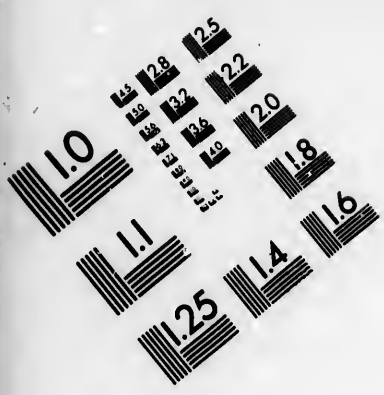

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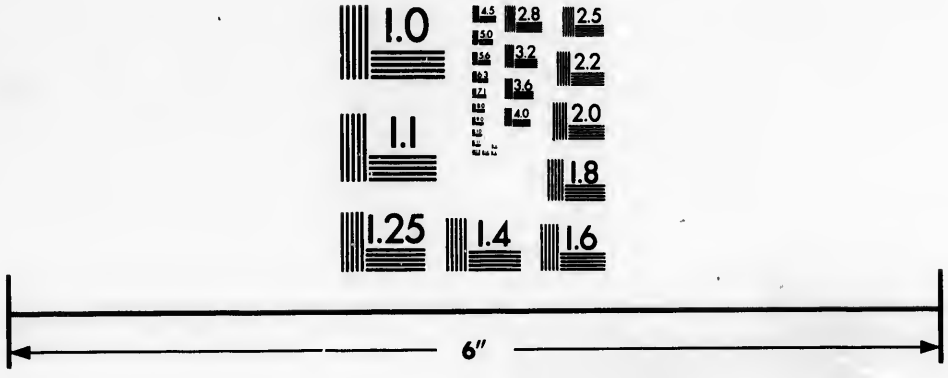

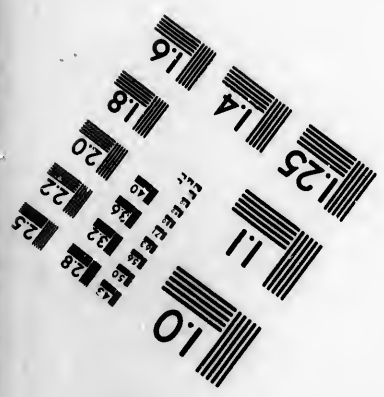

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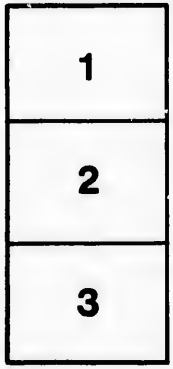

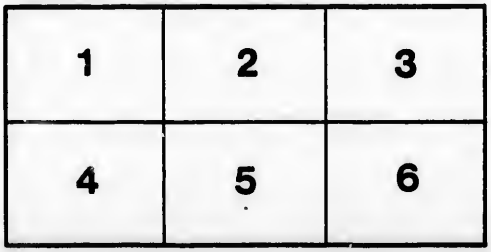

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A NEW METHOD OF DISTINGUISHING BETWEEN ORGANIC AND INORGANIC COMPOUNDS OF IRON.

By A. B. MACALLUM, M.B., Ph.D.

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A NEW METHOD OF DISTINGUISHING BETWEEN ORGANIC AND INORGANIC COMPOUNDS OF IRON.

By A. B. MACALLUM, M.B., PH.D. *Associate Professor of Physiology, University of Toronto.*

FIRM organic compounds of iron are, as shown by recent investigations, much more abundantly present in animal and vegetable organisms than was formerly supposed to be the case, and some of these, the chromatins, are of the highest importance in the life of the cells[1]. Bunge[2], whose investigations first stimulated interest in them, called them organic, but, beyond describing the reactions they give with ammonium sulphide and with acid ferrocyanide solutions, he furnished no method whereby these can, in the last resort, be definitely distinguished from inorganic and albuminate compounds. I have indeed found that in the great majority of these compounds the warm sulphide reagent only after a long application sets free their iron as ferrous sulphide, but there are organic compounds in which this result is obtained more readily. Bunge, however, used the diluted reagent on one of the latter class, namely, the hæmatogen of egg-yolk, and determined that it gave no reaction for iron until after half-an-hour, while a day was required for its full development, but when the quantity of the reagent used was great, the reaction appeared more quickly. If the acid ferrocyanide solution was allowed to act on the compound, the readiness with which the Prussian blue reaction appeared depended on the quantity of hydrochloric acid added. In this manner Bunge would distinguish between such organic compounds on the one hand and those of the inorganic and albuminate class on the other. He further claims that hydrochloric acid alcohol[3] extracts the iron of inorganic and albuminate compounds, while it leaves unaffected the iron of organic preparations.

[1] Macallum. *Quart. Jour. Mic. Sci.* XXXVIII. p. 175. 1895.

[2] *Zeit. f. physiol. Chemie*, IX. p. 49. 1895.

[3] This contains 10 volumes of hydrochloric acid of 25 per cent. strength and 90 volumes of 96 per cent. alcohol.

I have already shown (*op. cit.*) that hydrochloric acid alcohol affords no means of distinguishing between the two classes of iron compounds, and have pointed out that when a dilute solution of ammonium hydrogen sulphide is added to a quantity of fresh egg-yolk the greenish reaction of ferrous sulphide is at once developed. Further, if one takes a dilute solution of the organic compound of egg-yolk dissolved in ammonia and adds to it a quantity of ammonium sulphide, the green reaction obtains almost immediately. The acid ferrocyanide reagent does not bring out the Prussian blue reaction in ammoniacal solutions of this compound because the reagent precipitates the latter, and as, therefore, the iron in it is rendered much less liable to be attacked, it is obvious that the tardiness with which the reagent acts in this case cannot be considered as an indication that the compound is an organic one.

The method of distinguishing between the two classes of compounds offered by Bunge is therefore not as decisive by any means as one would wish it to be. His view, that only organic compounds of iron when absorbed go to form hæmoglobin, has directed the attention of physiological chemists to the possibility of preparing artificially organic compounds of iron in the laboratory, and it is of great importance consequently to have a decisive test for them. Already a number of iron compounds, for which an organic character is claimed, have been put on the market. If one relies on the method referred to, it would in some cases at least be difficult to disprove their organic nature, while it is equally difficult to believe that they are other than inorganic.

One of the best known of these compounds is ferratin, first prepared by Marfori[1] from egg-albumin and tartrate of iron, and which he and Schmiedeberg[2] believed to be similar to the iron compound, also called ferratin, which they have succeeded in extracting from the liver. The artificially prepared compound does not, it is claimed, readily react with ammonium sulphide, and it also gives the Prussian blue reaction only after a time when treated with acid ferrocyanide solutions which precipitate it. I have repeatedly examined quantities of this compound prepared by myself according to Marfori's method, or obtained from different manufacturers through the trade, and the result was always the same. In all cases an alkaline solution of the compound

[1] *Arch. f. exp. Path. u. Pharm.* XXIX. p. 212. 1891; *Arch. ital. de Biologie*, XXI. p. 62. 1894.

[2] *Arch. f. exp. Path. u. Pharm.* XXXIII. p. 102. 1894.

gave at once on the addition of strong ammonium sulphide the characteristic dark green reaction of ferrous sulphide, and when the reagent was added to the dry powder, the reaction developed in a few seconds.

It is indeed a fact that if the reagent is very dilute the reaction which may be obtained on adding it to the ferratin powder, or to an ammoniacal solution of it, does not develop as readily as when the reagent is added in a concentrated form, but neither in this, nor in the fact also that acid solutions of potassic ferrocyanide first precipitate the dissolved ferratin and afterwards give the precipitate the Prussian blue colour, can one find decisive indications that the iron compound is an organic one. It is true that in respect to these reactions there is between ferratin and hæmatogen of egg-yolk some resemblance, but it is one which is partly caused by physical conditions. Just as in the case of hæmatogen, the precipitate caused by the acid ferrocyanide solution in the solution of ferratin renders the iron in it less liable to be attacked by the reagent; on the other hand, the interval between the moment when dilute ammonium sulphide is added to a solution of ferratin and that when the reaction appears is so short that I cannot attribute any importance to it. It may be that ferratin is an organic iron compound, but the facts mentioned do not in any case decide this.

It is not alone of ferratin that one may make this statement. Carniferrin is a manufactured preparation which is claimed to be an organic iron compound, on evidence which is but slightly, if at all, stronger than illustrated in the case of ferratin.

There are also iron compounds in animal cells whose organic nature is uncertain. These, which may be found in the fœtal liver and spleen, in the placental villi of the rabbit, cat and man, as well as in the spleen, liver and kidney of the adult vertebrate, give not immediately a dark green reaction with ammonium sulphide, but if the latter is dilute, the appearance of the reaction is longer delayed. In the case of the placental villi of the human subject at the sixth week the reaction is slowly developed, but if the preparation containing the reagent and the villi is slightly heated, the reaction develops at once. Sections from the placenta of the cat treated in the same way require time for the development of the reaction, but this is shortened to several seconds on the application of heat to the preparations. Similarly with sections of the liver, spleen and kidney of *Necturus lateralis* and the frog. In the sections of the spleen of *Necturus*, especially of those

kept without food for a long time, the sulphide reagent will bring out a feeble reaction limited to a few of the cellular elements, but when the section is heated in a drop of the reagent, it acquires as a whole in a few seconds a dark green reaction more or less diffused in the walls of the blood vessels and fibrillar elements, but pronounced and localized in the lymphoid cells.

In the case of the placental iron compound, it might be supposed that as the organic compound of iron analogous to that of egg-yolk must be supplied to the mammalian embryo through the placenta, the compound shown to be present must belong to this class. On the other hand, it may quite as readily be held to be an inorganic iron compound in the process of excretion, although its occurrence in the chorionic villi on the seventeenth day would seem to oppose such an explanation. It may also be held, and not unreasonably, that the substance of the tissues holding the iron prevents a ready access of the sulphide to the iron compound, and thus delays the appearance of the reaction, although the compound may be an inorganic one.

It is thus seen that there are compounds of iron which, from their hitherto known reactions, it is difficult to classify as organic or inorganic; I have therefore thought it important to determine whether there is any other reagent which can be pressed into the service of distinguishing more markedly between the two classes of compounds.

After a great number of trials I succeeded in determining that hæmatoxylin is an extremely sensitive reagent for deciding whether a given compound of iron is organic or inorganic. For this purpose it must be absolutely pure and put up in a 0·5 per cent. solution with absolutely pure distilled water. In this case the solution as is well known is brownish yellow, but when alkalies or alkaline earths are added, the colour becomes violet or red. When mixed with solutions of a salt of iron, it becomes blue-black or bluish-black. The slightest trace of a salt of iron present will serve to convert the yellow into blue-black or bluish-black. On the other hand, if the iron compound is organic, the hæmatoxylin is unaffected. This is illustrated when solutions of hæmaglobin and hæmatin are mixed with solutions of hæmatoxylin, and also when the reagent is added to solutions of ferrocyanides and ferricyanides. In the latter instance, the hæmatoxylin may acquire a reddish tinge due to the presence of free alkali.

The method of employing the reagent must vary. When the compound of iron may be obtained in simple aqueous solution, it merely suffices to add the solution of hæmatoxylin directly. When it is to be obtained in solution only with the aid of alkalies, the hæmatoxylin solution may be added to the dry powder. This can be done in the case of ferratin and carniferrin, which are ochre-coloured, and the granules of the powders absorb the hæmatoxylin and become blue-black as may be demonstrated on examination with the microscope. Another method, which was found serviceable in the case of ferratin and carniferrin, consists in steeping iron-free filter paper in ammoniacal solutions of the substance to be tested, and, after the paper has thoroughly dried, moistening it with the hæmatoxylin solution. The production of a blue-black tinge is evidence that the iron compound examined is inorganic. In the application of the test to the iron compound of egg-yolk, which, according to Bunge, is organic, certain additional methods must be employed. If the solution of hæmatoxylin is applied directly to the yolk spherules, the iron-holding elements of these, and especially of that variety known as "white," appear faint rose red or violet[1]. This reaction is largely due to the presence of volatile compounds, for when the yolk is dried at the temperature of the room, the hæmatoxylin absorbed by the spherules is almost unaffected in colour. This is the case also when yolk spherules are coagulated with heat.

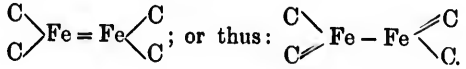
When the compounds of iron to be investigated are found in tissues, the latter are to be well hardened in alcohol, and their sections, or teased-out preparations of them, are covered for a few minutes with the hæmatoxylin solution. Wherever inorganic salts of iron occur, there the reaction is blue-black or bluish-violet, while in the remainder of the preparation the colour is yellowish brown, which may sometimes be so intense as to obscure in some parts, for example, nuclei, the occurrence of traces of inorganic iron. This difficulty is removed by steeping the preparation in a mixture of equal volumes of ether and absolute alcohol for at most an hour, the time varying with the size of the preparation. The mixture extracts the unaffected hæmatoxylin, but the blue-black compound strongly resists extraction, so much so that in some cases it may be found in the preparation after lying twenty-four hours in the mixture.

The section or teased-out tissue after treatment may be cleared

[1] A lime salt is present in the iron-holding constituent of the spherules, and this will partly account for the colour reaction given with hæmatoxylin solutions.

in oil of cloves and mounted in balsam. The preparations so made are permanent, and as infinitesimal traces of inorganic iron compounds are thus revealed, the method is much more advantageous than that of Prussian blue, which is apt to fade out when exposed to light, and demonstrates much less readily very minute traces of inorganic iron compounds.

The explanation for the differences in the action of inorganic and organic iron compounds on hæmatoxylin may possibly be found in the different relations of the units of affinity of the iron atoms in the two classes of compounds. A ferric salt converted into ferric ferrocyanide or a ferrous salt converted into ferrous ferricyanide becomes incapable of affecting the hæmatoxylin. The ferrous or the ferric elements are probably attached to the carbon atoms thus[1]:



If a direct attachment to the carbon atoms of all its bonds of union robs the iron atom of its power to affect hæmatoxylin, it is not unreasonable to suppose that in such compounds as hæmoglobin, hæmatin and chromatin, the iron atoms are similarly united.

The action of inorganic compounds of iron on hæmatoxylin would appear to be that of oxidation[2].

Results of the Application of the Method.

The application of this test has shown that there are inorganic iron compounds in the chorionic and placental villi of the human subject, in the fœtal liver and spleen and in the placenta of the cat, rabbit and guinea-pig. These compounds do not react immediately with ammonium sulphide, probably owing to the slow penetration of the reagent.

The existence of inorganic iron compounds in the human chorionic villi of the seventeenth day would seem to indicate that there is a transference of inorganic iron from the maternal to the fœtal tissues.

Ferratin artificially prepared always affects hæmatoxylin, and it must therefore be an inorganic compound of iron. On the other hand the ferratin which is derived from the ox liver does not bring about

[1] Roscoe and Schorlemmer. *Treatise on Chemistry*, II. Part 2, p. 112.
[2] Mayer. *Mitth. aus der Zool. Stat. zu Neapel*, x. p. 170.

a change in the colour of hæmatoxylin, and it must therefore be considered as an organic compound.

Carniferrin is also, as shown by this test, an inorganic compound.

The peptonates and albuminates of iron belong to the inorganic class.

Special Application of the Test.

As already indicated, organic iron compounds leave hæmatoxylin unaffected. It is, however, often important to know whether there is much or little organic iron in tissues, and for this purpose hæmatoxylin is very useful. It is necessary to convert the organic iron into an inorganic form, which may be done, as I have pointed out (*op. cit.*), by allowing acid alcohols, and especially sulphuric acid alcohol[1] to act on a piece of tissue for from one to twenty-four hours at 35° C., the length of time varying with the size of the preparation and the amount of the acid alcohol used. If after such treatment the preparation be freed from acid by washing it in alcohol, it may be covered for a few minutes with a solution of hæmatoxylin of not less than 0·5 per cent. strength. Wherever iron exists in such a preparation the hæmatoxylin becomes blue-black or blue. Very often sections of tissue thus treated appear as if stained with Ehrlich's hæmatoxylin. As the acid alcohol leaves the iron where it is set free, as an inorganic compound, the preparation demonstrates very sharply the original distribution of the organic iron, especially when it is contrasted with a preparation which has not been treated with acid alcohol, but yet has been stained with hæmatoxylin.

Extraordinarily minute traces of organic iron, with the exception of that in hæmoglobin and hæmatin, are thus demonstrated which could not be revealed by the Prussian blue method or by ammonium sulphide. There is a further advantage in the method. Prussian blue preparations made to show the presence of iron in tissues are apt to fade out unless kept away from the light. In the case of hæmatoxylin preparations, no deterioration is to be anticipated under any condition when they are properly made.

[1] It must be noted that the iron of hæmoglobin and hæmatin is not readily converted thus into an inorganic form.