MALARIAL PIGMENT (SO-CALLED MELANIN): ITS NATURE AND MODE OF PRODUCTION.*

By W. H. BROWN, M.D.

(From the Pathological Laboratory of the University of Wisconsin, Madison, Wisconsin.)

The pigment elaborated by the malarial parasite during its development in the red blood corpuscle is constantly referred to in the literature as a melanotic pigment, or melanin. Yet, if one seeks authority for such an implied relation to the melanins, there seems little to warrant such an assumption. It is equally difficult to trace the origin of this conception, though it is probably referable to the work of Meckel,[1] who first described malarial pigment in the blood as a melanemia. The marked difference between this pigment and the familiar hematogenous pigments has undoubtedly aided in the perpetuation of the melanin conception of malarial pigment.

Other Pigments.—The literature on malaria contains also numerous descriptions of other types of malarial pigment, though, in reality, none of these are peculiar to malaria—occurring more frequently and even in larger amounts in conditions other than malaria. These pigments, therefore, should not be considered as malarial pigments, this term being reserved for the dark brown or black pigment which is almost, if not entirely, peculiar to malaria.

Malarial Pigment Differentiated.—The so-called malarial melanin is readily differentiated from all other pigments by those familiar with the usual descriptions of the pigment and its quite characteristic distribution.

Unfortunately, the work of Ewing[2] has introduced much confusion. Ewing describes at some length, and illustrates with most excellent cuts, granular and crystalline pigments (occurring in

* Received for publication, November 5, 1910.

[1] Meckel, *Ztschr. f. Psychiat.*, 1847, v, 198; cited by Virchow, *Virchows Arch. f. path. Anat.*, 1849, ii, 587.

[2] Ewing, *Jour. Exper. Med.*, 1905, vi, 119.

malaria as well as in other conditions with jaundice) which he considers are differentiated with "great difficulty" from the pigment elaborated by the malarial parasite. He states that in malaria one may meet with granular or crystalline pigment "not giving the Prussian blue reaction, nor dissolving in chloroform, ether, or carbon bisulphide, but dissolving in ammonium sulphide, which may have any one of the following origins:

(1) Pigment elaborated by the intracellular parasite.

(2) Hæmatoidin derived from the remnants of infected red cells.

(3) Hæmatoidin or altered hemoglobin deposited in granular or crystalline form from red cells dissolved in the plasma . . .

(4) Bilirubin or urobilin granules or crystals."

The pigments represented in groups 2, 3, and 4, he considers are rendered abnormal by fixation.

It has been impossible for me to confirm these findings. For example, in none of my preparations have I been able to detect such alterations in the bile pigments, after alcohol or formalin fixation, as to render them insoluble in the usual solvents. In groups 2, 3, and 4, I see only the precipitate, granular and crystalline, produced by the action of formalin on dissolved hemoglobin and found in greater or less amounts in nearly all tissues fixed in formalin. As Ewing states that these pigments do not give the usual reactions for bile pigments, I cannot see his reasons for considering them as such.

Although I have not investigated the nature of this formalin precipitate, Browicz[3] describes it as the conversion of dissolved hemoglobin into methemoglobin and eventually into hematin. The precipitate follows closely the solubility of the malarial pigment and for this reason, in the study of malarial pigment, tissues fixed with formalin should never be used. Nevertheless, the general features and distribution of the formalin precipitate are such as should give one no difficulty in distinguishing it from the true malarial pigment.

After what has been said, it seems hardly necessary to state that this investigation deals solely with the so-called melanotic malarial

[3] Browicz, *Virchows Arch. f. path. Anat.*, 1900, clxii, 373.

pigment and is in no wise concerned with the associated hematogenous pigments.

In the investigation of the supposed melanin nature of malarial pigment it seemed advisable to institute a comparison between this pigment and certain well known melanins. I have used several specimens of melanin from negro's skin, from the choroid, and from melano-sarcomata. The malarial tissues represent principally spleen and liver from six cases of pernicious malaria in which there were abundant deposits of pigment. The comparison made has been based largely upon bleach reactions and solubility. While different melanins vary rather decidedly in both of these respects, as is well known, they are bleached to colorless compounds with comparative ease when subjected to the action of such oxidizing agents as potassium permanganate and hydrogen peroxide. On the other hand, their resistance to solution is pronounced—even with strong acids and alkalies.

The following technique and reagents have been used in carrying out this work. Celloidin sections were used almost exclusively. A ¼ per cent. aqueous solution of potassium permanganate and a 30 per cent. hydrogen peroxide[4] were employed as bleaching reagents. More dilute solutions than these are efficacious, though these act more rapidly and damage the section but little unless used longer than twenty-four hours. Sections treated with potassium permanganate require decolorization with oxalic acid or Pal's solution. The list of solvents was not intended to be exhaustive. It included the following: saturated aqueous solution of lithium carbonate; 0.2 per cent. aqueous potassium hydroxide; alcoholic potash made by adding four parts of 1 per cent. potassium hydroxide to 100 parts 80 per cent. alcohol (0.04 per cent. alcohol potash); ammonium hydroxide, diluted one-half; ammonium sulphide; 1 per cent. hydrochloric acid in 70 per cent. alcohol (by volume); 1 per cent. hydrochloric acid ether; 5 per cent. (by volume) aqueous hydrochloric and sulphuric acids. The alcoholic potash solution is one in common use in many laboratories for removing the formalin precipitate from sections and it was accidentally noted that it was also an excellent solvent for malarial pigment. All

[4] The preparation used is the perhydrol of Merck and Company.

sections, however treated, were dehydrated, cleared, and mounted for study.

The following table illustrates the essential differences in the bleach reactions and solubility of malarial pigment and melanins.

Tissue.	Bleach reagents.		Solvents.								
	½ per cent. $KMnO_4$.	30 per cent. H_2O_2.	Saturated Li_2CO_3.	0.2 per cent. KOH.	$(NH_4)OH$.	Alcohol KOH.	Acid alcohol.	Acid ether.	$(NH_4)_2S$.	HCl.	H_2SO_4
Negro's skin, No. 1.....	+	+	—	—	—	—	—	—	—	—	—
Negro's skin, No. 2.....	+	+	—	—	—	—	—	—	—	—	—
Negro's skin, No. 3.....	+	+	—	—	—	—	—	—	—	—	—
Choroid, No. 1.....	+	+	—	—	—	—	—	—	—	—	—
Choroid, No. 2.....	+	+	—	—	—	—	—	—	—	—	—
Melano-sarcoma, liver..	+	+	—	—	—	—	—	—	—	—	—
Melano-sarcoma, liver..	+	+	—	—	—	—	—	—	—	—	—
Melano-sarcoma, choroid.....	+	+	—	—	—	—	—	—	—	—	—
Melano-sarcoma, skin..	+	+	—	—	—	—	—	—	—	—	—
Melano-sarcoma, source unknown.....	+	+	—	—	—	—	—	—	—	—	—
Malaria, Case 1.....	—	—	+	+	+	+	+	+	+	—	—
Malaria, Case 2.....	—	—	+	+	+	+	+	+	+	—	—
Malaria, Case 3.....	—	—	+	+	+	+	+	+	+	—	—
Malaria, Case 4.....	—	—	+	+	+	+	+	+	+	—	—
Malaria, Case 5.....	—	—	+	+	+	+	+	+	+	—	—
Malaria, Case 6.....	—	—	+	+	+	+	+	+	+	—	—

Plus (+) indicates a positive change, and minus (—), no change.

So many factors influence the time required to secure complete bleaching or solution, that unqualified statements are of little value. The thickness of the section, the intensity of the color, the size of the granules, and the total amount of the pigment, all influence the time required to obtain the end reaction. Besides these factors, however, I have observed that melanins vary rather decidedly in the ease with which they bleach. In my experiments, therefore, no negative result has been recorded until the reagent has acted for forty-eight hours.

Hydrogen peroxide bleaches melanin much more slowly than potassium permanganate, and with all of my material, required four to sixteen hours to produce complete bleaching.

On malarial pigment, 30 per cent. hydrogen peroxide acts very rapidly. In some of my malarial sections there was a total disappearance of the pigment within thirty minutes, and in all cases

within two hours. The speed of reaction, in these instances, was due, not to the factors noted above, but to the nature of the pigment. On careful investigation, the change proved to be one of disintegration, no bleaching[5] being detectable.

Bleached melanin granules are readily demonstrable in stained sections, but no such granules could be detected after the action of peroxide of hydrogen on malarial pigment. Furthermore, if the sections from cases of malaria are studied from time to time during the process of dissolution, the decrease in size as well as in the intensity of the color of the pigment granules, can be observed.

Therefore, one is justified in the conclusion that, with the reagents here employed, the bleach reactions of melanin and of malarial pigment show no points in common—all melanins bleaching rapidly with potassium permanganate, while with this reagent malarial pigment manifests not the slightest sign of a true bleach reaction within forty-eight hours.

If we consider the question of solubility of malarial pigment and of melanin, the difference is equally pronounced. Neither substance is soluble in alcohol, ether, or chloroform. The resistance of melanins to solution by such reagents as were employed in these tests is not at all surprising. On the other hand, in view of the fact that Ewing and some others record only one solvent for malarial pigment, namely, ammonium sulphide, it is quite surprising to find such a list of substances that will dissolve this pigment with comparative ease, even in extreme dilution.

My own results, in this respect, are closely in accord with those reported by Carbone,[6] who pointed out the parallelism in the solubility of malarial pigment and of hematin. This parallelism of solubilities led me to make a spectroscopic examination of the malarial pigment.

Solution of Malarial Pigment.—The difficulty and possible source of error attendant upon such an examination was to obtain a solvent which, while dissolving the malarial pigment from fixed

[5] A distinct bleaching of the malarial pigment may, however, be seen to precede the disintegration provided a 3 per cent. solution of the hydrogen peroxide is used instead of the 30 per cent. solution employed in these experiments.

[6] Carbone, *Gior. d. r. Accad. di med. di Torino,* 1891, series iii, xxxix, 901.

tissues, would not affect the hemoglobin of the blood corpuscles. For this purpose, I employed the solution of alcoholic potash described above. In preparing the solution of malarial pigment, a piece of spleen (one cubic centimeter) fixed in Zenker's fluid was dried and ground in a mortar. Ten cubic centimeters of the alcohol potash, previously described, was poured over the powdered spleen and allowed to stand for twelve hours in a tightly corked flask and then filtered. Bile pigments, when present, should be extracted before dissolving the malarial pigment, though they may be separated subsequently. The solution thus obtained was of a brown color. As a control, a piece of spleen of the same size from an acute splenic tumor and a blood clot of the same size, both fixed in the same way as the malarial tissue, were treated in identically the same manner. As the filtrate from these was perfectly clear, the strength of the alcoholic potash was increased by the addition of two drops of 10 per cent. potassium hydroxide and this was allowed to act for twenty-four hours, after which the filtrate still showed no trace of color. From this, it was absolutely certain that the color in the filtrate from the malarial tissue was not derived from the red blood corpuscles or dissolved hemoglobin, but was referable to the malarial pigment in solution.

Spectroscopic Examination.—The solution of malarial pigment, when subjected to spectroscopic examination, showed a single band starting sharply at the D line and extending to the left. The left border was very indistinct, gradually shading into the red between C and D. On acidifying the solution with a drop of hydrochloric acid, the color became redder. This solution also presented a single banded spectrum. In this case, however, the band was moved markedly towards the left, the spectrum undoubtedly being that of acid hematin. On further treating this solution with Stoke's reagent, it assumed the characteristic color of a hemochromogen solution. Spectroscopically, it showed the two perfectly characteristic bands of hemochromogen in the green, the left band being much the darker and more distinct. On shaking with air, the color changed and no absorption bands could be detected. On again adding Stokes' solution, the two bands reappeared. A portion of the original solution added to concentrated sulphuric acid failed to

show any absorption bands, but when the solution was evaporated to dryness and the greenish black residue added to the concentrated acid, a spectrum with two well defined bands was obtained. The fainter and narrower band was situated just to the left of D, while the darker and broader band lay between D and E—the characteristic spectrum of hematoporphyrin.

The spectroscopic examination of malarial pigment confirms the results obtained by Carbone in 1891 and leaves no question as to the hematin nature of this pigment. It is rather remarkable that this conception of malarial pigment seems to have gained no confirmation or acceptance. In fact, the author finds this most excellent work of Carbone almost entirely overlooked or misquoted.[7]

When we consider the marked difference in the bleach reactions and solubility of melanins and malarial pigment, it seems almost impossible that the two substances could be placed in the same class. The statements found in the literature are not entirely in accord with my own findings in these particulars. For example, Thayer[8] speaks of the decolorizing of malarial pigment by alkalies. This is not a true bleach reaction but a solution. Again, Ewing states that the pigment "long resists the action of strong acids and alkalies." I cannot agree with this statement, for, while relatively strong aqueous solutions of sulphuric and hydrochloric acids do not dissolve malarial pigment, very dilute alcoholic and ether solutions of these acids are good solvents and alkalies in either aqueous or alcoholic solution dissolve malarial pigment even in extreme dilution.

So much emphasis has been placed upon the negative iron reaction of malarial pigment that I feel I cannot leave the question of the nature of the pigment without mentioning this reaction. It must be remembered that the iron reaction with potassium ferrocyanide and hydrochloric acid, as shown by hemosiderin, is the reaction of a proteid-iron compound in which the Prussian blue is largely deposited in the proteid matrix. Hematin is not such a

[7] During the progress of this work, the author relied upon reviews of Carbone's paper for his information of that investigator's work. The original work of Carbone was not obtained until this paper was in manuscript, and several changes have been necessary to give him full credit for his results.

[8] Thayer, Lectures on the Malarial Fevers, New York, 1897, p. 240.

compound, and on *a priori* grounds we should not expect the same type of reaction but rather the reaction of a soluble iron salt in which the Prussian blue is not fixed *in situ*. Milner[9] goes as far as to state that hematin does not give a microchemical reaction for iron. It is certainly not demonstrable by the usual technique.

The Iron Reaction Obtained.—If the test for iron in a celloidin section containing malarial pigment is made on a slide with a mixture of ferrocyanide and acid alcohol (2 per cent. or stronger), and if the cover is sealed with vaseline or paraffin to prevent evaporation, a spreading halo of Prussian blue can be demonstrated about many of the pigment granules. This is more pronounced after the preparation has been gently warmed or allowed to stand for some time. If there is any suspicion that this reaction is due to a collar of hemosiderin, such a possibility can be eliminated by the removal of all iron from the hemosiderin with Pal's solution or a 1 to 2 per cent. solution of oxalic acid allowed to act for twelve hours; usually a shorter time is sufficient and this treatment does not affect the malarial pigment. A preliminary oxidation, preferably with hydrogen peroxide, will facilitate the iron reaction. At all events, the residue from a solution of malarial pigment, neutralized with hydrochloric acid and evaporated to dryness yields when incinerated, an iron-containing ash.

Turning now to the mode of formation of malarial pigment and the possible biochemical factors concerned in its elaboration, the problem seems rather easy of solution. It is well known that many protozoa possess proteolytic enzymes analogous in their action to pepsin or trypsin. Pepsin and trypsin, acting upon hemoglobin, split it into a proteid (globin) and hematin. At present, no one doubts that malarial pigment is produced from hemoglobin by the malarial parasite. From these facts and from what we know of the nature of the pigment in question, it is only a logical assumption that the malarial parasite contains some proteolytic enzyme, analogous to pepsin or trypsin, capable of splitting hemoglobin into proteid and hematin, the proteid being further metabolized in the economy of the parasite, and the hematin being merely a waste product, accumulating as the parasite develops, to be finally extruded on the segmentation or disintegration of the parasite.

[9] Milner, *Virchows Arch. f. path. Anat.*, 1903, clxxiv, 475.

The ultimate fate of the malarial pigment is uncertain. That it remains for years in the spleen and other organs in which it is deposited indicates strongly that the human organism possesses no adequate means for its disposal. This fact is of much importance in its bearing on hemoglobin metabolism, indicating that hematin is not formed in any considerable amount as an intermediate product in the production of bile pigments, as is at present taught.

For years, the question of the extra-parasitic production of malarial pigment has been under discussion. Hemosiderin, present in the tissues, has been suggested as a possible source for such pigment. While this question has never been absolutely settled, it seems to the author quite certain that such a mode of formation plays no important rôle in the pigment production. The occurrence of black pigment in the centre of hemosiderin masses in malaria has added strength to the extra-parasitic hypothesis. Such pictures have been observed in a few instances in hemosiderin deposits in other conditions and the significance has been indicated by the author[10] in a previous publication. It is in reality a conversion of hemosiderin into hematin. As this rare change occurs in conditions other than malaria, I consider it of no particular importance, but only a concomitant process which probably has nothing to do with the production of malarial pigment. More frequently the black pigment is merely a nucleus about which the hemosiderin is deposited.[11]

In conclusion, it seems hardly necessary to state that I do not assume that the black malarial pigment is pure hematin. There are doubtless impurities, although I believe nothing of sufficient consequence can be demonstrated to warrant the perpetuation of the misnomer of melanin for a pigment that is in reality hematin.

SUMMARY.

1. Two important methods for the study of malarial pigment are described.

(*a*) A method for obtaining a solution of malarial pigment from fixed tissues without the removal of a trace of hemoglobin from the red blood corpuscles.

[10] Brown, *Jour. Exper. Med.*, 1910, xii, 623.

[11] Neumann, *Virchows Arch. f. path. Anat.*, 1900, clxi, 422.

(*b*) A method for obtaining an iron reaction in malarial pigment.

2. By comparing the bleach reactions and solubility of melanins and malarial pigment, the dissimilarity of the two classes of pigments has been demonstrated.

3. The spectroscopic examination of a solution of malarial pigment proves conclusively that the pigment is hematin.

4. It is suggested that the action of a proteolytic enzyme of the malarial parasite upon the hemoglobin of the red blood corpuscle is the most probable mode of elaboration of malarial pigment.

5. The difficulty with which the human organism disposes of malarial pigment indicates that the production of hematin cannot be considered as a normal intermediate process in the formation of bile pigments from hemoglobin.

My thanks are due to Dr. G. H. Whipple, of the Johns Hopkins Hospital, for supplying me with a part of the tissue used in this investigation, and to Dr. A. S. Loevenhart, of the University of Wisconsin, who has kindly aided me with the spectroscopic work and confirmed that part of the findings here reported.