanid and dilute aqueous hydrochloric acid, the red cells will be found to give a definite, though light, blue reaction. With clots fixed with alcohol, the corpuscles show various degrees of laking and the reaction color is proportional to the amount of hemoglobin in the cells or concentrated at any part of the section. The fibrin and leucocytes also manifest the iron reaction.

Some interesting features in the development of such iron reactions in red cells can be traced by interrupting the oxidation at various stages and applying the tests for iron. Such experiments show that, with the Prussian blue method, the early stages of oxidation yield only a pale green color deepening to an olive green, greenish blue, and ultimately a clear blue, as the oxidation proceeds. These atypical color reactions are familiar to all who have had extensive experience with microchemical tests for iron, particularly in old specimens of hemosiderin, and we here find a possible explanation for such reactions. With the optimum degree of oxidation, the intensity of the reaction color is never so great in hemoglobin as is that exhibited by hemosiderin granules. This is undoubtedly due to the relatively greater amount of iron present in the latter substance.

Turning next to such iron-containing derivatives of hemoglobin as hemin and hematin, chemically pure products were prepared from rabbit's blood, according to the method of Schalfijew.[3] This hematin, when placed on a slide with a small amount of hydrogen peroxid (3 per cent.) and observed with a microscope, was found

[3] See Schäfer's Text Book of Physiology, Edinburgh and London, 1898, i, 252.

to undergo the same changes of color noted in blood clots. The dark brown or black granular masses change to lighter shades of brown and bleach from the periphery towards the center. Many of the smaller masses are completely decolorized within an hour. The freshly precipitated and still moist hematin is much more readily oxidized than a preparation which has been thoroughly dried. The decolorized hematin masses, though appearing to swell slightly, preserve their sharpness of outline and are highly refractile glass-like bodies. As oxidation proceeds, however, these masses swell decidedly, lose their sharpness of outline and gradually disintegrate. If the progress of the oxidation is interrupted below the complete destruction of the hematin, by draining off the excess of hydrogen peroxid and carefully drying over a flame or in the air, such preparations can be tested for iron, preferably by the Prussian blue method. All completely decolorized hematin and decolorized portions of hematin masses will exhibit an intense Prussian blue reaction, which appears immediately upon the addition of the reagents. The partially decolorized hematin shows a less pronounced reaction, depending upon the degree of decolorization (oxidation). Hematin granules that show beginning disintegration will give a diffuse precipitate of Prussian blue or a spreading halo about such granules.

With hemin and hydrogen peroxid, identically the same changes are produced, though hemin is oxidized with much greater difficulty, requiring approximately three to four hours to show many crystals which are decolorized, while some of the larger crystals will show patches of color even after twenty-four hours. These statements with reference to hemin do not agree with the findings of Gardner and Buckmaster,[4] who found hemin much more resistant even to the strongest hydrogen peroxid (perhydrol). They make no reference to the phenomena of decolorization, but describe the action as a corrosion of the hemin crystals. Some crystals do undoubtedly show this type of change which is probably more pronounced with the stronger solutions of hydrogen peroxid, which I have not employed in this instance. With carefully handled preparations and dilute peroxid, the process of decolorization can be readily

[4] *Jour. Physiol.*, 1906–7, xxxv, p. xxxii.

demonstrated. This decolorization takes place in the most peculiar forms, some of which are shown in the accompanying plate (figure 1). It is easy to see that what is really a partial decolorization of the hemin crystal might readily be mistaken for a corrosion, though with reduced illumination one would have no difficulty in distinguishing the decolorized portion of the crystal. Such decolorized crystals preserve their sharpness of outline, for a time at least, as is the case with hematin, though the tendency to disintegration seems stronger. Here again, the decolorized crystals and decolorized portions of crystals give a beautiful Prussian blue reaction for iron, as shown in figure 2.

If either paraffin or celloidin sections of tissues are treated with hydrogen peroxid, prior to the application of the iron reagents, the red blood corpuscles, laked hemoglobin, and iron-containing derivatives of hemoglobin behave essentially as above described. Certain differences, however, are to be noted. For reasons which thus far I have been unable to explain, tissue sections require a longer oxidation than is necessary with the isolated elements which I have studied. Tissues fixed with formalin are more slowly oxidized than those fixed with alcohol, and old tissues are more resistant to oxidation and react more feebly than freshly fixed tissues. Occasionally the reaction is entirely negative in tissues which have been kept for several years.

The behavior of hemosiderin, when treated with hydrogen peroxid, is so peculiar as to deserve especial mention. Although hemoglobin, hematin, and hemin are all relatively easily decolorized and, in the latter instances at least, disintegrated by 3 per cent. hydrogen peroxid, hemosiderin seems very slightly, if at all, affected after three to four days' action of the reagent, even when kept at a temperature of 37° C. Some change, however, has been produced, as is shown by its behavior towards iron reagents. First, alcoholic solutions of hydrochloric acid give only the faintest reaction, while aqueous solutions will produce a most intense reaction by the Prussian blue method. Again, those specimens of hemosiderin which previously have reacted imperfectly, or with difficulty, will give an instantaneous and typical iron reaction after treatment with hydrogen peroxid.

It is with some hesitancy that I speak of the reaction obtainable in other tissue elements, such as the various tissue cells, their nuclei, and cell granules, as no exhaustive study of such elements has been made. The tissues which were studied by the peroxid method included liver, kidney, spleen, lymph nodes, and a few specimens each of chronic inflammatory tissues, striated muscle, and carcinomata. As a rule, the cell body in these tissues reacted very faintly for iron, if at all. The reaction was variable in all cases. Some cells, such as liver parenchyma, however, seemed more constant than others and frequently exhibited a well marked iron reaction. The same statements apply with equal force to the nuclei of these cells. While in some instances the nuclear reaction was intense, in others, it was very faint. In a few instances I have failed entirely to obtain anything which I could recognize as a positive iron reaction, particularly with some very old specimens. As to cell granules, I have studied only the eosinophil granules of cells found in chronic inflammatory and malignant conditions about the genito-urinary tract of the human subject, and the eosinophil granules in chronic inflammatory tissues from the horse. The granules from the latter source are extremely large and furnish excellent material for study. While with the human tissues I was not able, in all instances, to detect a positive iron reaction in the eosinophil granules, I found that the eosinophil granules of the horse react uniformly and sharply.

When it is stated that all the above tissue elements react for iron, as has been indicated, perhaps all has been said that can be said with certainty. However, there must still remain an element of doubt as to the significance of such reactions. The results reported by those workers who have employed acid alcohols, or ammonium sulphid and glycerin for prolonged intervals, or at high temperatures, to obtain microchemical reactions for iron in the chromatin of cell nuclei, are certainly not absolutely conclusive. The possibility of an ante- or post-mortem absorption of hemoglobin by these structures can not be entirely excluded. Further than this, the acid alcohols tend to dissolve any inorganic or albuminate iron that may be present, as well as to extract hematin from the red blood corpuscles. In view of the absorptive power of nuclear material for

iron compounds, the use of acid alcohols is certainly open to question.

While the trace of acid present in most preparations of hydrogen peroxid still leaves some legitimate ground for questioning the results obtained in some cases, still I believe the method is, on the whole, more reliable than other methods. Yet, at present, I am not at all prepared to claim more than the production of a positive iron reaction in the elements mentioned. In fact, the most pronounced nuclear reactions have been obtained in just those instances where the possibility of the absorption of hemoglobin by such nuclei seemed highly probable. As to the iron reaction in eosinophil granules, this was described by Barker[5] some years ago, the results being obtained by the prolonged use of warm ammonium sulphid and glycerin. Although I have seen no confirmation of this work, there can be no doubt as to the ability to obtain an iron reaction from the eosinophil granules of the horse, at least. The source or significance of this iron is another question and, in the tissues I have studied, might possibly be referable to hemoglobin absorbed from the erythrocytes of the inflammatory tissues. In this case, however, it must needs be modified by or united to the cell granules in some way. The best means I have found for determining the presence of dissolved hemoglobin in all these tissue elements is that used by Browicz.[6] According to Browicz, hemoglobin in solution, when fixed with formalin, is precipitated as brown or black granular or crystalline material and all structures containing dissolved hemoglobin show the presence of this precipitate. If this test is wholly reliable, and thus far I have found no reason to question it, tissue cells, cell nuclei, and eosinophil granules contain varying amounts of iron, not behaving as dissolved hemoglobin, which are demonstrable after an appropriate oxidation with hydrogen peroxid.

The technique for demonstrating iron in the various structures, as described above, must be varied to suit the demands of the particular case. Most workers prefer alcohol as a fixative in the study of iron in tissues, as it is claimed that the largest amount of demon-

[5] *Bull. Johns Hopkins Hosp.*, 1894, v, 93.
[6] *Virchows Arch. f. path. Anat.*, 1900, clxii, 373.

strable iron is obtained after this method of fixation. The method of fixation which shows the largest amount of iron, however, does not necessarily represent the true picture. This I am sure is, at times, the case with alcohol, which produces laking of corpuscles and diffusion of hemoglobin and may permit of post-mortem changes in such diffused hemoglobin. For these reasons, formalin is to be preferred for purposes of fixation in some cases. Except where very thin sections are to be used, celloidin sections are usually more satisfactory than paraffin sections. As to the proper length of time that sections should be oxidized with hydrogen peroxid no absolute statement can be made. The degree of oxidation necessary for the demonstration of iron in any particular element will vary slightly with different tissues, and there is a marked difference in the length of time necessary to render demonstrable the iron of such bodies as red blood corpuscles and that of cell nuclei.

The optimum time with 3 per cent. hydrogen peroxid will usually be found to be about as follows: hemoglobin and its modifications (not including hemosiderin) require twelve to twenty-four hours eosinophil granules require twenty-four to thirty-six hours; cell nuclei and protoplasm require thirty-six to forty-eight hours, though frequently seventy-two hours will be found to give still better results.

Wherever possible, the dilute solutions of hydrogen peroxid should be employed in preference to the strong, for the reasons previously noted. The treatment with hydrogen peroxid should be followed by thorough washing with distilled water. The application of the actual tests for iron may be carried out according to individual preference, except that alcoholic solutions of hydrochloric acid seem not to yield as good results as the aqueous solutions.

In addition to the experiments with hydrogen peroxid, which have been described in some detail, potassium bichromate, chromic acid, potassium permanganate, and potassium chlorate, in acid solutions, have been used as oxidizing reagents in the same manner as the peroxid. There are obvious objections to the use of such compounds. The chromium compounds might possibly give rise to confusing results unless completely removed from the section; and this is not readily accomplished. Likewise the necessity of using

FIG. 1.

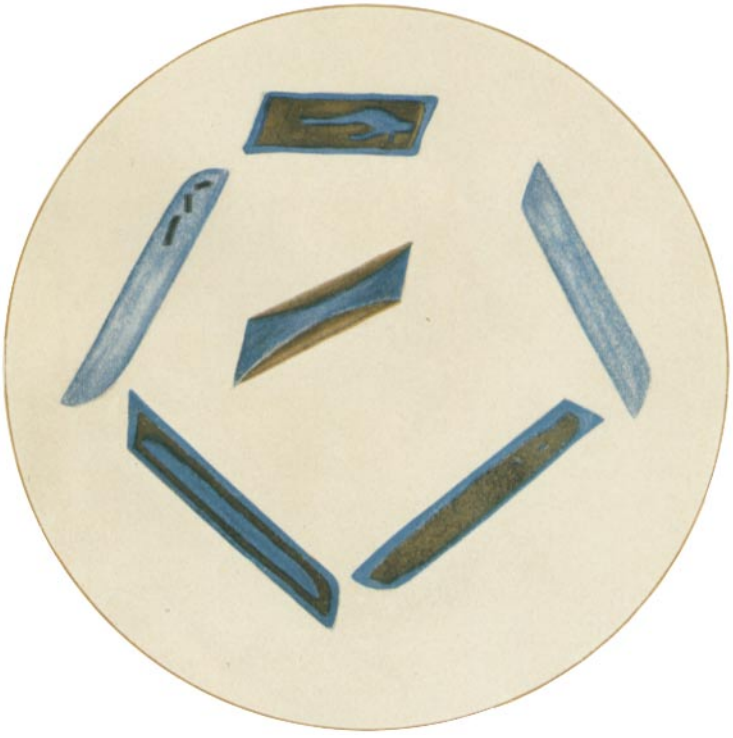

FIG. 2.

such substances as oxalic acid to decolorize the section after the use of the permanganate may result in the removal of much of the iron that has been liberated. Finally, the acid in the solution constantly tends to extract the iron as it is oxidized. Notwithstanding these objections, very good results have followed their use in some instances, although I see nothing to commend their use in preference to the peroxid of hydrogen. They are mentioned in this connection as means of confirming the results previously described and as furnishing additional evidence to the value of the principle of oxidation in the microchemical detection of iron.

SUMMARY.

1. It has been shown that hemoglobin in solution or within the red blood corpuscles can be oxidized by hydrogen peroxid to form a colorless substance capable of manifesting a microchemical reaction for iron, the type of the reaction depending upon the degree of oxidation.

2. It has been further shown that hematin and hemin, when treated with hydrogen peroxid, are decolorized. The colorless or nearly colorless body, preserving the form of the original body and being an intermediate product of its oxidation, will also exhibit a typical reaction for iron.

3. A method has been described for obtaining microchemical reaction for iron from hemoglobin and its various modifications and iron-containing derivatives, from tissue cells, from cell nuclei, and from eosinophil granules, by an appropriate degree of oxidation with hydrogen peroxid.

EXPLANATION OF PLATE LXII.

FIG. 1. Hemin crystals showing various types and stages of their oxidation by hydrogen peroxid.

FIG. 2. Similar crystals showing the Prussian blue reaction for iron.