SPECTROPHOTOMETRY OF FAIRLEY'S NEW BLOOD PIGMENT, METHEMALBUMIN

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In the course of the study of acute hemolytic anemia and hemoglobinuria during administration of sulfonamide drugs (1), the patients' sera were found to contain a pigment which, like methemoglobin and sulfhemoglobin, showed an absorption in the red region of the spectrum. The characteristic band of this pigment, around λ620 mμ and adjacent to that of methemoglobin, was not altered by cyanide, although that of methemoglobin was obliterated. Furthermore, comparisons with known mixtures and calculations based upon absorption coefficients for sulfhemoglobin, methemoglobin, and hemoglobin did not correspond with the observed spectrophotometric measurements.[2]

At this time (1940) Fairley's paper appeared describing a new pigment (3) with practically identical properties named methemalbumin (1937, 1938) (4). The pigment, which did not occur in washed, laked erythrocytes nor in urine, was readily formed by incubating laked human erythrocytes with human plasma at 37° C. for several days. Our observations were in accord with these.

Inasmuch as the sera in cases of acute hemolytic anemia contained hemoglobin, methemoglobin, and apparently methemalbumin (1), it was necessary to obtain absorption coefficients for this new pigment in order to determine its proportion in these sera. Fairley's procedure was, therefore, repeated and the resulting solutions (after centrifuging off the heavy precipitate to be described after further study) were analyzed with the Hardy recording spectrophotometer (5).

To establish the identity of the new pigment, two reagents were used: 5 per cent neutralized NaCN [3] and 3 per cent hydrogen peroxide. The cyanide was used first to obliterate the absorption caused by methemoglobin at λ630 mμ without changing absorption in this region attributed to methemalbumin. Hydrogen peroxide was next added to obliterate the absorption band of sulfhemoglobin, if present.

In Figure 1 are shown the transmission curves of the new pigment, methemalbumin, together with curves of methemoglobin, sulfhemoglobin, and oxyhemoglobin and the changes that negate the absorption in the region of interest.[4] In the case of methemalbumin after the addition of cyanide, the absorption in the red region is decreased slightly owing to the removal of small contaminating amounts of methemoglobin. The subsequent addition of hydrogen peroxide caused no further change. Table I summarizes these findings which correspond to Fairley's results.

TABLE I

Differential reactions of the blood pigments

Reagent*	Methemoglobin λ630 mμ band	Sulfhemoglobin λ620 mμ band	Methemalbumin λ620+ mμ band
Cyanide, 5 *per cent*.	obliterated	unaltered	unaltered
H_2O_2, 3 *per cent*....	obliterated	obliterated	unaltered

* Fairley also used several other reagents which disperse the band of methemoglobin but not the adjacent band of methemalbumin (3,4). Ammonium sulfide caused confusion by subsequently producing a sulfhemoglobin band at 620 mμ. Stokes' reagent, hydrazine hydrate and sodium fluoride might also be used but the results with cyanide alone are quite convincing. The major problem is differentiation from sulfhemoglobin; H_2O_2 is the only reagent known to disperse the sulfhemoglobin band but not the methemalbumin band. This test also excludes verdehemochromogens (8).

Determination of absorption coefficients

In Figure 2 is shown the transmission curve *A* of the supernatant of a plasma hemoglobin solu-

[1] Aided in part by the Dazian Foundation.

[2] Absorption coefficients for hemoglobin and methemoglobin taken from Fox and Cline (7) and for sulfhemoglobin taken from Drabkin (2).

[3] Ammonium sulfide may be used but this frequently causes confusion by subsequently producing sulfhemoglobin.

[4] For ease of comparison arbitrary concentrations have been selected. Before spectroscopy, all specimens were diluted about 10 times with M/4 phosphate buffer of pH 7.0 to prevent variations in the absorption by methemoglobin with varying pH (2).

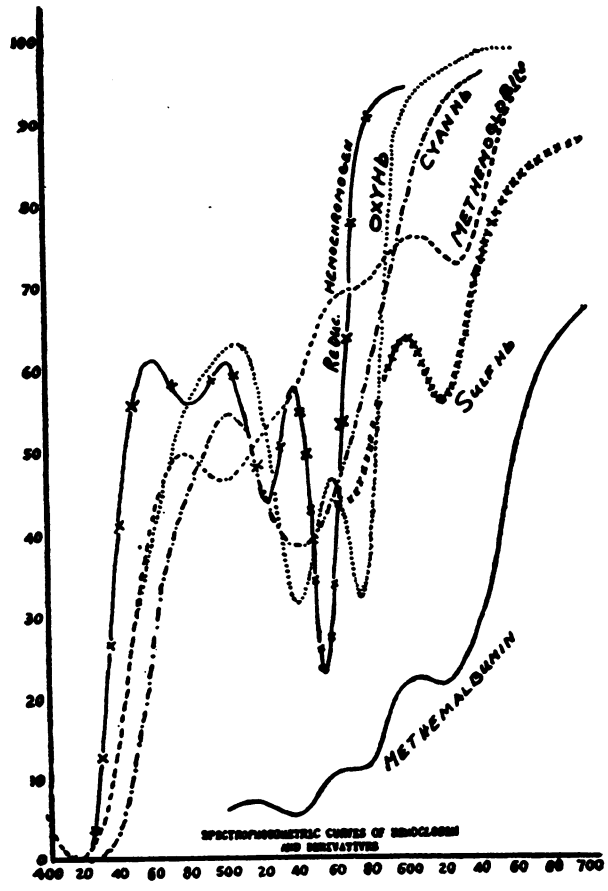

FIG. 1

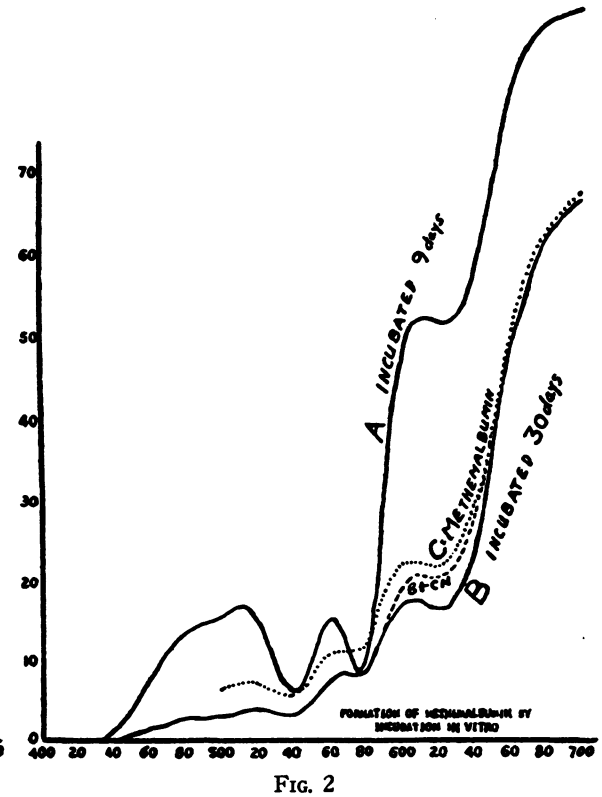

FIG. 2

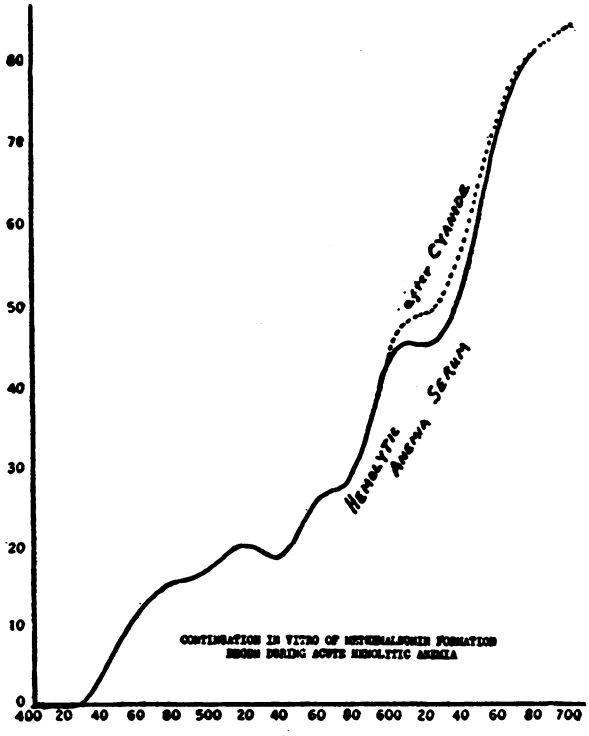

FIG. 3

tion that had not been incubated as long as the solution represented in curve *B*. The hemoglobin bands present in curve *A* had practically disappeared in curve *B*, indicating almost complete disappearance of hemoglobin from the solution. It was then assumed that the curve *B* represented only methemalbumin and methemoglobin. The total pigment was determined by iron analysis with alpha alpha' dipyridyl (6). The methemoglobin, determined independently by the change in optical density at λ630 mμ after the addition of cyanide, was then subtracted from the total to obtain the quantity of methemalbumin present. Since the absorption coefficients of methemoglobin had been determined previously on this instrument (7), it was possible to subtract its absorption from curve *B*, using this formula based on Beer's and Bouguer's laws:

$$\log \frac{1}{T} = kcd$$

d = thickness of cell in cm.
c = concentration in grams per cent
k = absorption coefficient
T = light transmission, per cent.

The calculations are summarized in Table II and the resulting curve *C* of methemalbumin is shown in Figure 2. By the same equation, the absorption coefficients for methemalbumin were computed and are listed in Table II.

TABLE II

Computation of absorption coefficients of methemalbumin

Wavelength	Experimental log 1/T	Methemoglobin to be subtracted ($0.0572 \times K_{MHb}$)	Methemalbumin computed absorption	Methemalbumin absorption coefficients
mμ				
700	0.177	0.007	0.170	0.72
660	0.323	0.027	0.296	1.25
630	0.751	0.133	0.618	2.61
620	0.788	0.125	0.663	2.79
600	0.770	0.113	0.657	2.77
580	1.059	0.137	0.922	3.89
560	1.112	0.144	0.968	4.08
540	1.456	0.222	1.234	5.2
520	1.414	0.272	1.142	4.83
500	1.502	0.319	1.183	5.0
480	1.55	0.286	1.264	5.34

In order to check the accuracy of the data, the resulting absorption coefficients were used to compute the concentrations of hemoglobin, methemoglobin, and methemalbumin in the solution containing all three represented by curve *A* in Figure 2. The method previously described (7) of solving three simultaneous equations was used. Equations were set up at the peak wavelength of each component λ540 mμ, λ630 mμ, λ620 mμ, and solved for the concentration of each component, *e.g.*, at λ630 mμ.

$$\log \frac{1}{T} = 0.133 \times \text{concentration Hb.} + 2.32 \times \text{concentration MHb.} + 2.55 \text{ concentration MHA1b.}$$

As a check on the reliability of the entire computation, the values for the concentration of each component were then substituted at 6 other wavelengths and values for the transmission of such a solution were computed. Comparison with the observed values are shown in Table III and serve to establish the relative accuracy of the coefficients of methemalbumin that were obtained.

The absorption coefficients are quite similar to those for methemoglobin and further confirm Fairley's suggestion that "the iron is held in the trivalent state, and is even more resistant to reducing agents than methemoglobin itself" (4).

It was also possible to apply these data to serum obtained from a patient who had suffered acute blood destruction during therapy with sulfonamide drugs (1). This specimen had been measured with the Hardy at the time of the hemolytic crisis.[5] It was then allowed to remain in the refrigerator for 2 months and again measured (Figure 3). The sharp hemoglobin bands originally present had almost entirely disappeared and the band in the red region that resisted both cyanide and hydrogen peroxide had increased markedly; apparently, much more methemalbumin had formed. The methemoglobin was computed by the change in density at λ630 mμ after adding cyanide. The concentrations of hemoglobin and methemalbumin

[5] Case 4 in Bibliography (1).

FIGS. 1, 2 AND 3. TRANSMISSION CURVES WITH THE HARDY RECORDING-SPECTROPHOTOMETER

Ordinates = Per cent of light transmission.
Abscissae = Wavelength of light in mμ.

TABLE III

Check on absorption coefficients of methemalbumin

Wavelength	Hemoglobin ($0.082 \times k_{Hb}$)	Methemoglobin ($0.04 \times k_{MHb}$)	Methemalbumin ($0.069 \times K_{MHalb.}$)	Calculated log $1/T$	Experimental log $1/T$
mμ	*gram per cent*	*gram per cent*	*gram per cent*		
600	0.046	0.078	0.191	0.316	0.312
580	0.585	0.076	0.27	0.951	0.969
560	0.476	0.101	0.282	0.859	0.824
520	0.338	0.19	0.333	0.861	0.824
500	0.271	0.223	0.345	0.839	0.817
480	0.340	0.200	0.368	0.908	0.88

were computed by two equations [6] at λ620 mμ and λ540 mμ. Using the concentrations obtained, values for log $1/T$ at 8 other wavelengths were computed and are compared with the experimental values (Table IV).

TABLE IV

Formation of methemalbumin at 10° C. after intravascular hemolysis

Wavelength	Experimental log $1/T$	Calculated log $1/T$
mμ		
700	0.077	0.078
660	0.140	0.142
630	0.328	0.329
620	**0.344**	**0.344**
600	0.359	0.342
580	0.538	0.544
560	0.594	0.552
540	**0.727**	**0.727**
520	0.699	0.665
500	0.770	0.752
480	0.809	0.724

Values calculated on basis of:
Hemoglobin = 0.01144 gram per cent.
Methemoglobin = 0.0244 gram per cent.
Methemalbumin = 0.1036 gram per cent.

There are some discrepancies between Table III and Table IV; it is recognized that the values for the absorption coefficients in the green are the least reliable and the discrepancies are most marked in this region. Furthermore, the serum used in Table IV was that of a case of acute hemolytic anemia, and did not give an absolutely clear solution for study.

[6] In view of the fact that the method of simultaneous equations is open to criticism when more than two pigments are present, the concentration of methemalbumin was also calculated more directly by using the residual absorption at λ620 mμ after cyanide and the coefficient for methemalbumin 2.79 (Table II). After subtracting for the absorption of cyanmethemoglobin, the concentration of methemalbumin found is 0.108 gram per cent. This is 4 per cent more than the result 0.104 gram per cent by the method of simultaneous equations. The difference is presumably due to the fact that, in the residual absorption method, no allowance can be made for the small amount of absorption at this wavelength from the hemoglobin present.

SUMMARY AND CONCLUSIONS

Fairley's new blood pigment methemalbumin was measured in the visible range with the recording spectrophotometer and preliminary values of its absorption coefficients were obtained.

The characteristic absorption curve of this new pigment is compared with that of hemoglobin, methemoglobin, and sulfhemoglobin.

The data obtained are utilized in measuring the formation of methemalbumin, *in vivo* and *in vitro*.

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