CCLXXXIV. THE ULTRAVIOLET SPECTRUM OF HAEMOGLOBIN AND ITS DERIVATIVES.

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SINCE Soret [1883] described an ultraviolet absorption band in three of the haemoglobin series, numerous investigations have been carried out on the ultraviolet absorption spectrum of the blood pigment and its immediate derivatives. The haemoglobin compounds were investigated by Gamgee [1895] who pointed out that there was a specific ultraviolet band common to oxyhaemoglobin, reduced haemoglobin, carbon monoxide haemoglobin, nitrous oxide haemoglobin, haemochromogens, haematin hydrochloride, methaemoglobin and haematoporphyrin which was absent from the spectra of bilirubin, hydrobilirubin and urobilin. Kobert [1900] investigated several haemoglobin compounds and demonstrated the existence of a band in the ultraviolet which was common to the spectra of oxyhaemoglobin, carbon monoxide haemoglobin, alkaline methaemoglobin and cyanide haemoglobin and concluded that there was a structure common to these compounds which possessed this band.

Subsequent investigations have fixed the position of the band as follows:

Investigator	Position of the ultraviolet band (Å.)
Rost <i>et al.</i> [1909]	4100
Lewin <i>et al.</i> [1907]	4150
Newcomer [1919]	4140
Schumm [1921]	4135-4140
Suhrmann & Kollath [1927]	4150
Hicks & Holden [1929]	4115
Berg & Schwarzscher [1930]	4134
Davis & Sheard [1934]	4180

Friedli [1926] investigated mesoporphyrin and haematoporphyrin and located a band common to both at 4000 Å. Triebs [1932] has made extensive investigations concerning spectra of fourteen porphyrins, including some made from haem, and has established the position of a common band at 4000 Å. Studies on bile and purified bile pigment yield a spectrum different from that of the haem and porphyrin pigments. Mann *et al.* [1926] studied the bile pigment, and found maximum absorption in the region of 4400 Å. Davis & Sheard [1934] published ultraviolet spectrum data of bile pigment in pure state. Their curve shows a heavy absorption from 5000 to 3600 Å. with a maximum at 4500-4600 Å.

The following investigations were undertaken in order to correlate the ultraviolet absorption spectra of haemoglobin compounds and derivatives with their chemical structures. These included a study of a wide variety of haemoglobin derivatives. Part of this work had been done previously, but the separate entities had been examined by different investigators with different apparatus and methods. In no instance could reference be found to a study of this kind having been carried out on the haemoglobin of a single species by one investigator and the same technique.

Methods. The instrument employed was a Bellingham and Stanley quartz spectrograph. The ultraviolet spectrum from 2100 to 5000 Å. was extended over 18 cm. upon the negative. The absorption measurements were determined by the use of a photoelectric recording photometer developed in this laboratory by Macallum & Coates [1933].

Recrystallized horse haemoglobin was used for the experiments. It was prepared by the usual methods for obtaining a purified haemoglobin solution and crystallized in an electrodialysis apparatus after a 4-hour period dialysis. It was recrystallized in the dialyser twice from a solution of cold water containing a small amount of bicarbonate. The crystals prepared in this way were dried in air and kept for periods of weeks without showing appreciable change to methaemoglobin.

The pure haem was prepared from solutions of the purified haemoglobin by a modification of the Schälfeff method. It was recrystallized twice by dissolving it in chloroform solution containing a small amount of pyridine and allowing it to reprecipitate slowly after the cautious addition of acid. The recrystallization was repeated twice and the crystals thoroughly dried.

Crystalline haematoporphyrin was prepared from the recrystallized haemin by dissolving it in glacial acetic acid and saturating the solution with dry hydrogen bromide gas at 10°. It required 3–4 days to dissolve 5 g. of haemin in 50 ml. of glacial acetic acid by this method. Reprecipitation of the haematoporphyrin was brought about by the addition of sodium acetate. The ferric oxide was removed by sodium hydroxide. The haematoporphyrin was repeatedly reprecipitated with acid and finally crystallized *in vacuo* over sulphuric acid. Recrystallization was made from 10% hydrochloric acid and the crystals were "air dried".

The oxyhaemoglobin solutions were prepared from the dried horse haemoglobin solutions by weighing out the required amount to give a 0.2% solution, and dissolving it in distilled water with sufficient shaking to aerate the mixture and convert all the haemoglobin into oxyhaemoglobin. The spectrograms were taken at once.

The reduced haemoglobin solutions were made in the same way from the dried crystalline haemoglobin; to each 100 ml. of these solutions there were added 10 ml. of 10% sodium hydrosulphite in 5% ammonia in order to bring about complete reduction of all the haemoglobin. The hand spectroscope was used to detect the complete transformation of oxy- to reduced haemoglobin.

Neutral methaemoglobin was prepared by adding 10 drops of 10% potassium ferricyanide to 100 ml. of 0.2% oxyhaemoglobin. Alkaline methaemoglobin was similarly prepared except that the 0.2% oxyhaemoglobin had been prepared in 0.01 *N* ammonia. The degree of the change from oxyhaemoglobin to methaemoglobin was measured by the hand spectroscope.

The haemoglobin was converted into carboxyhaemoglobin by pure carbon monoxide.

The stock alkaline haematin was prepared by dissolving 13 mg. of dried haemin in 25 ml. of 0.5 *N* NaOH. For spectrographic purposes 0.25 ml. of the stock solution was diluted with 4.75 ml. of water. An alcoholic solution of alkaline haematin was made from the stock solution by adding 0.25 ml. to 4.75 ml. of absolute alcohol. The stock solution of acid haematin was made up by dissolving 13 mg. of the dried haemin in 10.0 ml. of 0.5 *N* NaOH. 0.5% gum arabic was added to this solution as a protective colloid in order to prevent precipitation of the pigment when 1.0 ml. of concentrated hydrochloric acid was added. 0.3 ml. of this stock was diluted to 5.0 ml. for spectrographic purposes. Acid haematin in alcohol solution was made by adding 0.3 ml. of the stock solution to 4.7 ml. of absolute alcohol.

Pyridine haemochromogen was made by adding small amounts of pyridine and sodium hydrosulphite to alkaline haematin solution. Piperidine haemochromogen was similarly prepared. A preparation of alkaline haematin and pyridine without reduction was also made and the spectrogram taken.

The bilirubin obtained for this work was the preparation used for intravenous injection and of a fairly high purity. It was purified further and crystallized from chloroform. It was dissolved in 0.01 *N* ammonia for spectrographic work. A solution was also made in a 50:50 alcohol-acetone mixture to which a small amount of 0.1 *N* NaOH had been added.

The globin was prepared from horse blood by the method of Hill & Holden [1926]. For spectrographic purposes a 10% solution was made. To prepare a denatured globin solution, the native protein was heated with 0.5*N* hydrochloric acid for half an hour at 60°.

Fig. 1. $\times - \times$ Alkaline methaemoglobin. — Neutral methaemoglobin. $---$ Oxyhaemoglobin. $o - o$ Carboxyhaemoglobin.

Fig. 2. — Native globin. $---$ Denatured globin. $\bullet - \bullet$ Haemoglobin reduced in a tonometer. — Haemoglobin reduced by hydrosulphite.

Observations. Oxyhaemoglobin, reduced haemoglobin and carboxyhaemoglobin all give spectrum curves which are quite similar. All are characterized by the intense main band in the region of 4100 Å, while lesser absorption is shown at 3700 Å. There is another minor band at 3400 Å, which appears to be characteristic

of the haemoglobin molecule. There is also a band at 2750 Å. Methaemoglobin in neutral and alkaline solution shows the typical absorption at 4100 Å. and the

Fig. 3. ——— Acid haematin in alcohol. - - - - Acid haematin in water.
 × — × Alkaline haematin in alcohol. o — o Alkaline haematin in water.

Fig. 4. - - - - - Haematoporphyrin. ——— Pyridine haemochromogen.
 - - - - Piperidine haemochromogen. × — × Alkaline haematin (reduced).

haematin solutions show the same general type of curve. The haemochromogens have a very distinct band at 4100 Å. and a heavy general absorption below 3400 Å. The haematoporphyrin has a similar spectrum but the general absorption

is greater. The bilirubin spectrum is entirely different. The absorption is very high from 5000 to about 4000 Å., while there are no areas of selective absorption. The globin, both native and denatured, gives a band at 2750 Å.

Fig. 5. ——— Bilirubin in alcohol-acetone solution. - - - Bilirubin in dilute ammonia.

DISCUSSION.

All the haemoglobin compounds and derivatives display a similar selective absorption in the ultraviolet. The "great" absorption band at 4100 Å. is found in the same general form in oxy-, reduced, carboxy-, and met-haemoglobin. The exact centre of the band is difficult to locate, since its determination depends on the selection of the boundaries of the band. The bands of oxyhaemoglobin and CO-haemoglobin are very similar, oxyhaemoglobin showing more general absorption. All have a small peak at 2780 Å. and a rather poorly defined band centred at 3400 Å. At 2700 Å. there is another band which has been shown to be due to the globin fraction [Lewis, 1917]. The curves for purified globin, both native and denatured, show this characteristic band in the region of 2700 Å. Methaemoglobin in alkaline solution has the same general spectrum as oxyhaemoglobin and CO-haemoglobin. In neutral solution it exhibits greater general absorption but the same type of curve. Reduced haemoglobin has the same general form except for increased general absorption, due to the presence of sodium hydrosulphite. This reagent has such a high absorption in the ultraviolet that a cut-off occurs about 3300 Å. The reduction of the oxyhaemoglobin by shaking *in vacuo* in a tonometer was also tried as a means of making reduced haemoglobin, but this did not give very good practical results since the haemoglobin had a tendency to precipitate, thus rendering the solution unsuitable for satisfactory observations.

Alkaline haematin solutions are very similar to haemoglobin. There is a typical selective band centred at 4000 Å. which represents a slight shift towards the shorter wave-lengths. The general outline of the curve is unchanged. The small sharp peak at 3650 Å. also shifts towards the violet, and a broad band, which in centred at 3400 Å. in haemoglobin, appears at 3350 Å. The degree of absorption is about the same. The alkaline haematin in aqueous solutions gives a less clearly

defined curve than the one in alcoholic solution. In aqueous solutions, the band centred at 4000 Å. spreads over the adjacent wave-lengths and includes the small curve normally present at 3650 Å. The other band is shifted considerably to the violet and is centred about 3200 Å. Since the haematin compounds are protein-free and have a spectrum similar to haemoglobin it follows that the globin fraction is not responsible for any of the observed bands in the haemoglobin spectrum with the exception of the one at 2700 Å. Acid haematin is similar to alkaline haematin, although in aqueous solution the band at 4000 Å. is broad and not well defined. Dissolved in alcohol its spectrum resembles closely the other haemoglobin spectra. Haemochromogens are very similar to the haemoglobin compounds, the band being centred about 4150 Å. This is in contrast with the haematin bands which are centred about 4000 Å. The physical state has an influence in the spectrum, apparently association of the molecules as in acid haematin causes shifting toward the shorter wave-lengths.

Haematoporphyrin, the iron-free pigment, has a spectrum which resembles in general form that of the other haemoglobin derivatives. This would indicate that the iron is not concerned in the spectrum. It has been postulated [Hicks & Holden, 1932] that the presence of the intense band at 4100 Å. is due to the fully saturated iron valence, but the intense band in the iron-free pigment seems to show such a hypothesis to be incorrect. The valence of the iron is apparently not a factor even in the compounds where iron exists in different states. This is shown by the comparison of the spectra of haemoglobin and methaemoglobin. There appears to be no essential difference in the general form of the curve and the intense band at 4100 Å. is present in both. Small differences in degree of absorption do not indicate much difference in chemical structure.

The bile pigment spectrum is different from that of haemoglobin. There is a region of heavy absorption from 5000 to 4000 Å. In alcohol and acetone there is a slight curve at 3400 Å., which does not appear in the ammoniacal solution. In the haemoglobin compounds and derivatives there must be a common feature which is responsible for the characteristic ultraviolet spectrum. In the bile pigment this structure or molecular arrangement is so changed that the spectrum is radically different. It has been shown that the haem radical is the common factor which apparently gives the characteristic spectrum which is not fundamentally altered by union with the globin, as in haemoglobin. Nor is it influenced by the valence of the iron as there is little difference between the spectra of oxyhaemoglobin and methaemoglobin and it is not altered radically by change in physical state, as in acid haematin in water solution. In the bile pigment, however, which is derived from haemoglobin by chemical rearrangement, the haem nucleus is no longer present. The porphyrin ring system made up of four substituted pyrrole nuclei is rearranged and altered to give four pyrrole nuclei, changed in substitution slightly and arranged in a straight chain. A study of the ultraviolet spectra leads to the conclusion that the absorption band at 4100 Å. in haem compounds is due to the porphyrin ring arrangement and that the change of the ring to a straight chain involved in bile pigment formation from blood pigment is responsible for the lack of the specific absorption band at 4100 Å. in bile pigments.

SUMMARY.

1. Ultraviolet absorption curves have been described for oxyhaemoglobin, carboxyhaemoglobin, reduced haemoglobin, neutral and alkaline methaemoglobin, alkaline and acid haematin in aqueous and organic solvents, reduced alkaline haematin, pyridine and piperidine haemochromogens, haematoporphyrin.

phyrin, bilirubin in ammoniacal and organic solvents and also for native and denatured horse globin.

2. It has been shown that oxyhaemoglobin and its compounds and derivatives have a main ultraviolet band in the region of 4000 Å., a small band at 3750 Å. and a broad shallow band in the region of 3400 Å.

3. The band at 2700 Å. found in the haemoglobin compounds which still contain globin is due to the globin part of the molecule. Curves are presented for native and denatured globin solutions and there is only the band in the region of 2700 Å. There is very little spectral difference between the native and denatured globin.

4. Haematin compounds in organic solvents have greater ultraviolet absorption and the peak of the band is shifted towards the violet.

5. Bilirubin has a broad absorption band extending from 5000 Å. to about 4100 Å. There is no band at 3400 Å. The spectrum is quite dissimilar from that of the haemoglobin derivatives.

6. There is no basis for the theory that the intense band at 4000 Å. in haemoglobin compounds is related to the valence of the iron. Haematoporphyrin, an iron-free pigment, shows the band at 4000 Å.

7. It is believed that sufficient evidence is presented to show that the main violet absorption band in the region of 4000 Å. only occurs when the porphyrin pyrrole ring system is present and that it is absent when this ring system becomes a straight chain as in bilirubin.

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