The Pigment of the Malarial Parasites *Plasmodium knowlesi* and *Plasmodium gallinaceum*

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The pigment produced by different malarial parasites has been extensively studied, the latest contributions relative to its chemical nature being those of Sinton & Ghosh (1934 *a*, *b*), Ghosh & Sinton (1934), Ghosh & Nath (1934), Devine & Fulton (1941, 1942) and Morrison & Anderson (1942). This aspect of the question is summarized historically in the first mentioned publication in which a close similarity if not identity was established between the pigment and haematin prepared from crystalline haemin and alkali. Ghosh & Sinton (1934) describe (without illustrations) the preparation of crystals of pyridine haemochromogen and of haemin from washed pigmented *P. knowlesi* parasites. The same crystalline derivatives (unillustrated) were obtained by Morrison & Anderson (1942) using portions of the parasite mass. Devine & Fulton (1941, 1942) present more extended spectroscopic data in support of the identity of *P. knowlesi* and *P. gallinaceum* pigment with haematin, including an examination in the visible region of met- and fluoromethaemoglobins, and oxy and reduced haemoglobins prepared by combining the amorphous malarial pigment with native ox globin. No measurements of the Soret bands were performed. Heilmeyer (1943), from a spectrophotometric study, has shown that all preparations of malarial pigment available to him contained variable amounts of contaminating substances of high molecular weight.

All the above workers relied upon dilute alkali (Na_2CO_3 or NaOH) to extract the pigment from the parasites, and crystalline derivatives, where described, were formed only from the crude parasite mass before such extraction. It is well known that dilute NaOH frequently so alters haematin during the process of dissolution and extraction from natural sources that it is subsequently well-nigh impossible to prepare crystalline derivatives (Fischer & Schneller, 1923). Keilin (1943) has also recorded the rapid change in properties brought about by the action of dilute Na_2CO_3 upon protohaemin.

Having available a new technique for the extraction of haematin without at any stage using alkalis, a method successfully applied by Schwabacher, Lucas & Rimington (1947) to the isolation and identification of haematin from *Fusiformis melaninogenicus*, it seemed desirable to re-examine malarial pigment from different sources and to prepare crystalline derivatives for identification from the isolated pigment, thus excluding any possibility that these were derived from a cellular material of the parasite other than the pigment itself. Moreover, the identification of this substance with haematin (ferriprotoporphyrin) depends, according to published work, upon comparison of the spectrophotometric absorption curves, usually obtained with the use of serial filters, of the pigment and haematin in dilute NaOH. These curves show only broad maxima of low intensity and the agreement in some cases is far from good. The absorption curves for the acid acetone solutions of malarial pigment and of haematin from haemoglobin, published by Morrison & Anderson (1942), show general similarity but not identity and examination is not recorded beyond 500 mμ. No author, so far as we are aware, has seriously considered the possibility that the vinyl groups of the protoporphyrin nucleus might be reduced by the activity of the parasite to form mesohaematin or even eliminated entirely, with the resultant production of deuterohaematin. That such changes may be brought about by micro-organisms is evident from the detection of both meso- and deuteroporphyrins in faeces by Zeile & Rau (1937). In the present study particular attention has been paid to this point.

EXPERIMENTAL

Pigment from P. knowlesi. Malarial parasites (Devine & Fulton, 1941) from a *P. knowlesi* infection of monkeys were used. After thorough washing, the parasite mass was stirred with 90 % phenol solution, prepared by adding 10 ml. water to 90 g. phenol, and extraction allowed to proceed at room temperature with occasional stirring for 12 hr. The mixture was then centrifuged and the residue re-extracted with successive amounts of the phenol solution at 2–3 hr. intervals until an absorption band at 627 mμ. was no longer visible in the extract. To the pooled solutions of phenol, 2 vol. of ethanol were then added to precipitate small quantities of dissolved protein and after filtration through a no. 4

sintered glass funnel the solution was dialyzed against tap water in a stout 'Visking' bag, the contents being frequently mixed as two phases rapidly separated. Dialysis was finally continued against distilled water and as soon as turbidity or precipitation was noticed in the lower, pigmented layer, the latter was removed into a centrifuge tube and diluted liberally with water, whereupon the pigment separated in a granular form. It was collected on the centrifuge, washed repeatedly with water and then dried *in vacuo*.

The pigment is obtained at this stage as an amorphous black powder almost completely soluble in pyridine-chloroform mixture (3:8) but insoluble in ether or ethanol; acid ether or acid acetone, however, dissolve it readily. That such preparations are free from protein is demonstrated by the absence of any haemochromogen spectrum when sodium hyposulphite (sodium hydrosulphite, $Na_2S_2O_4$) is added to a solution in dilute NaOH. Small amounts of impurity were removed and crystallization was effected as follows. For every 50 mg. of pigment was added 0·15 ml. of pyridine, then 0·4 ml. of chloroform and the very concentrated solution was filtered through a small sintered glass funnel; the filtrate was then added drop by drop to 2·5 ml. of acetic acid-chloride mixture (25 ml. acetic acid + 0·5 ml. saturated NaCl + 0·4 ml. conc. HCl) maintained in a small tube at boiling water-bath temperature. After 0·5 hr. the mixture was allowed to cool and placed in the ice chest overnight. The crystals were collected and washed on the centrifuge by 50 % (v/v) acetic acid, water, ethanol and finally ether. Recrystallization was effected in the same manner (Fig. 1).

Pigment from P. gallinaceum. For this preparation, blood from parasitized fowls was used (Devine & Fulton, 1942) and the method of extraction differed in no way from that described above. Extraction of the pigment by phenol was, however, slower and a considerable proportion of the crude material was insoluble in pyridine-chloroform, no doubt owing to the impossibility in this case of separating the parasites from erythrocyte debris. This impurity was not protein in nature, it was only sparingly soluble in sodium hydroxide solution and was not further investigated. One recrystallization afforded well-formed crystals of the pigment (Fig. 2). Haemin from blood, similarly recrystallized, is shown for comparison in Fig. 3.

That the malarial pigment derivative which closely resembled protohaemin in appearance, was in fact a haem pigment, was readily demonstrated by spectroscopic examination of its solutions in different solvents (Hartridge reversion spectroscope), by the formation of a pyridine haemochromogen, of a porphyrin when the pigment was treated with concentrated sulphuric acid and of methaemoglobin (and thence HbO_2, Hb and HbCO)* when it was combined in faintly alkaline solution with native ox globin (Tables 1 and 2).

Table 1. *Absorption maxima shown by malarial pigment and by crystalline haemin*

Solvent, treatment, etc.	Malarial pigment (mμ.)	Haemin (mμ.)
0·1N-NaOH	615·4	615·7
Above + $Na_2S_2O_4$	*c.* 583–535	575–540
Above + CO	569·3	565·0
	533·9	536·0
0·1N-NaOH + pyridine (10 % by vol.) + $Na_2S_2O_4$	556·6	556·3
	528·3	528·0
90 % phenol	627·4	627·6
	540·3	540·0
Ether: HCl	637·1	637·6
	543·6	543·7
Ether: acetic acid	634·6	633·1
	542·9	541·4
Conc. H_2SO_4	601·9	601·7
	557·7	556·6
Porphyrin from above in pyridine	623·6	625·8
	576·3	579·3
	535·6	540·3
	505·3	507·8
Haematoporphyrin in H_2SO_4	—	601·6
	—	554·3
Mesoporphyrin in H_2SO_4	—	594·5
	—	556·9
	—	551·9

Table 2. *Absorption maxima shown by reconstituted haem-ox globin pigments*

Pigment type	Malarial pigment-ox globin (mμ.)	Haematin-ox globin (mμ.)
Met Hb	630·9	631·8
HbO_2	576·3	577·7
	539·4	539·4
HbCO	572·1	572·1
	537·4	537·8
	Soret bands	
HbO_2	405·5	405·8
HbCO	416·5	416·0

On mixing a small portion of the pigment on a glass slide with Takayama's reagent (Harrison, 1944), it dissolved with a red colour and under the microscope crystal masses of pyridine haemochromogen were visible. The shape of these crystals, even when prepared from pure haematin, is variable, ranging from prismatic needles grouped in stellate

* For the measurement of the Soret bands of these pigments we are indebted to Mr E. M. Jope of the M.R.C. Spectroscopy Unit, London Hospital, who used a Hilger medium quartz spectrograph.

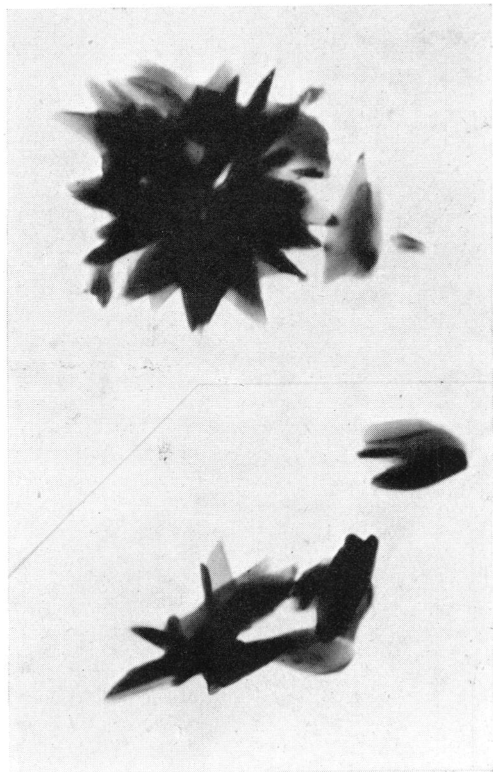

Fig. 2. Haemin from pigment of *P. gallinaceum*. ×540.

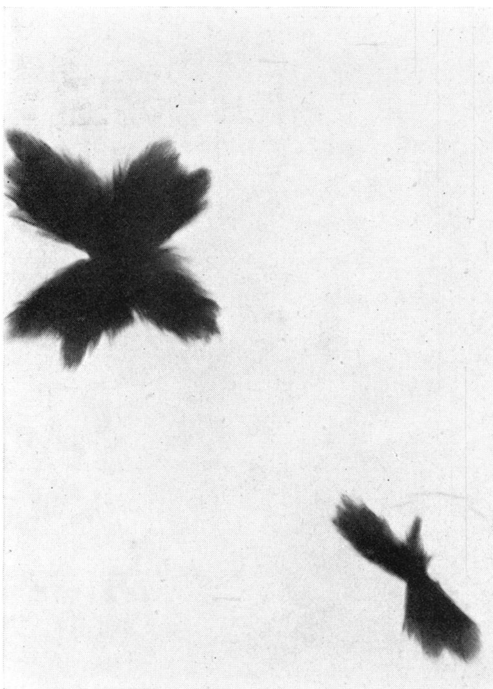

Fig. 1. Haemin from pigment of *P. knowlesi*. ×540.

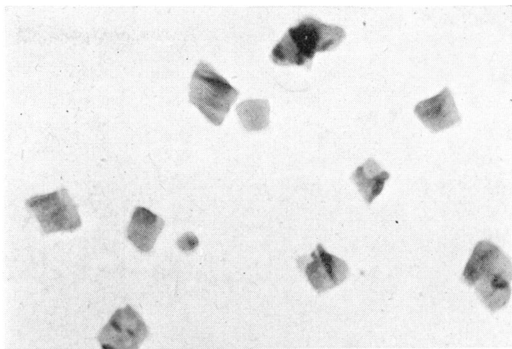

Fig. 5. Pyridine haemochromogen from haemin. ×875.

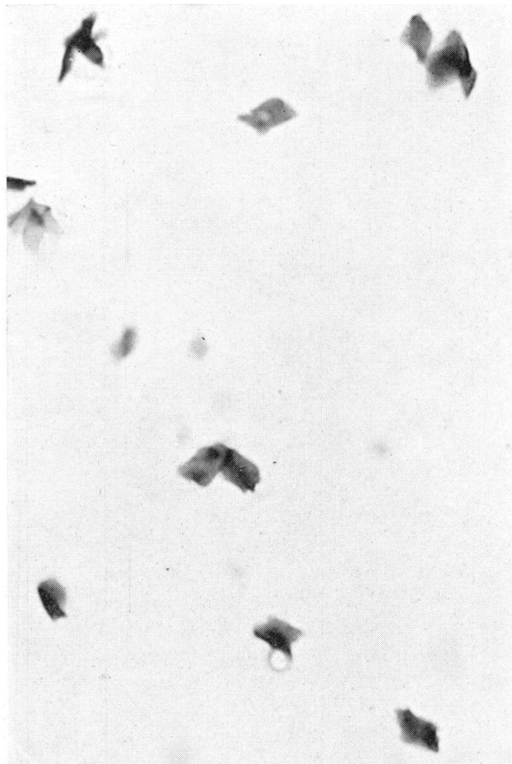

Fig. 4. Pyridine haemochromogen from malarial pigment. ×875.

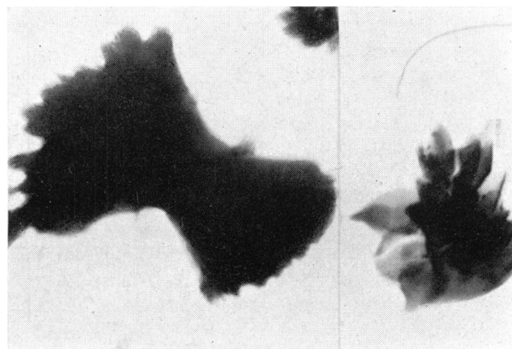

Fig. 3. Haemin. ×540.

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clusters to flat, overlapping platelike structures. It was observed that the type of crystal produced depended somewhat upon the maturity of the Takayama's reagent and hence the velocity of reduction and also that needles first formed tended to become transformed on the slide into plates. Control experiments were therefore always done at the same time with pure crystalline haemin (see Figs. 4 and 5).

The preparation of the reconstituted methaemoglobin, etc. was accomplished as follows:

Globin hydrochloride was prepared from ox blood (ox globin being relatively more stable than horse or human globin) and partially renatured by dialysis for 24 hr. against running tap water according to the method of Anson & Mirsky (1929–30). Dialysis was completed against M/30-K_2HPO_4 solution at the pH of which denatured globin is insoluble. After centrifugation, the supernatant liquid containing the renatured protein was carefully adjusted by KH_2PO_4 solution to pH *c.* 6·0. To separate portions of this solution were added solutions in dilute NaOH of malarial pigment and of pure crystalline haemin from blood (Rimington, 1942) respectively until the band of methaemoglobin at about 630 mμ. was clearly visible. After this was measured, freshly prepared Stokes reagent (Harrison, 1944) was added drop by drop until the colour changed from brown to red and the methaemoglobin spectrum had given place to that of oxyhaemoglobin. Over-reduction led to the single, broad band of reduced haemoglobin (purple coloured solution), but shaking with air restored the two-banded spectrum of oxyhaemoglobin. This demonstrated, incidentally, the capacity of the synthetic globin-pigment complex to function as an oxygen carrier as does haemoglobin.

It was observed that during the addition of the Stokes reagent some unavoidable denaturation occurred, so the solutions were centrifuged before proceeding further from this stage. For measurement of the Soret bands, the reconstituted haemoglobins were equilibrated to pH 7·2 by dialysis against M/30 phosphate buffer, since the exact position of this maximum is influenced by the pH of the solution. Good agreement between the reconstituted malarial pigment and that from haemin was found for both oxy and carboxy derivatives, the intensity of the Soret bands being of the order of ten times that of the visible bands as is the case with normal native oxyhaemoglobin. The actual maxima of the Soret bands were in each case at a wavelength shorter than that usually observed in the case of native oxyhaemoglobin and carboxyhaemoglobin, 405·5 mμ. as compared with the usual 414·5 mμ. for oxyhaemoglobin and 416·5 mμ. as compared with the usual 420·0 mμ. for carboxyhaemoglobin.

These shorter wave-lengths are those observed for the Soret bands on solutions of normal native oxyhaemoglobin and carboxyhaemoglobin respectively which have been kept for some time and have apparently undergone some preliminary form of denaturation, which does not, however, appear to affect the visible band wave-lengths. In the case of the reconstituted malarial pigments, the visible bands agree closely with those of normal native oxyhaemoglobin and carboxyhaemoglobin. The wave-lengths observed for the Soret band in these reduced reconstituted pigments and in partially denatured haemoglobins almost coincide with that of methaemoglobin. The presence of a Soret band is in itself strong evidence for the existence of the intact porphyrin ring.

DISCUSSION

The close correspondence of the absorption spectra of the malarial pigment with haematin and the porphyrin derived from it with haematoporphyrin rather than mesoporphyrin (see Table 1) or deuteroporphyrin indicates that malarial pigment from both sources is indeed haematin in which the vinyl groups have remained intact. Drabkin (1942) has shown that the presence of free vinyl groups in haem pigments displaces their absorption maxima about 11 mμ. towards the red, the α band of pyridine haemochromogen, for example, lying at 558 mμ. and that of pyridine mesohaemochromogen at 547 mμ. Further evidence of the identity of each pigment with haematin (ferriprotoporphyrin) is afforded by comparison of the spectral absorption in the visible and ultra-violet region of the haemoglobins and their derivatives made by combining these pigments with renatured ox globin.

SUMMARY

1. The pigment of malarial parasites (*P. knowlesi* and *P. gallinaceum*) has been extracted by a technique which avoids the use of alkali at all stages.

2. From this material, crystalline haemin and pyridine haemochromogen have been prepared.

3. A comparison of the spectral absorption of malarial pigment and of haemin in different solvents and of the visible bands and Soret bands of haemoglobin derivatives made by combining these pigments with renatured ox globin confirms the identity of both malarial pigments with haematin (ferriprotoporphyrin).

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Acetylation and Deacetylation of the *p*-Amino Group of Sulphonamide Drugs in Animal Tissues

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The work reported in this paper was originally intended to deal with the mechanism of the acetylation of sulphonamides in animal tissues, with special reference to the source of the acetyl radical and the conditions controlling the rate and degree of acetylation. We thought it would be advantageous to use for this work isolated tissues, sliced or minced, but soon found that the degree of acetylation in isolated tissues was much lower than was to be expected on the basis of observations on the intact organism. Whilst in the intact guinea-pig, for example, 75 % of the sulphamezathine excreted in the urine was conjugated, conjugation by slices of guinea-pig tissues never exceeded 20 % and was usually below 10 %, even when the added amount of drug was small. Such acetylation as did occur in isolated tissues was usually completed within 30 min. of incubation. An investigation of this apparent discrepancy in the metabolic behaviour of tissues *in situ* and *in vitro* occupies the main part of this paper. At least a partial explanation for the discrepancy offered itself when it was found that certain tissues contain an enzyme as already reported by Kohl & Flynn (1940) which hydrolyzes N^4-acetylated (or, generally, N^4-acylated) sulphonamides. It appears that the relative activity of this enzyme is altered by slicing or mincing. The activity is apparently low, perhaps even negligible, in the intact rabbit or guinea-pig, though it is operative in the intact pigeon, dog and cat. The enzyme activity is increased, either in absolute terms or relative to conjugating systems, by the conditions prevailing in the *in vitro* experiments. *In vitro*, therefore, the process of acetylation appears to be largely counterbalanced by reactions causing deacetylation.

EXPERIMENTAL

Determination of sulphonamides

The methods of Bratton & Marshall (1939) and of Rose & Bevan (1944) were used.

Blood. A suitable volume, usually 0·1 ml., was measured into 0·5 ml. of 0·5 % saponin solution in a 10 ml. measuring tube. Water was added to make up to 9 ml. and 1 ml. 15 % trichloroacetic acid solution was used for deproteinization. One 4 ml. portion of the filtrate was used for the determination of the free sulphonamide and another 4 ml. portion for the determination of total sulphonamide. A visual colorimeter was used; the standard contained 0·02 mg. of the sulphonamide. When total sulphonamide was determined the corresponding acetyl compound, treated in the same way as the unknown, was used as a standard.

Urine. Usually 0·1 ml. of urine, if necessary diluted, was used and treated in the same way as blood, except that the saponin was left out.

Faeces. Faeces were mixed with 10 vol. of water. A sample was deproteinized with trichloroacetic acid and the filtrate treated in the same way as the blood filtrate.

Tissue extracts. Media in which tissues had been suspended or extracts of tissues were treated in the same way