THE RELATION OF HEMATIN TO PATHOLOGICAL PIGMENT FORMATION.*

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Much of the literature dealing with the subject of the hematogenous pigments, hemosiderin and hematoidin, is peculiarly silent upon the question of the relation of these pigments to hematin. In considering the origin of hemosiderin and hematoidin, most authors have contented themselves with establishing the fact that these pigments are derived from hemoglobin, and, although the formation of hemosiderin is usually ascribed to a process of vital oxidation, there is little agreement as to the exact mode of their production, or as to their genetic relation to other pigments.[1]

Nevertheless, definite statements of the relation of hematin to these pigments are not entirely lacking, particularly in text-books. To present the matter more exactly, a recent text-book in dealing with this subject contains the following statement:[2] "As a pathological pigment, however, hematin is by no means so frequently found as its derivatives. Wherever formed, its duration is transient, for it gradually splits up into an iron-free pigment (hematoidin) and an iron-containing pigment (hemosiderin). This change may be represented by the following equation, according to Nencki and Sieber:

$$\underset{\text{(Hematin)}}{C_{32}H_{32}N_4O_4Fe} + 2H_2O = 2\underset{\text{(Hematoidin)}}{C_{16}H_{18}N_2O_3} + Fe."$$

The evidence in support of this conception might be summarized as follows: (1) that increased destruction of red blood corpuscles

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[1] For a full discussion of this question, see Neumann, *Virchows Arch. f. path. Anat.*, 1904, clxxvii, 401.

[2] Wells, Chemical Pathology, Philadelphia and London, 1907, 401.

or of hemoglobin, within the circulation, leads inevitably to an increased production of bile pigments and a deposit of hemosiderin in the spleen, bone marrow, liver, and other organs; (2) that the local metabolism of red blood corpuscles or of hemoglobin results in the formation of hemosiderin or hematoidin, or both; (3) that proteolytic enzymes split hemoglobin into proteid and hematin; and finally, (4) that the hydrolytic cleavage of hematin, by splitting off the iron, results in the formation of a pigment (hematoporphyrin) essentially the same as hematoidin.

Although there are obvious defects in such a chain of reasoning, it must be admitted that, knowing the fate of hemoglobin and that it is capable of cleavage into proteid and hematin and that the hydrolytic cleavage of hematin results in the formation of hematoidin plus iron, it is difficult to escape the conclusion that both hemosiderin and hematoidin are at least potential derivatives of hematin. The conclusive proof of the truth or fallacy of this statement would seem possible of experimental demonstration, and yet it appears that the crucial test has never been applied. In the present investigation, an attempt has been made to determine the relation of hematin to hemosiderin and hematoidin upon a more accurate experimental basis. With this object in view, experiments were carried out along three different lines: (1) the intravenous injection of alkaline hematin solutions in the rabbit, and intraperitoneal injection in the guinea pig; (2) the subcutaneous and intraperitoneal injection of powdered hematin and crystalline hemin in the guinea pig; and (3) the intraperitoneal injection of powdered parahemoglobin in the guinea pig.

The hematin and hemin used were prepared, according to the Schalfijew process,[3] from rabbit and from guinea pig blood. The parahemoglobin was prepared from rabbit's blood by thoroughly washing the red blood corpuscles with 0.85 per cent. salt solution, shaking the corpuscles in a separatory funnel with ether and distilled water, and then filtering the hemoglobin solution. This was evaporated to dryness at a temperature between 37° and 40° C. The dry residue was then ground to a coarse powder in a mortar and placed under absolute alcohol for one week, at room tempera-

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

ture. At the end of this time the alcohol was decanted, the residue washed with ether and, after drying thoroughly, ground to a fine powder. This powder was absolutely insoluble in either distilled water or 0.85 per cent. salt solution, and very sparingly soluble in dilute alkalies.

The injections of the powdered hematin, crystalline hemin, and powdered parahemoglobin were all accomplished by suspending these substances in sterile 0.85 per cent. salt solution, the injections being made under aseptic precautions.

While the first series of experiments, that of intravenous injection of dissolved hematin, gives valuable information as to the toxicity and fate of the pigment thus injected, complications are introduced by the action of the solvent and the extreme toxicity of such hematin solutions, which render the series of experiments unsuitable for the object of this inquiry. I shall, accordingly, devote this paper to recording the results obtained by the other two methods.

The above complicating circumstances are reduced to a minimum by the use of powdered hematin suspended in salt solution. Therefore, as extravasated blood and hemoglobin injected into the tissues have been shown to be subject to the same metabolic changes as red blood corpuscles destroyed within the circulation, or as hemoglobin injected into the circulation, the use of hematin suspended in salt solution would seem to be the method apt to give the most reliable results. It might be objected, however, that the use of powdered hematin does not simulate normal or pathological conditions upon which we have reliable data as to the fate of hemoglobin. To meet such objections, the powdered parahemoglobin was introduced as a control. It remains, therefore, only to determine whether this parahemoglobin can be converted into hemosiderin or hematoidin by the tissue cells, and finally, whether or not powdered hematin is subject to the same metabolic changes.

When powdered parahemoglobin suspended in salt solution is injected, under aseptic conditions, into the peritoneal cavity of the guinea pig, it collects principally in the omentum and about the point of puncture in the abdominal wall, although, to a less degree, it is distributed throughout the abdominal cavity. The ensuing reaction about the particles of pigment is essentially that which occurs

about a sterile, non-absorbable, foreign body, the giant cell being a prominent feature of the early stages of the reaction. No appreciable absorption of the pigment seems to take place, and although much of the finer pigment is picked up by phagocytic cells, these appear so sharply focalized as to render it doubtful if much removal of pigment from the peritoneal cavity is accomplished through this agency. At the end of five days, many granules of parahemoglobin have been partially or wholly converted into copper-colored pigment which exhibits a typical iron reaction, while other granules remain unchanged in character, as far as can be determined. As time progresses, more and more of the parahemoglobin is converted into definite hemosiderin granules. Under these conditions, however, in no instance has any demonstrable hematoidin been noted as a product of the action of the tissue cells upon powdered parahemoglobin.

It is certain, therefore, that the physical condition of parahemoglobin cannot prevent its transformation into hemosiderin. There is undoubtedly a retardation of the process and possibly also a less perfect conversion into hemosiderin, both of which are readily ascribable to the physical condition of parahemoglobin as compared with the natural state of this pigment.

An attempt was next made to determine whether a like change was produced in powdered hematin in the peritoneal cavity and subcutaneous tissues. In a series of experiments, the duration of which ranged from five to forty-seven days, the results were so uniform that they can be rendered sufficiently clear by extracts from a few protocols of typical cases from various stages of the experiments.

Experiment 1.—White female guinea pig, weight 500 grams. On January 7, 1911, 15 mg. of guinea pig hematin, suspended in salt solution, were injected into the peritoneal cavity. The animal remained normal, and after five days was killed by a blow on the head.

Autopsy.—The subcutaneous tissues about the site of the injection showed slight edema and ecchymosis, and a small amount of black pigment. The abdominal cavity contained a large amount of black pigment distributed irregularly over all the peritoneal surfaces, but collected especially about the site of puncture in the abdominal wall, and in the rolled up omentum. The aggregates of pigment varied from a diffuse smear to perfectly discrete nodules, and the diameters of the latter varied from that of a pin point to 0.5 cm. The larger foci, espe-

cially on the abdominal wall, showed an areola of redness about the black pigment. The pigmented nodules were adherent to the surfaces upon which they rested, though they could be rubbed off with force. With the exception of a slight enlargement of the spleen and lymph nodes, other conditions were normal.

Microscopic examination showed that the larger nodules of pigment were composed of fine and coarse, irregular masses of black pigment thickly imbedded in newly formed granulation tissue. There was pronounced hemorrhage throughout the nodules and in the tissues about their periphery. The granulation tissue contained many giant cells encircling the coarser masses of pigment, while there were numerous polymorphonuclear leucocytes at the centers of the nodules and many mononuclear phagocytes containing fine granules of brown and black pigment. Here and there through the nodules, but more especially in the adjacent tissues, were phagocytic cells containing yellowish brown pigment. Very few phagocytes containing black pigment could be detected in the surrounding tissues.

The smaller nodules differed from the larger ones only in showing little or no hemorrhage, very few leucocytes, and a very slight amount of yellowish brown pigment.

On applying the Prussian blue test for iron, a diffuse blue color was developed throughout the larger nodules of pigment, usually more pronounced at the periphery than at the center of the nodule. There were many fine granules of pigment which gave a positive iron reaction within the nodule, while most of the yellowish brown pigment in the surrounding tissues reacted positively. The smaller nodules of pigment showed only a few scattered granules which manifested an iron reaction, and the diffuse reaction was very slight indeed. The black pigment in all nodules still showed the characteristic solubility of hematin and did not react for iron by the usual technique.

Experiment 2.—Male guinea pig, weight 289 grams. On January 27, 1911, 15 mg. of rabbit hematin were injected into the peritoneal cavity. The animal remained normal and was killed after fifteen days.

Autopsy.—A large amount of black pigment was found distributed through the peritoneal cavity as in experiment 1, but there were fewer large nodules of pigment. The omentum was not rolled up, and there were no reddened zones about the nodules of black pigment.

Microscopically, the pigmented nodules were composed principally of masses of black granular pigment imbedded in granulation tissue and immediately surrounded by giant cells. There were numerous mononuclear phagocytes present which contained either fine granules of yellowish brown pigment or granules of black pigment, the latter being far more numerous than the former. Some of the larger masses of black pigment showed paler spots or borders and there were occasional granules of a greenish brown color. There were also a few almost colorless refractile bodies, the general morphology of which resembled that of the hematin masses. There was no appreciable hemorrhage and but few polymorphonuclear leucocytes.

The Prussian blue test for iron, on a slide, showed in addition to a very slight diffuse bluish coloration, two types of pigmented bodies manifesting a positive reaction for iron. Bodies of the first type consisted of yellowish brown granules and gave a deep Prussian blue color to the entire granule.

These were few in number and were found particularly about the periphery of the nodules. Granules of the second type had a similar color, but were slightly more refractile, and gave a reaction in which the blue precipitate spread away from the granule, or was precipitated about the granule, the body of the granule not becoming colored, but retaining its original yellowish brown color. The almost colorless refractile bodies referred to above, exhibited this type of iron reaction, though some of these (the least pigmented) showed the blue body of the granule. Sections, washed and mounted, showed a persistence of the Prussian blue in the pigments of the first class, though the blue was largely washed away from those of the second class. Such masses of hematin as showed evidence of bleaching, as above described, and those granules which had assumed a greenish brown color, showed varying degrees of an iron reaction of the second type, perfectly colorless areas giving an intense blue reaction.

The black pigment throughout the section still showed the characteristics of hematin.

Experiment 3.—Male guinea pig, weight 370 grams. On January 27, 1911, 20 mg. of rabbit hematin were injected into the peritoneal cavity. The animal remained normal for forty-five days and died from pneumonia on the forty-seventh day.

Autopsy.—The autopsy was done two hours after death. The peritoneal cavity presented an appearance similar to that of experiment 2, except that there appeared to be a greater amount of black pigment which was collected in larger nodules.

Microscopically, the pigmented nodules, although similar in general appearance, showed certain differences from those previously described. The granulation tissue was denser and formed a definite fibrous capsule about many clumps of pigment. The larger nodules again showed hemorrhage and a considerable number of polymorphonuclear leucocytes. For the most part, the pigment masses were broken up into smaller granules although there were still numbers of large masses surrounded by giant cells. There seemed to be no reduction in the total amount of hematin present, although the changes were more evident and there were numerous small granules which were almost completely decolorized, as well as larger pigment masses showing partially or completely decolorized margins. In addition to the hematin, the hemorrhagic nodules showed a small amount of yellowish brown pigment which was very scarce in the nodules without hemorrhage.

The Prussian blue reaction again showed a diffuse blue in the hemorrhagic nodules which was much less pronounced where there was no hemorrhage. The yellowish brown granules of hemosiderin were most numerous where there was hemorrhage, and almost completely absent where there was no hemorrhage. The partially and completely decolorized granules and portions of hematin masses gave an iron reaction, the nature and intensity of which was proportional to the degree of decolorization, as explained in experiment 2.

The changes produced in hematin in the subcutaneous tissues are absolutely identical with those produced in the peritoneal cavity, and, therefore, require no detailed presentation.

To reduce these experimental results to more general statements,

it can be said that, post mortem, the hematin introduced into the peritoneal cavity appears as black pigment distributed over all the peritoneal surfaces, being most abundant in the omentum and about the point of injection, frequently forming nodules as large as 0.5 of a centimeter in diameter. Wherever the hematin lodges, it is rapidly walled in by new growth of connective tissue and foreign body giant cells. The reaction on the part of the tissues is further characterized by a tendency to hemorrhage, both around and within the connective tissue nodules formed. This hemorrhage may persist in the larger nodules or recur at intervals for weeks, but it does not involve all nodules, especially the smaller ones where, very early, the hemorrhage usually disappears completely. The leucocytes present in the early stages of the experiments gradually give place to mononuclear cells of the polyblastic series. At the same time, there is an increase in the density of the connective tissue and the formation of a definite fibrous capsule about some nodules. Grossly, there is no apparent difference in the amount of hematin found at different stages of the experiments.

Microscopically, the hematin stands out as the most prominent feature of the section in all stages of the experiments. This pigment occurs as large irregular masses, mostly surrounded by giant cells, and as a finely granular black pigment, much of which is being picked up by phagocytes. There is but slight evidence, however, of removal of pigment from the peritoneal cavity, as only a very small amount can be found in the mesenteric lymph nodes, spleen, liver, or other organs.

In the early stages, no change is demonstrable in the hematin. Later, the larger masses are slowly broken up and there is increased phagocytosis. At the same time, the small granules of hematin and the margins of the larger masses begin to show a diminution of color intensity, giving rise to a series of pigmented bodies varying from dark brown or greenish brown through lighter shades of brown—yellowish brown and straw yellow—ultimately becoming perfectly colorless and highly refractile. At the end of forty seven days, the total change produced is but slight, by far the greater part of the hematin showing no demonstrable alteration.

Morphologically, these pigmented derivatives of hematin are

practically indistinguishable from certain varieties of hemosiderin, which, according to Schmidt[4] and to Neumann,[5] may show an even greater variety of color changes in its development and subsequent decomposition. The analogy becomes closer when to these morphological characters is added the fact that these modified products of hematin exhibit a microchemical reaction for iron by the Prussian blue method. This reaction, however, shows certain differences from that of hemosiderin. It will be recalled that the typical hemosiderin reaction results in the formation of a solid blue granule in which none of the original color of the pigment is perceptible. In addition to this type of reaction, Schmidt describes a second type, exhibited by what might be termed incompletely developed hemosiderin. This reaction is characterized by a precipitation of Prussian blue about the pigment, the original color of which is still distinctly visible. It is this second type of reaction that is shown by the colored products derived from hematin, the reaction approximating the typical hemosiderin reaction as the color of the granule decreases, and becoming typical only in the completely decolorized products of hematin, in which respect the reaction is decidedly different from that of hemosiderin.

This peculiarity of the iron reaction led to further comparison of the properties of hemosiderin and the pigmented hematin derivatives. On testing the solubility of these pigments in fixed tissue, it was found that whereas hemosiderin is insoluble in dilute alkalies (0.2 per cent. potassium hydrate), and, except for a slight brightening in color, shows no demonstrable change, the darker hematin derivatives are practically completely dissolved and the color is extracted from the lighter bodies, leaving a colorless residue which reacts for iron exactly as the originally colorless derivatives. A further difference between the two classes of pigment is shown by their behavior in the presence of hydrogen peroxid.[6] While the hematin derivatives suffer complete destruction after a few hours' action of this reagent, hemosiderin remains unaffected for several days. These tests, then, furnish further means for the

[4] Schmidt, *Virchows Arch. f. path. Anat.*, 1889, cxv, 397.
[5] Neumann, *loc. cit.*
[6] Brown, *Jour. Exper. Med.*, 1911, xiii, 477.

positive identification of hemosiderin, and, in the present instance, serve to show conclusively that these pigmented derivatives of hematin, though closely analogous, are not true hemosiderin but are bodies of a distinctly different nature which, for convenience, might be termed hemosideroid pigments.

Intimately associated with the hematin and the hemosideroid pigments in the peritoneal cavity and the subcutaneous tissues, there is usually present a variable amount of typical hemosiderin. This pigment is more constant in the early stages of the experiments, while later it appears only in such nodules as show the presence of hemorrhage. In no instance, however, has there been any evidence of the production of hematoidin.

The series of animals in which crystalline hemin was injected subcutaneously and intraperitoneally gave results which agree in all essential respects with those obtained with hematin. The hemin seems more irritating, and the tendency to hemorrhage in and about the pigment nodules is more pronounced. The hemin injections proved of great value in confirming results obtained with hematin, as the crystalline substance is so characteristic that it can be quite easily identified in the tissues, even after the color has been markedly altered. A single difficulty was encountered in the study of these crystals, and that was the preservation of the crystals in fixing the tissues in which they were imbedded. Tissues, such as the omentum, which permit of microscopic examination, while still fresh may show perfect preservation of the crystalline form, but after fixation with either alcohol or formalin, the crystalline hemin may be largely replaced by a fine brownish black precipitate or fine crystalline needles of the same color. There are usually enough crystals preserved, however, to enable one to trace the changes produced by the tissues, and this can be done best from portions of omentum or mesentery stretched over cover slips before fixation. With such preparations or with the usual sections, it is not difficult to recognize single crystals and groups of crystals with typical hemin morphology which are partially or completely decolorized and exhibit an iron reaction identical with that described for hematin. These altered crystals are also easily soluble in dilute caustic alkalies and easily destroyed by hydrogen peroxid,

as in the case of hematin. It seems certain, therefore, that both of these substances suffer the same type of change in animal tissues.[7]

Another point of interest that should be noted is that there is no difference whatsoever in the action of these products (hematin and hemin) from rabbit blood or from guinea pig blood.

In conclusion, it must be said that hematin, when injected into animal tissues, is not a substance of transient duration but, on the contrary, is metabolized very slowly and with great difficulty. It is of further importance to emphasize the fact that the sole demonstrable change occurring in either hematin or hemin within the tissues is that indicated by a slowly progressing decolorization of these pigments. As this change is absolutely identical with that produced by the oxidation of these substances by hydrogen peroxid, it is but natural to conclude that the process here involved is an oxidation and not a cleavage. Again, as the total amount of hemosiderin produced has always been exceedingly slight compared with the hematin injected, and as this hemosiderin has appeared only where there has been hemorrhage, it is quite clear that the hemosiderin has arisen from extravasated red blood corpuscles and not from the hematin injected. In so far as tissue cells are capable of metabolizing hemoglobin with the production of hemosiderin and hematoidin, while these cells seem wholly unable to form these pigments from hematin, the view that hemosiderin and hematoidin are derived from hematin appears to be untenable, and we are confronted by the necessity of seeking other explanations for the mode of formation of these pigments.

This series of observations, therefore, is significant on account of the light which is thrown on certain aspects of hemoglobin metabolism, especially the production of hemosiderin, as the hemosideroid products of hematin are probably to be regarded as closely analogous to true hemosiderin, the main difference being the presence of a protein group in the latter pigment. This difference, in turn, seems to be due to the fact that one series of pigments is

[7] The author has recently noted that many cover-glass preparations of omentum containing hemin and hematin left in dilute alcohol and exposed to air and sunlight develop these changes more rapidly than in the animal body.

produced by the oxidation of the non-protein portion of hemoglobin, while the other, hemosiderin, is produced by a similar process from the entire hemoglobin molecule, both the protein and hematin being essential to the formation of typical hemosiderin.

As regards hematoidin, so little is known of the circumstances connected with the formation of this pigment that any positive statement, based on these experiments, would seem unwarranted. However, the failure of the tissues to produce any demonstrable hematoidin when coupled with the fact that hematin undergoes a destructive oxidation would seem sufficient to cause some doubt as to the production of hematoidin from hematin according to the scheme of Nencki and Sieber.

SUMMARY.

1. It has been shown that powdered hematin is exceedingly resistant to the metabolic action of tissue cells, and wherever injected it will remain for weeks with but slight and slowly progressing alteration.

2. The changes produced in hematin by tissue cells are identical with those changes resulting from the oxidation of hematin by hydrogen peroxid, forming a series of bodies of decreasing color intensity, which manifest an iron reaction in inverse proportion to their color.

3. These hemosideroid pigments are distinguished from true hemosiderin by the type of their iron reaction, by their solubility in dilute alkalies, and by their destructive oxidation by hydrogen peroxid.

4. Crystalline hematin injected into the tissues is subject to the same changes as powdered hematin.

5. Whereas powdered parahemosiderin is converted into hemosiderin by the tissue cells with comparative ease, powdered hematin shows no such conversion within forty-seven days, and as the only demonstrable change produced in hematin by the tissue cells does not result in the formation of either hemosiderin or hematoidin, the assumption that hematin is the progenitor of these pigments seems unwarranted.