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METHEMALBUMIN. I. APPEARANCE DURING ADMINISTRATION OF PAMAQUINE AND QUININE

BY MORRIS ROSENFELD, CHARLES G. ZUBROD, WILLIAM D. BLAKE, AND JAMES A. SHANNON

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Hematin was first identified by Schumm (1) as an abnormal constituent of blood plasma occurring in certain hemolytic states. The fact that this pigment does not circulate as free hematin but is combined with serum protein was suspected by Heilmeyer (2) on the basis of a displacement of one of the hematin absorption bands in the presence of serum. The serum albumin-hematin complex was first adequately described and differentiated from methemoglobin by Fairley and Bromfield (3) who studied the pigment in cases of blackwater fever. Fairley (4) named the compound "methemalbumin" and presented a detailed characterization of its spectrophotometric properties, synthesis and chemical behavior. Spectrograms of the pigment as it occurs in serum of patients with blackwater fever were published by Foy and Kondi (5). Fox (6) based an analytical procedure for the estimation of methemalbumin on the intensity of the absorption band at 623 mμ and its response to certain reagents, sodium cyanide and hydrogen peroxide. The nature of the reaction between hematin and serum albumin was recently examined by J. Keilin (7). The metabolic disposition of methemalbumin is treated in the paper of Pass, Schwartz and Watson (8) on the fate of intravenously injected hematin.

Methemalbumin has not been demonstrated in normal serum as an intermediary in the metabolism of hemoglobin. It has been observed only as an abnormal serum constituent. In addition to his findings in blackwater fever Fairley (9) demonstrated methemalbuminemia in cases of severe malaria, nocturnal hemoglobinuria, gas bacillus sepsis and pernicious anemia. More recently it has been observed in hemolytic reactions to sulfonamides by Fox and Ottenberg (10) and by Ross and Paegel (11) and in pamaquine hemoglobinuria by Dimson and McMartin (12).

Early in the course of studies conducted at the Goldwater Memorial Hospital on the use of pamaquine in malarial therapy there was encountered an acute hemolytic reaction clinically indistinguishable from blackwater fever. Methemalbumin, in addition to free hemoglobin, was detected in the serum of this patient. This observation directed attention to the abnormal blood pigment.

The antimalarial program included an evaluation of the curative action of pamaquine-quinine therapy. The subjects receiving therapy under this program afforded an opportunity for more detailed study which led to the observation that individuals receiving the combined drug therapy consistently developed methemalbuminemia. Controls receiving either drug alone failed to do so. Whereas in previous studies the pigment appeared in sporadic cases in which the precipitating factors were ill-defined, the present work brings to light the uniform occurrence of the pigment under readily reproducible conditions of drug administration.

1 This work was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

2 The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to, the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, and Wyeth, Inc., for contributing toward the publication costs.

3 Captain, MC, AUS.
TABLE I
Concentration of serum methemalbumin, methemoglobin, hemoglobin, and serum bilirubin during administration of antimalarial drugs

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum methemalbumin* as hemin (mg./L)</th>
<th>Methemoglobin as per cent of total hemoglobin</th>
<th>Hemoglobin (gm./100 ml.)</th>
<th>Total serum bilirubin (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day of treatment</td>
<td>Day of treatment</td>
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<td></td>
<td></td>
<td>4</td>
<td>14</td>
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<tr>
<td>Group I</td>
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<tr>
<td>Quinine-</td>
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<tr>
<td>Pamaquine</td>
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<td></td>
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<tr>
<td>Fl</td>
<td>20</td>
<td>19</td>
<td>14.5</td>
<td>10.0</td>
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<td>Bu</td>
<td>22</td>
<td>21</td>
<td>13.0</td>
<td>11.5</td>
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<td>Sw</td>
<td>24</td>
<td>20</td>
<td>13.5</td>
<td>12.5</td>
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<td>Cl</td>
<td>9</td>
<td>11</td>
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<tr>
<td>Mo</td>
<td>4</td>
<td>6</td>
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<td>Pamaquine</td>
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<td>Group III</td>
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<td>Quinine</td>
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<td>Ba</td>
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<td>13.5</td>
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<td>0.2</td>
<td>13.5</td>
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<td>Group IV</td>
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<td></td>
<td></td>
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<tr>
<td>Quinacrine-</td>
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<tr>
<td>Pamaquine</td>
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<td>We</td>
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<td>6</td>
<td>15.0</td>
<td>12.5</td>
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</table>

* Serum methemalbumin measurements were made on the 14th day of combined therapy.
† Methemoglobin concentrations represent maximal values obtained during treatment, usually attained in seven to ten days.

MATERIALS AND METHODS

The experiments were conducted on four groups of white male volunteers infected by mosquito inoculation with P. vivax malaria, Chesson strain. Group I, Table I, received combined therapy of 90 mg. pamaquine and 1.8 gm. quinine daily, dosage in each case being expressed as the free base. Group II received 90 mg. pamaquine per day and Group III 1.8 gm. quinine per day. Group IV received combined therapy of 30 mg. pamaquine and 0.25 gm. quinacrine (as base) daily. Drug therapy by oral administration was started on the fifth day of fever and was continued for a two-week period. In the combined treatment quinine or quinacrine started on the fifth day of fever, pamaquine was added 48 hours later and the two drugs were continued for the two weeks.

Blood specimens were withdrawn prior to therapy for control studies and samples were taken at various times during and after treatment. Fasting serum was employed. To avoid mechanical hemolysis blood was drawn through a No. 18 needle into an oiled syringe containing no anticoagulant. The blood was immediately centrifuged at 2000 r.p.m. for one-half hour. By this procedure a clear layer of serum is obtained separated from the red cells by a deposit of fibrin. Absorption curves in the range of 300 μm to 650 μm were measured in the Beckman spectrophotometer, using a cuvette of 10 mm. light-path. The serum was either undiluted or in the presence of high concentrations of pigment it was diluted three- or five-fold. An aliquot was taken for bilirubin estimation.

The analytical procedure for methemalbumin makes use of the 405 μm absorption band of the pigment.

![Fig. 1. Spectrophotometric Curve of Methemalbumin Formed in Vitro by the Addition of Hematin to Purified Serum Albumin](image)

Protein concentration 5 gm. per liter, hemin concentration 20 mg. per liter, pH 7.5.
The optical density at 405 μm attributable to methemalbumin is computed by subtracting the optical density of normal serum from the measured optical density of the test serum. The serum blank for this computation is taken from Figure 3 which relates the optical density of serum at 405 μm to bilirubin content. Independent chemical measurement of bilirubin in each serum under test provides the data necessary for the use of this chart. Additional information regarding the serum blank was obtained from two other sources, namely, measurement of serum taken before the institution of drug therapy, and direct estimation of the serum blank from the shape of the spectrophotometric curve of the test serum.

The optical density data are converted to methemalbumin concentration which is expressed in terms of hemin (ferri protoporphyrin chloride) equivalent. One unit of optical density in a 10 mm. solution depth corresponds to 8.4 ± .2 mg. per liter of hemin. This value was derived from experiments on the in vitro combination of hematin with normal serum, with purified serum albumin, and with crystalline, human serum albumin. Figure 4 illustrates such an experiment in which varying amounts of hemin have been added to a constant concentration of purified serum albumin. It is evident that Beer's law is

![Chart for Estimation of Serum Blank](chart.png)

**Fig. 3. Chart for Estimation of Serum Blank**

Optical density at 405 μm of human sera containing different concentrations of bilirubin but no methemalbumin.
followed closely within the range of measurement. The
dissociation constant of methemalbumin is such that
possible fluctuations in serum protein concentration do not
affect the measurements. Similarly a five-fold dilution
of serum for purposes of measurement does not introduce
appreciable error. The absorption at 405 m\(\mu\) by methem-
albumin is independent of pH within the range of pH
7.0 to 9.0.

A typical experiment on the in vitro combination of
hematin with serum albumin was conducted as follows.
Hemin (Eastman Kodak Co.) was recrystallized in the
laboratory from pyridine and chloroform according to
the procedure of Hans Fischer (13). The human serum
albumin was very kindly supplied by Dr. Edwin J. Cohn
and Dr. Laurence E. Strong of the Physical Chemistry
Department, Harvard University. The samples used in
the present study were identified as human serum albumin,
fraction V, run 164 and five-times recrystallized
human serum albumin, run 179. Serum albumin, 125
mg., was dissolved in 10 ml. of 0.2 M phosphate buffer,
pH 7.5. Hemin, 50 mg., was dissolved in 10 ml. of
N/10 sodium hydroxide and was made up to 100 ml. with
distilled water. From this stock solution of hematin
sub dilutions were made in N/100 sodium hydroxide, usu-
ally starting with a five- or ten-fold dilution. The final
test solution was made by adding 2 ml. of the protein
solution and 1 ml. of the hematin solution to 2 ml. of
0.2 M phosphate buffer, pH 7.5. The mixtures were
held in a water bath for 15 minutes at 38°, then were
cooled to room temperature and read in the Beckman
spectrophotometer using cells of 10 mm. light-path. The
reference solution contained the protein, buffer, and N/100
sodium hydroxide but no hematin. Figure 1 and Figure
4 are based upon data obtained by this procedure.

Although spectrophotometric identification of methem-
albumin has been relied on chiefly, the chemical reactions
recommended by Fairley (9) have also been applied to
identify the pigment. The behavior of the pigment was
tested with Stoke's reagent, dilute (10 per cent), and
concentrated ammonium sulfide, sodium hydroxilte, cy-
ande, and hydrogen peroxide. The differential reactions
which identify the pigment were, in general, satisfied
though they were not all applied in every case of
methemalbuminemia.

No free hemoglobin was encountered in the sera of
experimental subjects. If present, oxyhemoglobin could
be readily detected spectrophotometrically by the aid of
the band at 576 m\(\mu\). Oxyhemoglobin has a strong ab-
sorption band at 415 m\(\mu\) which would seriously compi-
icate measurements on the 405 m\(\mu\) band of methemalu-
min. Under such circumstances the less sensitive met-
hemalbumin band at 623 m\(\mu\) could be used for the
estimation.

Serum bilirubin was measured according to Malloy and
Evelyn (14). Methemoglobin was estimated by the pro-
cedure of Horecker and Brackett (15), total hemoglobin
by the method of Evelyn and Malloy (16).

RESULTS

Methemalbumin appeared in the serum of all
individuals receiving the combined pamaquine-
quine regime, Group I. There was consider-
able variability in the levels attained at the end of
the two-week period of therapy, the values rang-
ing between 4 and 24 mg. per liter, Table I.

No methemalbumin appeared in the serum of
any individual receiving either pamaquine alone,
Group II, quinine alone, Group III, or pamaquine
in combination with quinacrine, Group IV. It
should be noted that pamaquine dosage was low,
30 mg. per day, in Group IV.

Methemoglobin developed in all patients receiv-
ing pamaquine either alone, Group II, in conjunc-
tion with quinine, Group I, or in conjunction with
quinacrine, Group IV. The methemoglobin concen-
trations were somewhat higher under the com-
bined therapy than under pamaquine alone.
None of the methemoglobin appeared free in the
serum; it was confined to red cells. The indi-
viduals receiving quinine alone, Group III, showed
no significant increment in methemoglobin.

Bilirubin levels were slightly elevated in Group
I on combined therapy but were normal in the
other three groups when measured at the end of
the two-week period of drug administration.

Hemoglobin concentrations dropped during ma-
larial parasitemia but did not appear to be ap-
cycles of quinine administration superimposed upon a continuous pamaquine regimen. As can be seen in Figure 6, methemalbumin appeared only during the periods of combined drug. This patient was a special case to whom the drugs were administered in the absence of malaria.

**DISCUSSION**

Methemalbumin exhibits characteristic spectrophotometric properties which distinguish it from other blood pigments such as hematin and methemoglobin. The absorption band at 623 m\(\mu\) has been utilized in previous work for the identification and estimation of the pigment. In the present study a band at 405 m\(\mu\) has been described which, because of its great intensity, permits more sensitive and more quantitative measurements.

In a series of individuals receiving pamaquine and quinine concurrently it has been possible to demonstrate a systematic production of methemalbumin. Such methemalbuminemia was not elicited by the administration of either drug alone or by pamaquine given in conjunction with quinacrine. Production of the pigment in these cases was related to a synergistic combination of the two drugs. It is important to note that the methemalbuminemia developed in the absence of frank hemolysis. The occurrence of the pigment has usually been associated with a massive hemolytic reaction though there have been instances reported, for example in pernicious anemia, where this has not been the case. The relationship of methemoglobin production to the generation of methemalbumin is not clear. Pamaquine alone gave rise to methemoglobin within the red blood cells but caused no methemalbumin to appear in the serum. The combination of pamaquine with quinine enhanced the methemoglobinemia to a moderate degree but this rise in itself certainly could not account for the generation of a totally new pigment, methemalbumin, in the serum.

The antimalarial drugs pamaquine and quinine have long been associated with disturbances in hemoglobin metabolism. Pamaquine therapy consistently gives rise to methemoglobinemia and more rarely in susceptible individuals to acute hemolytic reactions (12). There are a few cases in the literature in which hemoglobinuria has resulted from toxic doses of quinine (17). Quinine

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**Fig. 6. Serum Methemalbumin Concentrations in Patient J. K. Receiving Two Cycles of Quinine Administration (1.8 gm. per day) Superimposed upon Continuous Dosage with Pamaquine (90 mg. per day)**
has been incriminated as one of the contributing factors in the pathogenesis of blackwater fever (18).

These reported instances of acute hemolysis are uncommon. They have been detected in the rare individual case by virtue of the spectacular appearance of free hemoglobin in the urine and serum. The regular appearance of methemalbumin in all the individuals receiving combined pamaquine-quinine therapy reveals a more subtle disturbance in pigment metabolism which is consistently evoked by the drug combination.

SUMMARY AND CONCLUSIONS

A procedure is described for the estimation of methemalbumin in serum. The method makes use of an absorption band at 405 mμ and is applicable only in the absence of free hemoglobin.

Methemalbumin consistently appeared in the serum of individuals receiving quinine and pamaquine concurrently. Methemalbuminemia was not elicited by administration of either drug alone or of pamaquine in conjunction with quinacrine.

BIBLIOGRAPHY