(6) Fauré-Fremiet, 'Comptes Rendus Soc. Biol.,' vol. 85, p. 1051 (1923).
(7) Herlant, 'Archives de Biol.,' p. 519 (1920).
(8) Meyerhof, 'Bioch. Zeit.,' vol. 35, pp. 246, 280, 316 (1911).
(9) von Mollendorff, 'Ergebnisse d. Physiol.,' vol. 18, p. 142 (1920).
(10) Needham and Needham, 'Roy. Soc. Proc.,' B, vol. 98, p. 259 (1925).
(11) Reiss, 'Archives de Physique Biologique,' vol. 4, p. 35 (1924).
(12) Shearer, 'Roy. Soc. Proc.,' B, vol. 93, p. 213 (1922).
(13) Spaulding, 'Biol. Bull.,' vol. 6, p. 224 (1924).
(14) Tschachotin, 'Comptes Rendus Soc. Biol.,' vol. 84, p. 85 (1921).
(15) Vlès, 'Archives de Physique Biologique,' vol. 4, p. 1 (1924).
(16) Vlès, Reiss and Vellinger, 'Comptes Rendus Acad. Sci.,' vol. 179, p. 349 (1924).
(17) Warburg, 'Zeit. Physiol. Chem.,' vol. 57, p. 1 (1908).
(18) Warburg and Meyerhof, 'Pflüger's Archiv,' vol. 148 (1912).
(19) Schmidtmann, 'Bioch. Zeit.,' vol. 150, p. 253 (1924), and 'Klin. Woch.,' vol. 4, p. 759 (1925).

Chlorocruorin: A Pigment allied to Hæmoglobin.

By H. Munro Fox, Fellow of Gonville and Caius College, Cambridge, and Balfour Student in the University.

(Communicated by Prof. J. Stanley Gardiner, F.R.S.—Received October 14, 1925.)

[Plate 1.]

The name chlorocruorin was given by Ray Lankester (1867) to a pigment dissolved in the blood plasma of Sabellid, Serpulid and Chlorhæmid polychæte worms. Lankester showed that chlorocruorin is related to hæmoglobin, since he obtained a hæmochromogen from it. The pigment is burgundy-red when in concentrated solution, green when dilute. It will be shown below that while chlorocruorin is constructed on the same plan as hæmoglobin, the differences between the two are very much greater than the differences between specific hæmoglobins.

Chlorocruorin exists in an oxidised and a reduced state which are of almost the same colour. The reduced form in dilute solution is a slightly yellower green than the oxidised. The oxidised differs, however, spectroscopically from the reduced form in the same manner as oxyhæmoglobin differs from hæmoglobin. Oxychlorocruorin has two bands in the red-green part of the visible spectrum and reduced chlorocruorin one broader one. These bands are situated to the red of the corresponding hæmoglobin bands. Chlorocruorin like hæmoglobin, can act as a peroxidase (Lankester, 1870).

The present communication deals solely with the chemical aspects of chlorocruorin. The pigment will be treated from the biological standpoint in a following paper.

The work reported below was done in 1924 and 1925 partly at the Marine Biological Station, Roscoff, Brittany, and partly in the Zoological Laboratory, Cambridge. The ultra-violet spectrophotometry was carried out at Strasburg in Prof. Vlès' Laboratory of Biophysics. *Spirographis Spallanzanii* from Roscoff was studied, and *Branchiomma vesiculosum*, *Sabella pavonina*, *Myxicola infundibulum*, and *Pomatoceros triqueter* from Plymouth. At Cambridge living animals from Roscoff and from Plymouth were used, and at Strasburg *Spirographis* blood, sent from Roscoff.

I wish to thank Prof. J. Barcroft and Prof. Fred Vlès most sincerely for their valuable advice and help. I am grateful to the Directors of the Roscoff and Plymouth Marine Biological Stations for an abundant supply of material, and to Messrs. Keilin, Anson, and Mirsky for their continual interest. The expenses of the research were in part defrayed by Royal Society Grants.

Oxygen Affinity of Chlorocruorin.

In a previous communication (Fox, 1924) it was shown that oxychlorocruorin can be reduced both by a vacuum and by living tissues.* It may function, therefore, as a respiratory pigment, in the sense of absorbing a greater amount of oxygen than does water and giving up this oxygen to tissues.

The dissociation curve of oxychlorocruorin has not yet been made, but it can be shown qualitatively that chlorocruorin has a lesser affinity for oxygen than has hæmoglobin. In a mixed solution of oxychlorocruorin and oxyhæmoglobin the α-bands of each are sufficiently far apart to be seen separately (see p. 203 below). A mixture is made in which the intensities of the two α-bands are the same, and this mixture is reduced by sealing it up with a piece of living tissue. The oxychlorocruorin α-band is seen to vanish before the oxyhæmoglobin α-band. Further, if separate mixtures are made of polychæte blood containing oxychlorocruorin with (1) frog's blood, (2) human blood, and

* The inner layers of the mucous tube of *Myxicola* are coloured green by oxychlorocruorin. The latter, which is contained not only in the blood of Sabellids, but in much maller amount in the cœlomic fluid, probably exudes through the nephridia. The oxychlorocruorin in the mucus cannot be reduced by a vacuum nor by living tissue, even when the mucus is cut into thin slices. It can only be reduced by hydrosulphite when the mucus is ground in a mortar with this reducer. This suggests a colloidal protection effect, the oxychlorocruorin being protected by the mucin. Wurmser (1921) demonstrated a similar protective effect of colloids against the photo-oxidation of chlorophyll.

(3) earthworm's blood, the time interval between the disappearance of the oxychlorocruorin α-band and that of the oxyhæmoglobin is different in each case. It is shortest in the case of the frog, longer with human blood, and longest for the earthworm. This is the order of the oxygen affinities of the three hæmoglobins in question.

The oxygen affinity of chlorocruorin is affected in the same way as that of hæmoglobin by the H-ion concentration. This could not be deduced *a priori*, for Redfield and Hurd (1925) have shown that, while the oxygen affinity of the hæmocyanin of *Loligo* is decreased, that of *Limulus* hæmocyanin is increased by CO_2.

In order to study the *p*H effect, oxychlorocruorin was first put into a series of buffers to find within what limits of *p*H it is stable. Below *p*H 7·0 and above *p*H 8·5 the intensity of the α-band fades, presumably owing to its changing into the derivative corresponding to hæmatin, although the first fading immediately below *p*H 7·0 must occur because at low *p*H values the chlorocruorin is incompletely saturated with oxygen. The wave-length of the band is uninfluenced by [H]. Equal concentrations of oxychlorocruorin (from *Spirographis* blood) were next put into buffers (1) of *p*H 7·0 and (2) of *p*H 8·5. These were exposed to varying pressures of oxygen in tonometers, and the pigment examined with a reversion spectrometer (Hartridge, 1912 and 1922) while inside the tonometer. The tonometer was first evacuated so that the oxychlorocruorin α-band vanished. Measured quantities of air were then successively admitted until the oxychlorocruorin α-band again acquired the same intensity as it has in atmospheric air. At *p*H 8·5 this was attained with 9 mm. oxygen pressure; at *p*H 7·0 between $\frac{3}{4}$ and 1 atmosphere of oxygen pressure was required. The temperature was 16°.

Since chlorocruorin has a lower oxygen affinity than hæmoglobin, and its dissociation curve is correspondingly shifted to the right, the question next arose as to whether chlorocruorin is fully saturated with oxygen at the atmospheric tension of the gas or whether a higher tension is required for the curve to reach its top and flatten out. Oxygen was bubbled through *Branchiomma* and *Sabella* bloods diluted with tap water, and it was found in both cases that the intensity of the α-band of oxychlorocruorin was increased by this procedure; the increase was more marked for *Branchiomma* than for *Sabella*. The experiment was then repeated with blood diluted with a buffer solution of *p*H 8·5, the upper *p*H limit at which chlorocruorin is stable. The result of bubbling oxygen through was the same as before. Thus at the highest possible *p*H, and consequently greatest oxygen affinity, oxychlorocruorin in these animals

is still unsaturated with oxygen at the atmospheric tension. Bubbling oxygen through diluted *Spirographis* blood again causes an increase in intensity of the α-band, but in this case the change is very slight. For *Myxicola*, however, oxygen causes no appreciable darkening of the band, so that the oxychlorocruorin in this case is saturated.

Just as there are different hæmoglobins, there exist then a series of specific chlorocruorins differing from one another in their affinities for oxygen. The four forms studied can be arranged in the following order of oxygen affinities, the first-named having the greatest and the last the smallest: *Myxicola*, *Spirographis*, *Sabella*, *Branchiomma*.

Total Oxygen Capacity of Spirographis Blood.

The quantity of oxygen bound by chlorocruorin in the blood of *Spirographis* was determined with a Barcroft (1914) micro-differential manometer.* This was possible since oxychlorocruorin, like oxyhæmoglobin, gives up its oxygen on the addition of ferricyanide.

Each determination was made with 0·1 c.c. of blood. Undiluted *Spirographis* blood was obtained by inserting a capillary pipette into each of the two blood vessels at the base of the branchiæ. This situation was chosen owing to the absence of any cœlomic cavity here. From eight individuals 0·1 c.c. of blood can thus be extracted. The value for the total oxygen capacity given below is higher than that announced in my preliminary communication (Fox, 1924). The reason is that previously the blood had been taken from the body of the worm, where, in spite of the greatest precautions, some admixture of cœlomic fluid is unavoidable.

Determinations were first made of the oxygen capacity of my own blood. Seven estimations gave 20·4, 18·3, 19·0, 20·2, 19·3, 19·0, and 18·6 c.mm. O_2 at N.T.P. from 100 c.mm. blood. The average of these values is 19·3.

The results of five estimations of *Spirographis* blood were 8·1, 9·2, 9·1, 10·0 and 9·2 vol. per cent. at N.T.P., the average being 9·1. The blood thus binds 15 times the quantity of oxygen dissolved in sea water (barometer during experiments, 759 mm.).

It has been shown above that the oxychlorocruorin of *Spirographis* is not saturated with oxygen at the atmospheric partial pressure. Further determinations were accordingly made with *Spirographis* blood, this time saturated with oxygen (barometer, 760 mm.). Under these conditions the total oxygen

* The apparatus was calibrated by liberating in one bottle a known quantity of oxygen from H_2O_2.

capacity was found, in three estimations, to be 10·6, 10·1 and 9·8 vol. per cent. at N.T.P., with an average of 10·2. This means that at atmospheric oxygen pressure *Spirographis* oxychlorocruorin is 10·2/9·1 = 90 per cent. saturated.

For comparison, determinations were next made of the oxygen capacity of the blood of a polychæte containing hæmoglobin. *Arenicola* was chosen. It is possible here to extract 0·1 c.c. blood from one worm, and each determination was made with a single individual. The values obtained differed widely from one another, which means that the hæmoglobin content of the blood is very variable. Barcroft (1924) had already noted this in colorimetric estimations. The oxygen capacities of six individuals were found to be 8·2, 5·7, 8·7, 6·8, 7·4, and 6·8 c.mm. O_2 in 100 c.mm. blood. These values are seen to be lower than those for *Spirographis*.

The oxygen capacities recorded above for the bloods of *Spirographis* (9·1, vol. per cent.) and of *Arenicola* (5·7 to 8·7 vol. per cent.) are the highest known among invertebrates. The highest value for blood containing hæmocyanin is 4 to 5 vol. per cent. for *Octopus* (Winterstein, 1909; Dhéré, 1919).

Absorption Spectrum of Oxychlorocruorin.

It has been pointed out above that the absorption spectra of oxychlorocruorin and chlorocruorin resemble those of oxyhæmoglobin and hæmoglobin.

Fig. 1 shows the appearance of the spectrum of oxychlorocruorin in the visible region. The α-band is much more intense than the β, and both are

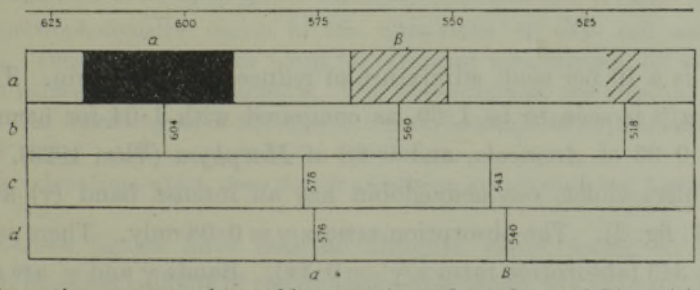

Fig. 1.—Absorption spectra of oxychlorocruorin and oxyhæmoglobin. (*a*) Oxychlorocruorin of *Spirographis*, general aspect of spectrum. (*b*) Axes of bands of (*a*). (*c* and *d*) Axes of bands of oxyhæmoglobin of the horse (*c*) and *Arenicola* (*d*). (*a* and *b*): spectrometer readings; (*c* and *d*) spectrophotometric determinations by Vlès (1923, p. 7).

shifted to the red of the corresponding oxyhæmoglobin bands. This shift is much greater than the differences between specific hæmoglobins (*see* fig. 1,

c and *d*). The axis of α is at 604 μμ, of β at 560 (spectrometer measurements*). Oxychlorocruorin has a third very light band at 518. This has no representative in oxyhæmoglobin. Fig. 2 (*b*) gives a photograph of the spectrum (the band at 518 here appearing exaggerated).

Fig. 3 (*a*) gives the spectrophotometric curve in the visible of oxychlorocruorin. The measurements were made at atmospheric oxygen tension, so

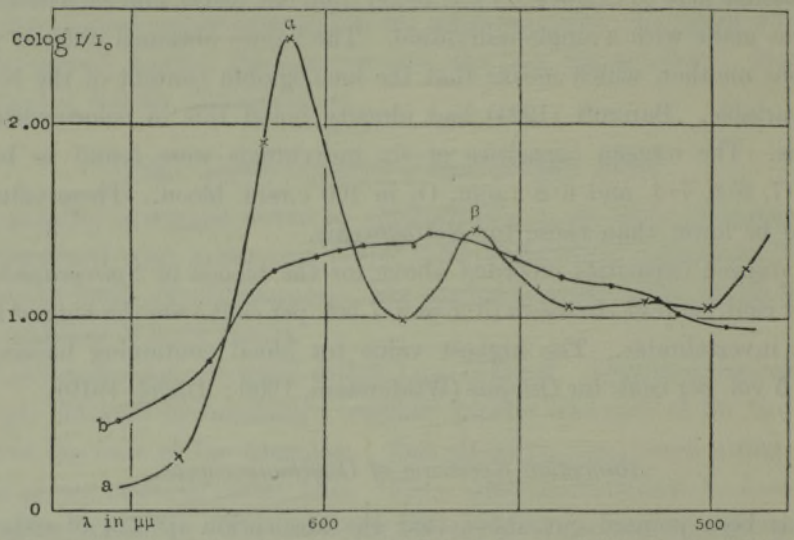

FIG. 3.—Spectrophotometric curves in the visible of (*a*) oxychlorocruorin and (*b*) chlorocruorin of *Spirographis*. The oxychlorocruorin solution is saturated with air. After the oxychlorocruorin had been determined it was reduced in the trough with hydrosulphite so that the molecular concentrations are the same for the two curves.

The values from which these curves are drawn are given in the Appendix (p. 217).

that there is a 10 per cent. admixture of reduced chlorocruorin. The absorption ratio α/β is seen to be 1·69, as compared with 1·04 for hæmoglobin of the horse, 0·95 of *Arenicola*, and 0·88 of *Marphysa* (Vlès, 1923).

In the ultra-violet, oxyhæmoglobin has an intense band (γ) at 416 (see (Vlès, 1921, fig. 2). The absorption ratio α/γ is 0·08 only. There is a smaller band γ′ at 343 (absorption ratio $\alpha/\gamma' = 0{\cdot}34$). Bands γ and γ′ are due to the pyrrol nucleus. Finally, there is a band, φ, at 275, due to the protein (globin).

The photograph fig. 2 (*c*) (Plate 1), shows the γ-band of oxychlorocruorin.

* The wave-lengths of the axes of bands determined with the spectrophotometer (fig. 3 and Table 1), the ordinary spectrometer (fig. 1), and Hartridge's reversion spectrometer (Table II) differ from one another. This is to be expected from the differences in methods used (Hartridge, 1913, and Vlès, 1921, p. 15).

The spectrophotometric* curves of fig. 4 show the γ-, γ'-, and ϕ-bands of oxychlorocruorin saturated (*a*) with oxygen and (*b*) with air. Solution (*b*) consequently contains a 10 per cent. admixture of reduced chlorocruorin. The absorption ratio γ/γ' appears to be 1·5 from curve (*a*) or 2·3 from curve (*b*).

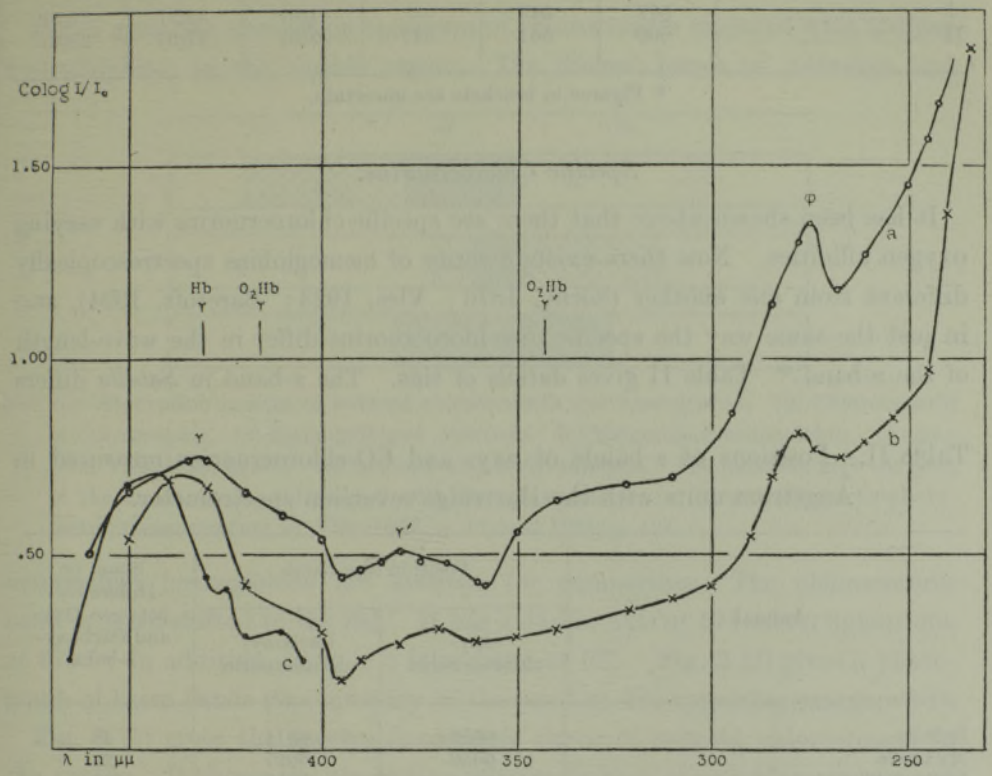

FIG. 4.—Spectrophotometric curves in the ultra-violet of oxy- and reduced chlorocruorin. Diluted *Spirographis* blood (*a*) saturated with oxygen, (*b*) saturated with air, (*c*) reduced with hydrosulphite. The concentrations are identical in the three cases. The wave-lengths of the axes of the oxyhæmoglobin (O_2Hb) and hæmoglobin (Hb) γ- and γ'-bands are inserted for comparison.

The values from which these curves are drawn are given in the Appendix (p. 218).

Although these figures have here no absolute value, owing to the general absorption by other colloids of the diluted whole blood, yet the ratio is evidently lower than that for oxyhæmoglobin. In the latter case the γ-band is considerably more intense relatively to the γ', the ratio being 4·1 (Vlès, 1921, p. 9). Table I compares the positions of the axes with those of the corresponding hæmoglobin bands. As in the case of α and β, γ and γ' are shifted to the red in oxychlorocruorin as compared with oxyhæmoglobin.

* For the ultra-violet spectrophotometry the technique of Prof. Vlès (1925) was used.

Table I.—Spectrophotometric axes of the bands of (I) oxyhæmoglobin of the horse (Vlès, 1921, p. 7), and (II) oxychlorocruorin of *Spirographis*.

Designation of Band	α	β	—	γ	γ'	ϕ
I	578	543	—	41(6)*	34(3)	27(5)
II	609	561	517	43(2)	37(9)	27(5)

* Figures in brackets are uncertain.

Specific Chlorocruorins.

It has been shown above that there are specific chlorocruorins with varying oxygen affinities. Now there exists a series of hæmoglobins spectroscopically different from one another (Sorby, 1876; Vlès, 1923; Barcroft, 1924), and in just the same way the specific oxychlorocruorins differ in the wave-length of the α-band.* Table II gives details of this. The α-band in *Sabella* differs

Table II.—Positions of α-bands of oxy- and CO-chlorocruorins measured in Ångstrom units with the Hartridge reversion spectrometer.

Animal	Position of α-band		Span, or Difference between Oxy- and Carboxy-α-band
	Oxy-chlorocruorin	Carboxy-chlorocruorin	
Sabella	6059	6011	48
Myxicola	6056	6027	29
Spirographis	6048	6016	32
Branchiomma	6029	5987	42
Pomatoceros	6025	6008	17
	Oxy-hæmoglobin	Carboxy-hæmoglobin	
Man	5763	5700	63
Planorbis	5745	5695	50

* MacMunn (1885) described specific chlorocruorins of Serpulids, differing in colour, some red, some brown, others green. In my experience chlorocruorin is always of the same colour, red concentrated, green dilute, although the tint seems to vary slightly from one species to another. The diluted blood of *Sabella*, for instance, is of a more yellowish green than that of *Spirographis*. This, however, may be due to an additional pigment. MacMunn's assertion can perhaps be explained by such additional pigments in the tissues of Serpulids. When *Pomatoceros* is cut up in fresh water a pink solution is formed. In addition to oxychlorocruorin there is present a substance with two bands at 539 and 499 (reversion spectrometer). Sodium hydrosulphite and living tissues both reduce this

from that in *Pomatoceros* by 34 Ångstrom units. This is a greater variation than that between two of the most divergent hæmoglobins, human and *Planorbis*, which differ by 18 Å only.

Absorption Spectrum of Reduced Chlorocruorin.

Fig. 5 gives the absorption spectrum of chlorocruorin (reduced with sodium hydrosulphite) in the visible region. The Stokes' bands of *Arenicola* and

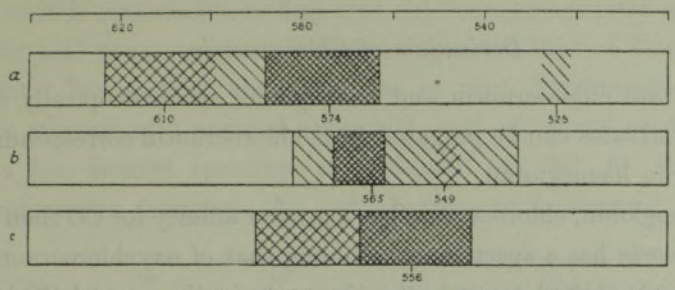

Fig. 5.—Absorption spectra of reduced chlorocruorin and hæmoglobin. (*a*) Chlorocruorin of *Spirographis*. (*b*) Hæmoglobin of *Arenicola*. (*c*) Mammalian hæmoglobin. (*b* and *c* from Vlès, 1923, fig. 5.) All reduced with hydrosulphite. The numbers give the axes of the bands, in (*a*) determined with the spectrometer, in (*b*) and (*c*) spectrophotometric measurements by Vlès (1923, p. 17, and 1921, p. 12).

mammalian hæmoglobins are inserted for comparison. The chlorocruorin band is again shifted to the red. It has a darker axis at 574 and a lighter one at 610.* In addition, there is a faint band at 525. Fig. 2 (*d*) gives a photograph of these bands (the intensity of the band at 525 appearing exaggerated).

Fig. 3 (*b*) gives the spectrophotometric curve of reduced chlorocruorin in the visible. It shows the absorption relative to that of oxychlorocruorin and explains the appearances seen in figs. 5 (*a*) and 2 (*d*). There is, in reality, a main summit at 572 with a buttress on either side. The Stokes' band of mammalian hæmoglobin has a simple summit (Vlès, 1921, fig. 3) and that of *Arenicola* hæmoglobin a double apex (Vlès, 1923, fig. 4).

Reduced hæmoglobin has a γ-band situated to the red of that of oxyhæmoglobin. Its axis is at 431 (Henri et Wurmser, 1912), while that of oxyhæmoglobin is at 416. Similarly, the γ-band of reduced chlorocruorin is to the red

pink pigment to a colourless substance which permits the green colour of chlorocruorin to appear in the solution. The reduced substance re-oxidises in air. This pink pigment is at present under investigation. The normal green of chlorocruorin, however, can be seen under the microscope in the blood-vessels of *Pomatoceros*.

* Reduced with ammonium sulphide the main band has a single axis at 589. Vlès (1923, p. 17) has shown that the spectrum of Arenicolan hæmoglobin, too, varies with the reducing agent employed. Mammalian hæmoglobin, on the other hand, is invariable.

of that of oxychlorocruorin. This band is seen in the photograph, fig. 2 (*d* and *e*). The spectrophotometric curve is given in fig. 4 (*c*). The axis of the γ-band of reduced chlorocruorin is at 442 as compared with 432 for oxychlorocruorin. The absorption is the same for both forms. Fig. 2 (*e*) shows another band on the side of γ away from the visible. This is seen at 425 on the curve (fig. 4 (*c*)).

The γ′- and φ-bands of reduced chlorocruorin could not be studied owing to the general absorption in this region by the colloidal hydrosulphite.

Derivatives of Chlorocruorin.

Not only are chlorocruorin and hæmoglobin spectroscopically similar, but a series of derivates can be prepared from chlorocruorin corresponding to those obtained from hæmoglobin.

Like hæmoglobin, chlorocruorin has a greater affinity for CO than for oxygen. *CO-chlorocruorin* has a spectrum resembling that of oxychlorocruorin with the α- and β-bands shifted towards the blue. As in the case of CO-hæmoglobin, the CO-chlorocruorin α-band is fainter and broader than the oxychlorocruorin α-band. The amount of shift of the α-band when CO displaces O_2 is called by Barcroft the "span." Table II gives the spans of several specific chlorocruorins, determined with the reversion spectrometer. The spans of human and *Planorbis* hæmoglobins are inserted for comparison.* They are greater than the chlorocruorin spans. Light dissociates CO-hæmoglobin. It has a similar effect on CO-chlorocruorin but to a more pronounced degree.

Metachlorocruorin of a brown-green colour is formed on the addition of potassium ferricyanide to oxychlorocruorin. The spectrum shows three bands at 604, 569 and 518 (spectrometer readings). There is no band in the red. The band at 569 is more intense than that at 604. On the addition of sodium carbonate the band at 604 becomes more intense than that at 569, which moves to 564. On acidifying again there is no further change, so that the alteration on addition of sodium carbonate may have been due to a change in turbidity, not to the appearance of an alkaline metachlorocruorin. Vlès (1923, p. 19) has shown that the methæmoglobin of *Arenicola*, too, is aberrant, but in a different sense.

Weak acids convert oxychlorocruorin into acid *chlorocruorohæmatin*. When *Spirographis* blood is diluted with sea water the addition of citric acid gives a heavy precipitate, but when diluted with distilled water a clear solution is

* The values given here differ from those of Anson, Barcroft, Mirsky and Oinuma (1924, p. 62), using the same instrument. The values of all spans given by these authors require modification.

obtained. It is reddish brown. The absorption spectrum shows a well-defined band with its axis at 562. In addition, there are less defined bands at 670 and 504.* On making the solution alkaline, it takes on a greenish-brown colour. Its spectrum shows two well-defined bands at 630 and 588. In addition, there are less defined bands at 683 and 475.

It is thus apparent that the spectra of acid and alkaline chlorocruorohæmatin have a character different from those of acid and alkaline hæmatin. This being so it is remarkable to find that chlorocruorohæmatin in ether plus glacial acetic acid has a spectrum quite analogous to that of hæmatin in the same solvent. The method employed was to drop *Spirographis* or human blood into a mixture of 2 parts of ether to 1 part of glacial acetic acid. In each case a four-banded spectrum is obtained (*see* fig. 6), the bands of chlorocruorohæmatin being to the red of those of hæmatin. In chloro-

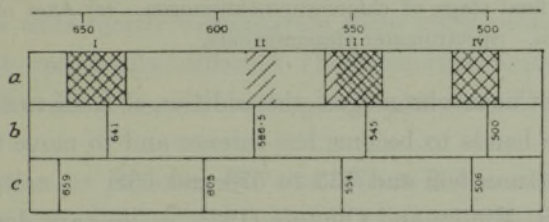

FIG. 6.—Absorption spectra of hæmatin and chlorocruorohæmatin prepared with ether and acetic acid. (*a*) General aspect of the hæmatin spectrum, determined with the ordinary spectrometer. (*b*) and (*c*) Wave-lengths of the axes of the bands of hæmatin (*b*) and chlorocruorohæmatin (*c*), determined with the reversion spectrometer.

cruorohæmatin band I is darker than III, in hæmatin the reverse is the case. Band II is very light in both.

Spirographis blood dried on a microscope slide and treated with the usual procedure for preparing *hæmin* gives crystals which resemble the latter.

While, however, the derivatives corresponding to methæmoglobin and hæmatin in watery solution do not closely resemble these substances, a typical hæmochromogen can be prepared from chlorocruorin. The method used was either to reduce the oxychlorocruorin with hydrosulphite and then add alkali, or to add acid, then alkali, and lastly to reduce. The spectrum of this *chlorocruorochromogen* is shown in fig. 7. The bands are to the red of those of hæmochromogen prepared from hæmoglobin, but the difference is less than that between oxychlorocruorin and chlorocruorin compared with

* This implies a correction to the absorption spectrum given in my preliminary communication. The reason is that previously the clear solution in distilled water had not been obtained.

oxyhæmoglobin and hæmoglobin. The α-band of chlorocruorochromogen is asymmetrical and less intense than that of hæmochromogen. Further, unlike hæmochromogen, the bands do not immediately attain their definitive positions. There is an initial stage of chlorocruorochromogen which only gradually changes into the final state (*see* fig. 7).

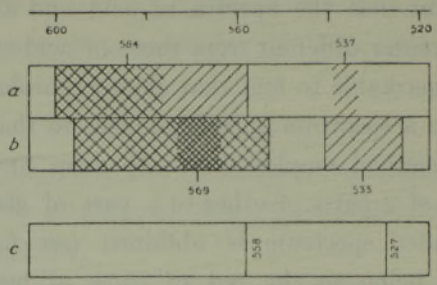

Fig. 7.—Absorption spectra of chlorocruorochromogen and hæmochromogen. (*a*) Initial stage, and (*b*) final stage of chlorocruorochromogen. (*c*) Axes of bands of globin-hæmochromogen. Spectrometer measurements.

As in the case of hæmochromogen, the addition of KCN to chlorocruorochromogen causes the bands to become less intense and to move towards the red. Their axes move from 569 and 533 to 573 and 538.

Anson, Barcroft, Mirsky and Oinuma (1925, p. 75) have demonstrated with Hartridge's reversion spectrometer that the hæmochromogens prepared from the hæmoglobins of various vertebrates and insect larvæ all have the axis of the α-band at the same wave-length. This does not necessarily mean that all of these hæmochromogens are identical, for Vlès (1923, p. 20) has shown spectrophotometrically that the hæmochromogen of *Arenicola* absorbs less than that of the horse. It was of interest, however, to know whether the specific chlorocruorins, too, would give chlorocruorochromogens all alike as regards the wave-length of the α-band. Such was found to be the case. Preparations were made from the bloods of *Branchiomma*, *Sabella* and *Myxicola*. For all three forms the position of the axis of the α-band, measured with the reversion spectrometer, was the same. The wave-length was : (*a*) in the initial stage, immediately after preparation, 5810 Å, and (*b*) in the final state, measured next morning, 5657 Å.

The Protein and the Coloured Group of the Chlorocruorin Molecule.

Protein precipitants were used to test for the presence of a protein in chlorocruorin. Chloracetic acid brings down a precipitate in *Spirographis* blood. On centrifuging it is seen that the supernatant liquid is colourless.

The following procedure was adopted to confirm the presence of a protein. It allowed of a colour reaction being used, which is of course impossible in presence of the coloured portion of the chlorocruorin molecule. Schulz's classical method of splitting hæmoglobin into globin and the coloured group, consists in first converting the hæmoglobin into acid hæmatin and then adding ether and a little alcohol. The hæmatin then all passes over into the ether while the globin remains in the watery phase. *Spirographis* blood was treated in this way, chlorocruorohæmatin being first prepared from the oxychlorocruorin with a dilute acid. When ether was added, the coloured group passed over into the latter. The acid watery phase, which was colourless, could now be tested for a protein, and was found, in fact, to contain one. The biuret test was positive and tungstic acid gave a precipitate.

Chlorocruorin appears thus to be a conjugated protein, although it may be objected that the tests merely demonstrate some other protein in the blood. The existence of specifically different chlorocruorins argues, however, in favour of a protein rather than some simpler constituent of the chlorocruorin molecule.

The next question to be answered was, What is the relationship of the coloured group in the chlorocruorin molecule to that in hæmoglobin?

Bartin-Sans and Moitessier (1893) showed that the hæmochromogens are a family of substances formed when egg-albumin, amines or ammonia are added to reduced hæmatin, and that while all have the typical hæmochromogen spectrum the wave-lengths of the bands vary a little in each case. Anson and Mirsky (1925, p. 50) have taken up the question again, and, using Hartidge's reversion spectrometer, have fixed the axis of the α-band for a series of artificial hæmochromogens containing different nitrogen compounds attached to the hæmatin nucleus. One of their hæmochromogens was formed by adding globin to hæmin dissolved in NaOH and reduced. This gave an α-band in an identical position with that of ordinary hæmochromogen prepared directly from hæmoglobin. Hence, they conclude, ordinary hæmochromogen contains globin.*

Now, there exist several natural pigments other than hæmoglobin which belong to the same hæmatin family. Such are (1) cytochrome, a mixture of hæmochromogens present in most animal and plant cells (Keilin, 1925);

* Anson and Mirsky consider that hæmoglobin is a polymer of hæmochromogen. If this be so, the initial stage of chlorocruorochromogen may consist of, say, two molecules of the final stage, while chlorocruorin would consist of four molecules of the latter.

(2) helicorubin, a hæmochromogen in the gut of snails; and (3) actiniohæmatin in the anemone *Actinia equina*.

Anson and Mirsky (1925, p. 161) attacked the problem next as to whether these different pigments have the same or a different hæmatin nucleus (called by them hæm). They did not examine the hæmatins directly, but from each pigment prepared an artificial ammonia-hæmochromogen. This procedure was adopted because the wave-length of the hæmochromogen α-band can be very accurately fixed (to within 2 Å) with Hartridge's reversion spectrometer. It was found that the wave-length was identical for the ammonia-hæmochromogens of (1) various hæmoglobins, (2) a portion of the cytochrome complex, (3) helicorubin, and (4) actiniohæmatin.

Hence these authors conclude that the coloured group (hæmatin, or, as they term it, hæm) of all these pigments is identical. It should be pointed out, however, that while the fact just recorded shows a very close similarity between the hæmatins in question, it does not imply identity. In order to demonstrate this, the spectrophotometric curves must be prepared and the ratios between the absorption constants at different wave-lengths must be shown to be identical for each substance.

Chlorocruorin was next examined with the object of seeing whether its hæmatin nucleus is similar to those of the pigments mentioned above. In view of the fact that the various pigments of the hæmatin family studied by Anson and Mirsky turned out to have hæmatin nuclei either identical or very similar to one another, it might reasonably have been expected that the coloured group of the chlorocruorin molecule would fall in the same category. It was therefore surprising to discover that the hæmatin nucleus of chlorocruorin is very different.

The procedure was as follows: Ammonia-chlorocruorochromogen was prepared from *Spirographis* blood and the wave-length of its α-band compared on Hartridge's reversion spectrometer with that of ammonia-hæmochromogen. The wave-lengths of the two turned out to be far from alike. The α-band of ammonia-chlorocruorochromogen is 20 Å to the red of that of ordinary chlorocruorochromogen prepared directly from chlorocruorin. On the other hand, the α-band of ammonia-hæmochromogen is 27 Å to the blue of ordinary hæmochromogen made from hæmoglobin. The α-bands of ordinary chlorocruorochromogen and ordinary hæmochromogen are themselves 85 Å apart.*

* Chlorocruorochromogen 5657 Å, hæmochromogen from hæmoglobin 5572 Å (reversion spectrometer measurements).

Thus the ammonia-hæmochromogens are not at all similar, and hence the hæmatins of chlorocruorin and of hæmoglobin are unlike.

As to the mode of preparation, ammonia-hæmochromogen can easily be made by adding ammonia to ordinary globin-hæmochromogen. Ammonia has a great affinity for hæmatin and it displaces the globin. It was found, however, that this cannot be done with chlorocruorochromogen. Its protein has too great an affinity for its hæmatin group to be displaced by ammonia. Recourse must be had to another procedure. Chlorocruorin is split in Schulz's way. Ammonia is then added to the ether phase and the hæmatin passes over into the ammonia. A reducer (hydrosulphite) is added, ammonia-chlorocruorochromogen being thus formed. Naturally, the ammonia-chlorocruorochromogen was compared with ammonia-hæmochromogen prepared from hæmoglobin by an identical procedure. Ammonia-chlorocruorochromogen is a greenish-yellow solution, while ammonia-hæmochromogen is pink.

Concerning the protein part of the chlorocruorin molecule, all that can be said at present is that relatively to ammonia it has a greater affinity for its coloured group than has globin for hæmatin, since ammonia displaces globin from globin-hæmochromogen but not the protein from chlorocruorochromogen. The nitrogen compound of helicorubin has a similar high affinity for its hæmatin group (Anson and Mirsky, 1925, p. 221). Further, the protein of chlorocruorin can be made to combine with the hæmatin of hæmoglobin. To bring this about chlorocruorin was split in Schulz's way and the acid watery portion containing the protein made alkaline. Hæmatin was then prepared by dissolving hæmin crystals in NaOH. Hydrosulphite was added and the product mixed with the protein from chlorocruorin. This hybrid has a typical hæmochromogen spectrum with its α-band within 3 Å of that of ordinary globin-hæmochromogen. The chlorocruorin protein has a good affinity for ordinary hæmatin, for it was necessary to add to the latter a smaller quantity of this protein to form a hæmochromogen than of any other protein except globin.

Constitution of the Coloured Group of the Chlorocruorin Molecule.

The hæmatin group of chlorocruorin being different from that of hæmoglobin, etc., the next question asked was whether it is an iron compound or perhaps contains some other metal. The test for masked iron, however, was positive. The iron is at present being estimated quantitatively in order to establish

whether or not oxychlorocruorin resembles oxyhæmoglobin in having two atoms of oxygen united to one of iron.

Since the coloured group of the chlorocruorin molecule is an iron compound, the difference between chlorocruorohæmatin and the hæmatin of hæmoglobin must be due not to the metal but to the porphyrin with which the metal is united. In my previous publication (1924) it was left undecided whether or not chlorocruoroporphyrin is identical with hæmatoporphyrin. The method employed did not give sufficiently constant results for either product. Since, then, other methods have been adopted which show that, while chlorocruoroporphyrin and hæmatoporphyrin have the same patterns of spectra, the bands of the former are situated considerably to the red of those of the latter. The porphyrins, then, are different from one another. This means that, whereas both ordinary hæmatin and chlorocruorohæmatin contain iron, they differ in being built up from different groups containing pyrrol nuclei.

The method used in the preparation of the porphyrins was the following:—Blood of *Spirographis* and human blood were each dropped into concentrated H_2SO_4 kept cool under the tap. In both cases porphyrins were obtained having a broad unsymmetrical band in the green and a narrower symmetrical band in the red. The wave-lengths of the bands were measured six hours after

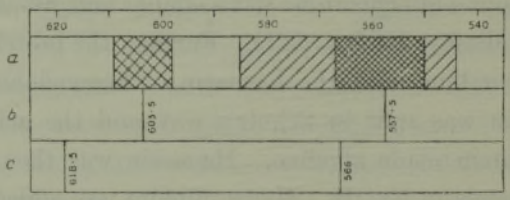

Fig. 8.—Absorption spectra of porphyrins prepared with concentrated H_2SO (*a*) General aspect of the hæmatoporphyrin spectrum, determined with the ordinary spectrometer. (*b*) and (*c*) Wave-lengths of axes of bands of hæmatoporphyrin (*b*) and chlorocruoroporphyrin (*c*), measured with the reversion spectrometer.

preparation, since the positions are unsteady at first. The red band is more intense relative to the green band in chlorocruoroporphyrin than it is in hæmatoporphyrin. Fig. 8 gives the axes of the bands in both cases.

Hæmatoporphyrin is fluorescent (Dhéré and Sobolewski, 1916). Chlorocruoroporphyrin exhibits the same phenomenon.

Conclusion.

The hæmatin nucleus found in hæmoglobin, helicorubin and actiniohæmatin has a very wide distribution both in animals and plants, since it is also a constituent of cytochrome. Chlorocruorin stands apart in having a peculiar pyrrol

group, which is the more remarkable since there is a much greater similarity between the chemical structure of hæmoglobin and that of chlorocruorin than there is between hæmoglobin and any of the other pigments mentioned. Chlorocruorin, with its peculiar hæmatin, has a most restricted zoological distribution. It is found only in certain families of polychæte worms, while other families of these annelids have hæmoglobin. Now, the sporadic occurrence of hæmoglobin and related pigments in the animal kingdom may perhaps be accounted for by a common development with the almost omnipresent cytochrome. If this be the case, however, what is the origin of chlorocruorin? This is one of the principal problems connected with this substance which still remain to be solved.

Summary.

1. Chlorocruorin is a pigment dissolved in the blood plasma of certain polychæte worms. It is red in concentrated, green in dilute, solution.

2. Chlorocruorin exists in an oxidised and a reduced form, having absorption spectra analogous to those of oxy- and reduced hæmoglobin. The oxidised form can be reduced (*a*) by a vacuum and (*b*) by living tissue, and then reoxidised by air.

3. Chlorocruorin has a lesser affinity for oxygen than has hæmoglobin.

4. The oxygen affinity of chlorocruorin is increased by an increase in *p*H.

5. There is a series of specific chlorocruorins differing in oxygen affinities. The following are arranged in order of oxygen affinity from highest to lowest: *Myxicola*, *Spirographis*, *Sabella*, *Branchiomma*.

6. The chlorocruorins of *Branchiomma*, *Sabella* and *Spirographis* are unsaturated with oxygen when this gas is at the normal atmospheric pressure, while that of *Myxicola* is saturated.

7. The total oxygen capacity of the blood of *Spirographis* is (*a*) in contact with air, 9·1 vol. per cent.; (*b*) in oxygen, 10·2 vol. per cent. *Spirographis* oxychlorocruorin is thus 90 per cent. saturated at atmospheric oxygen pressure.

8. The absorption bands of oxychlorocruorin and reduced chlorocruorin resemble those of oxy- and reduced hæmoglobin shifted towards the red end of the spectrum.

9. In addition to this shift, the principal difference in the oxychlorocruorin spectrum compared with that of oxyhæmoglobin consists (1) in the high value of the absorption ratio α/β and the low value of γ/γ', and (2) in the presence of an extra small band in the visible region between β and γ.

10. The specific oxychlorocruorins differ in the wave-length of the α-band axis.

11. The Stokes' band of chlorocruorin (reduced with hydrosulphite) has a summit with a buttress on either side, differing thus from the simple summit of the mammalian and the double apex of the Arenicolan hæmoglobin band. In reduced chlorocruorin the γ-band is shifted to the red of its position in oxychlorocruorin. (*Cf.* the same shift in reduced compared with oxy-hæmoglobin.)

12. Chlorocruorin has a greater affinity for CO than for oxygen. The spans of different chlorocruorins vary, but are always smaller than those of hæmoglobins. Light has a greater effect in dissociating CO-chlorocruorin than CO-hæmoglobin.

13. The spectrum of metachlorocruorin differs considerably from that of methæmoglobin.

14. Chlorocruorohæmatin in both acid and alkaline watery solution shows spectra unlike those of acid and alkaline hæmatin. On the other hand, chlorocruorohæmatin in ether-acetic has bands similar to those of hæmatin in the same solvent, but moved to the red.

15. Spectroscopically, chlorocruorochromogen closely resembles hæmochromogen. The former differs from the latter in that (1) its bands are nearer the red, (2) the final situation of the bands is not attained immediately after preparation; there is an initial form which gradually changes into the final one. The different specific chlorocruorins yield chlorocruorochromogens, all of which have the axis of the α-band at an identical wave-length.

16. Anson and Mirsky have demonstrated that the ammonia-hæmochromogens of hæmoglobin, helicorubin, actiniohæmatin and part of the cytochrome complex all have the axis of the α-band at the same wave-length, showing that the hæmatin group is either the same or very similar in all of these pigments. Ammonia-chlorocruorochromogen, on the other hand, has its α-band at a different wave-length, so that the hæmatin group of chlorocruorin is not the same as that of the pigments just mentioned.

17. The hæmatin group of the chlorocruorin molecule contains iron. It differs from the hæmatin group of hæmoglobin in that the iron is united to a different porphyrin. The latter has a similar spectrum to that of hæmatoprophyrin, with the bands shifted to the red.

REFERENCES.

Anson, M. L., Barcroft, J., Mirsky, A. E., and Oinuma, S., 'Roy. Soc. Proc.,' B, vol. 97, p. 61 (1924).

Anson, M. L., and Mirsky, A. E., 'Journ. of Physiol.,' vol. 60, pp. 50, 161, and 221 (1925).

Barcroft, J., 'The Respiratory Function of the Blood,' Cambridge (1914).

Barcroft, J., and Barcroft, H., 'Roy. Soc. Proc.,' B, vol. 96, p. 28 (1924).

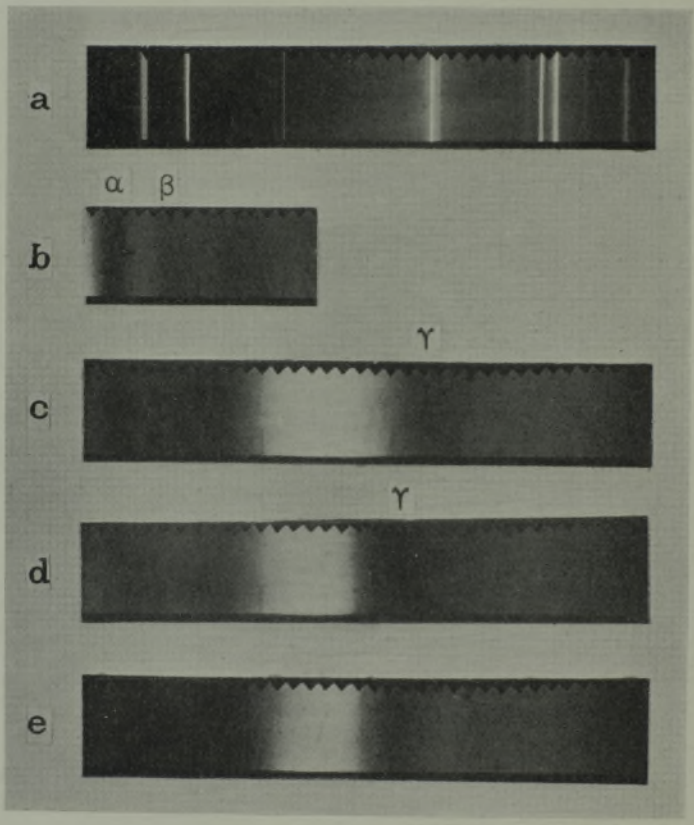

Fig. 2.

(*Facing p.* 217.)

Bertin-Sans, H., et Moitessier, J., 'C.R. Acad. Sci.,' vol. 116, p. 401 (1893).
Dhéré, Ch., 'J. Physiol. et Path. gén.,' vol. 18, p. 221 (1919).
Dhéré, Ch., et Sobolewski, S., 'C.R. Soc. Biol.,' vol. 70, p. 511 (1911).
Fox, H. Munro, 'Proc. Camb. Phil. Soc. (Biol. Sci.),' vol. 1, p. 204 (1924).
Hartridge, H., 'Journ. of Physiol.,' vol. 44, p. 1 (1912) and vol. 57, p. 47 (1922); 'Roy. Soc. Proc.,' B, vol. 86, p. 128 (1913).
Henri, V., et Wurmser, R., 'C.R. Soc. Biol.,' vol. 72, p. 1039 (1912).
Keilin, D., 'Roy. Soc. Proc.,' B, vol. 98, p. 312 (1925).
Lankester, E. Ray, 'Journ. Anat. Physiol.,' vol. 2, p. 114 (1867), and vol. 3, p. 119 (1870).
MacMunn, C. A., 'Quart. Journ. Micr. Sci.,' vol. 25, p. 469 (1885).
Redfield, A. C., and Hurd, A. L., 'Proc. Nat. Acad. Sci.,' vol. 11, No. 2 (1925).
Sorby, 'Quart. Journ. Micr. Sci.,' vol. 16, p. 76 (1876).
Vlès, Fred, 'Arch. Phys. Biol.,' vol. 1, No. 1 (1921); vol. 2, No. 6 (1923); and vol. 4, No. 3 (1925).
Winterstein, H., 'Bioch. Z.,' vol. 19, p. 384 (1909).
Wurmser, R., 'Arch. Phys. Biol.,' vol. 1, No. 3 (1921).

DESCRIPTION OF PLATE 1.

FIG. 2.—Photographs of the absorption spectra of oxy- and reduced chlorocruorin of *Spirographis*. (*a*) Mercury vapour lamp spectrum. (*b* and *c*) Oxychlorocruorin. (*d* and *e*) Chlorocruorin. (*b* is photographed with a plate sensitive to the red.)

APPENDIX.

Spectrophotometric values from which the curves of Figs. 3 and 4 were constructed.

(Fig. 3—p. 204.)

Oxychlorocruorin.

λ in μμ	Colog I/I_0	λ in μμ	Colog I/I_0
638	0·282	537	1·061
616	1·896	517	1·086
609	2·45	502	1·064
580	0·994	491	1·259
561	1·450		

Reduced chlorocruorin

λ	Colog I/I_0	λ	Colog I/I_0
653	0·46(7)	573	1·434
630	0·76(9)	551	1·30
613	1·240	539	1·20
604	1·341	526	1·17
596	1·34(5)	514	1·10
590	1·380	509	1·02
577	1·367	496	0·96

(Fig. 4—p. 205.)

Oxychlorocruorin (oxygen).

λ	Colog I/I_0	λ	Colog I/I_0
460	0·50	350	0·56
450	0·66	340	0·64
435	0·74	322	0·68
430	0·74	310	0·70
420	0·66	295	0·86
410	0·60	285	1·14
400	0·54	278	1·30
395	0·44	275	1·35
390	0·46	268	1·18
385	0·48	261	1·27
380	0·51	250	1·45
375	0·48	245	1·57
368	0·48	242	1·66
360	0·43	238	2·24
358	0·42	234	3·74

Oxychlorocruorin (air).

λ	Colog I/I_0	λ	Colog I/I_0
450	0·54	310	0·39
440	0·69	300	0·42
430	0·67	290	0·55
420	0·42	285	0·70
400	0·30	278	0·81
395	0·18	274	0·77
390	0·23	267	0·75
380	0·27	261	0·79
370	0·31	254	0·85
360	0·28	245	0·97
350	0·29	240	1·38
340	0·31	234	1·80
321	0·36		

Reduced chlorocruorin.

λ	Colog I/I_0	λ	Colog I/I_0
465	0·23	425	0·41
450	0·68	420	0·29
440	0·69	410	0·30
430	0·44	405	0·23

[*Addendum, December*, 1924.—A further investigation has been made of the porphyrins derived from chlorocruorin.

Chlorocruoroporphyrin was first prepared by Nencki's procedure for the preparation of hæmatoporphyrin.* Diluted *Spirographis* blood was treated with acetic acid (1 part) plus ether (3 parts). The acetic was washed out with water and the remaining ether solution of chlorocruorohæmatin was evaporated to dryness. The residue (with some sodium sulphite, to prevent oxidation of the porphyrin) was dissolved in hydrobromic-acetic acid. The porphyrin thus formed was driven by sodium acetate into ether and then taken back into hydrochloric acid (1 part HCl plus 2 parts water). Hæmatoporphyrin, for comparison, was prepared from hæmin with hydrobromic-acetic acid. The axes of the bands of the two porphyrins (both in HCl, 1 in 3), measured with Hartridge's reversion spectrometer, were as follows :—

Nencki's hæmatoporphyrin	593	549
Chlorocruoroporphyrin (Nencki's method)	613	553

H. Fischer and O. Schumm ('Zeit. Physiol. Chemie,' numerous publications in recent years) have added much to our knowledge of porphyrins. They show that porphyrins fall into two groups.

Group I comprises porphyrins insoluble in chloroform. It contains: (1) Coproporphyrin, extracted from human fæces and from yeast by ether-acetic acid (Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130, p. 302 (1923), and Schumm, vol. 136, p. 243 (1924)); (2) Uroporphyrin, found in pathological human urine (Fischer, 'Zeit. Physiol. Chemie,' vol. 97, p. 125 (1916), and Schumm, *loc. cit.*); and (3) Turacin, the Cu salt of a porphyrin, found in the feathers of certain birds (Fischer and Hilger, 'Zeit. Physiol. Chemie,' vol. 128, p. 167 (1923)). Coproporphyrin is the porphyrin derived from a part of cytochrome (Keilin, *loc. cit.*). Cytochrome is present in relatively considerable amounts in yeast and in bacteria. Cytochrome (in yeast) gives, in addition, a porphyrin of Group II (Fischer, 'Zeit. Physiol. Chemie,' vol. 138, p. 288 (1924)).

Group II is formed by porphyrins soluble in chloroform. It contains (1) the porphyrin prepared from reduced hæmoglobin in whole blood by the action of HCl (Laidlaw, 'Journ. Physiol.,' vol. 31, p. 467 (1904), and Schumm, 'Zeit. Physiol. Chemie,' vol. 132, p. 34 (1924)); (2) Kämmerer's porphyrin, extracted from putrefying blood by ether and acetic acid (Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130, p. 302 (1923)); (3) Papendieck's porphyrin, obtain-

* I am indebted to Mr. R. Hill of the Biochemical Laboratory, Cambridge, for valuable help.

able from fæces after a meat meal (Papendieck, 'Zeit. Physiol. Chemie,' vol. 128, p. 109 (1923)); and (4) Oöporphyrin, extracted by HCl from egg-shells (Fischer and Kögl, 'Zeit. Physiol. Chemie,' vol. 131, p. 241 (1923)).

The following table shows the wave-lengths of the band-axes of the principal of these porphyrins :—

Porphyrin.	Reference.	Axes of bands in $\mu\mu$.	
		In ether.	In 25 per cent. HCl.
Group I.			
Coproporphyrin	Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130	624 571 528 499	594 553
Ditto	Schumm, 'Zeit. Physiol. Chemie,' vol. 136	623 569 526 495	593 550
Group II.			
Laidlaw's porphyrin	Schumm, 'Zeit. Physiol. Chemie,' vol. 132	631 575 535 502	602 557
Kämmerer's porphyrin	Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130	633 575 533 498	602 557
Oöporphyrin	Fischer and Kögl, 'Zeit. Physiol. Chemie,' vol. 131	631 581 537 502	603 557

I next submitted chlorocruorin to a modification of Laidlaw's procedure for porphyrin preparation. *Spirographis* blood was treated with ether-acetic acid. The resulting chlorocruorohæmatin was taken into sodium carbonate solution and reduced with hydrosulphite. In a current of CO_2, conc. HCl was then added. The porphyrin thus formed was driven by sodium acetate into ether, and then taken back into HCl (1 in 3). For comparison, (1) the porphyrin from hæmin was prepared by a similar modification of Laidlaw's method, and (2) oöporphyrin was extracted from egg-shells with HCl. The measurements of the bands with Hartridge's spectrometer are given below :—

	Axes of bands in $\mu\mu$.	
	In ether.	In 1 part conc. HCl + 2 parts water.
Porphyrin from hæmin	632 585 536 503	600 555
Oöporphyrin	634 587 536 504	601 556
Porphyrin from chlorocruorohæmatin	641 580 553 512	614 559

It is seen that the bands of the porphyrin from chlorocruorin are situated considerably to the red of those of any of the above-mentioned porphyrins. This new porphyrin resembles the members of Group II in being soluble in chloroform.]