a Research into the Spectroscopy of Blood-pigment and its derivatives in Health and Disease

by

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The subject of the following Research was suggested to me by Professor M^c^Kendrick through whose kindness I was privileged to avail myself of the resources of his splendidly equipped Laboratory: I would thus, at the outset, gratefully acknowledge my indebtedness to him.

The scope and details of the work are of my own choosing; Spectroscopy being only one of several methods of research into the presence and amount of pigmentary material in animal fluids and tissues in health and disease, I have availed myself, as occasions arose, of other means of detecting these bodies and of yielding, when necessary, confirmatory evidence.

I

On the details of the preparation of Haemoglobin and its derivatives for the purpose of spectroscopic demonstration to a large class.

The following directions are for preparing solutions to be placed in the (standard Haemautinometer 1c 1cm wide, to be illuminated by the electric or "oxy-hydrogen" lime-light passed through a large Carbon-disulphide Prism* (base 5 inches) yielding, at a distance of 12 feet, a spectrum 4 ft long, sufficiently "pure" to exhibit the necessary absorption-bands.

Based on Rollet's calculations, a very instructive demonstration can be given as to the Strength or Percentage of Haemoglobin in watery solutions.

(1) Begin with ordinary defibrinated blood of ox or sheep as received from the slaughter-house, and so show that without any dilution no light can pass to the prism: the blood is "opaque." According to Preyer (Quantitative Bestimmung des Farbstoffs im Blute durch das Spectrum.

* Refracting angle 45°.

Ann: d. Chem: and Pharm: 1866) we can say the Percentage of HbO_2 is at least greater than 7·3%

(2) Dilute now with water until a zone of red light is visible at the extreme left of the spectrum: at this degree of concentration the HbO_2 is certainly greater than 1%.

(3) Continue the dilution till the first glimmer of extra-linear green light appears; it now contains about ·9%.

(4) Dilute farther, till you can just see the intra-linear green light; the Percentage is at this point about ·7%

(5) Reduce this concentration to one half, when the Percentage will of necessity fall to ·35%

(6) Continue the process until both the absorption bands are just visible (or to the point of vanishing of the right-hand band, which is the fainter) and there is present ·01% of HbO_2.

(7) Conclude the attenuation until only the α band is left, when the Percentage will have fallen to something less than ·01%.

Reduction of the oxyhaemoglobin with the vanishing of the two bands to be replaced by the one of Haemoglobin is, on a large scale, a very striking phenomenon and may be easily performed by adding to a dilute solution of Oxyhaemoglobin in a Haematometer a quantity of a test-tubeful of warm Sulphide of ammonium to be gently stirred the while.

Seeing that the single band is always fainter than either of the bands which have been replaced by it, it comes to be a question what strength of HbO_2 is the most suitable to use in order that (1) the two bands may be well seen as two (if the intra-linear green must be visible) and (2) the resulting one band be not too faint.

From numerous trials with this end in view, I believe a Percentage of ·6 the most serviceable.

The relative faintness of the Haemoglobin band may be merely due to the dilution of the fluid by the optically inert Ammonium sulphide.

To make Carbon-monoxide-Haemoglobin (CO-Hb)

Pass a stream of coal-gas straight from a gas-pipe by an india-rubber tube through any quantity of a 4% solution of HbO_2 for 20', when the solution becomes of a slightly more "laky" colour (dilute cherry-juice colour) than the original which is rather of a dilute vermilion. Certain persons, I find, can detect no difference between these two tints of "Red". The spectrum, as is well-known, is exceedingly like that of HbO_2, but besides the fact that when very accurately measured the two bands are found to be both slightly nearer the violet end in the case of CO-Hb, there is, in the latter pigment, a rather greater degree of diffuse absorption or dimness throughout the entire green region. As further diagnostic characters, the solution of CO-Hb is never quite so transparent as that of HbO_2 from which it was prepared, even after several filtrations, and the two bands are of a more equal tint, whereas in the case of HbO_2 the γ band is distinctly the darker. The additional point as to the difficulty of reducing CO-Hb by $NH_4 \cdot HS$ will be alluded to under the section upon the decomposition of the Haemoglobin-derivatives.

To make Neutral Met-Haemoglobin.

For the present purpose (large class demonstration) use a 10% solution of Potassium Ferricyanide: if it is neutral. Of defibrinated sheep's blood, diluted with one and a half times its volume of water, take 140 cc, and, when warm

(35°C) add to it 15 c.c. of the Potassium Ferrocyanide.
If unoiluted blood be used and heated somewhat more highly it becomes thick like paste, but shows the "chocolate colour" mentioned in the text-books as characteristic of met-Haemoglobin. The filtrate from this mass gives a spectrum indistinguishable from alkali-Haematin.
One must not expect to be able to demonstrate in spectra of greater dispersion certain bands which are under the most favourable conditions for viewing them, decided by faint. The absorption-spectrum of neutral met-Haemoglobin (made as above) is identical with that of acid met-Haemoglobin, the band in the Red being very well seen and that immediately to the right of the D line also, but much better in a pocket spectroscope than in a large Carbon Disulphide Prism.
Acid met-Haemoglobin cannot be produced by acidifying the above-made neutral Pigment by glacial acetic acid on account of an abundant precipitate, the filtrate from which yields no spectrum.
One can now demonstrate that the spectrum is not due to the mechanical presence of Potassium Ferricyanide, for if the solution be placed in the Haematinometer it gives no absorption-bands but merely cuts off all light from the right-hand extremity of the green to the violet end.
Similarly if Potassium Permanganate have been used to make the met-Haemoglobin, its spectrum can be shown to be very different from that of the blood-pigment, yielding as it does, five absorption-bands in the green region, three being internal and darker, one being on the left, and the other on the right of the group: these outer bands are much fainter.

To make alkaline met-Haemoglobin.

This pigment can be at once made by adding 5 c.c of Liquor Ammoniae to 140 c.c of the neutral met-Haemoglobin as made with Potass: Ferricyanide.
The two bands, one on each side of the D line, as well as the broad band in the green, are well seen, the fourth band is lost in the comparatively diffuse spectrum amidst the general absorption towards the blue end.
I think it misleading to tell Students that either of these pigments (neutral or alkaline met-Haemoglobin) is "chocolate coloured" in solution, for the neutral solution has an extremely dark Portwine colour, while the alkaline has a distinctly ruby glow in it when viewed by transmitted light: "chocolate-coloured" suggests something opaque, both of these are translucent.

To make acid Haematin.

Dilute defibrinated sheep's blood with its own volume of water, and to 140 c.c. of this fluid at 35° C add 5 c.c. of glacial acetic acid. Thus prepared, the characteristic band in the red is very distinct, and it should be noted that it is situated farther to the left than the "band in the red" of met-Haemoglob: it is also a somewhat broader band and seems to be slightly more intense than that of the other pigment.
The solution of acid Haematin in Ether to show the other three bands (whence Thudicum's name for it "Four-banded Haematin" is derived) is not feasible with the end we have here in view.
The shaking up with the Ether takes some time, and the more thoroughly it is done the more air-bubbles one gets to interfere with the light passing through the solution.
I find with Stirling ("Outlines of Practical Physiology" (1890)

p.49) and entirely to what Mc-Munn indicates ("The Spectroscope in Medicine". 1880. p 125) that Acid-Haematin (prepared as above) cannot be reduced by NH_4HS to any other pigment.
Mc-Munn is indeed speaking of pigment pathologically in urine, and he gives the above as a means of determining whether a band in the red indicates Met-Haemoglobin or Acid Haematin, for the former will become "reduced haemoglobin," while the latter becomes "reduced haematin".
Using solutions obtained as above-described I find, on the contrary, that while Met-Haemoglobin, whether neutral or alkaline, does become reduced to Haemoglobin via HbO_2, the Acid-Haematin does certainly not become Haemochromogen (by which term "Reduced Haematin" is now known).

To make Alkali-Haematin

To 140 cc of a ·7% of HbO_2 while at 35°C add a stick of solid KHO 2·5cm long.
This clear, dark, ~~port~~ olive-brown ~~wine~~-coloured solution shows the broad band over D fairly well with the large prism, but very much better with a small pocket-spectroscope on account of the ill-defined band (as it is relatively to many others) not being nearly so striking in the four foot spectrum as are bands of less width & better defined margins. The cutting out of light towards the violet end is, of course, sufficiently well marked.

To make Haemochromogen.

To 140 cc of Alkali-Haematin (diluted to three times its volume) add, while at 35°C, 15 cc of NH_4HS, when the dark olive-brown solution changes almost immediately to deep ruddy

port-wine tint. This is Reduced alkali-Haematin with two absorption bands, the left of which, as in HbO_2, is the darker but both are distinctly nearer to the violet end of the spectrum.

A very striking experiment may now be performed. Oxygenate by shaking up with air a little of this Haemochromogen, and examine at once. The two bands have disappeared, having given place to the one broad band of alkali-Haematin! But alkali-Haematin so made is by deoxygenating Haemochromogen, is very unstable, and if it be carefully watched with the spectroscope, in about thirty seconds there will be seen to arise out of the general dimness one dark line, the first of a dark band which every moment becomes more intense & better defined being joined a few seconds later by its right-hand fainter neighbour separated from it by a clear interval of beautiful green light.

To make Acid Haematoporphyrin.

To 100 c cm of concentrated sulphuric acid add, in the cold, 5 c cm of undiluted fresh filtered defibrinated blood. This must be done in a wide-mouthed glass vessel so that the blood, as soon as it comes in contact with the denser acid on the surface of which it would naturally float, may be at once stirred up so that any charring of it is prevented. Should a little of it become charred, the black specks of carbon must be removed by the whole being filtered thro' a mass of asbestos.

The dark-brown, oily liquid may now be slowly poured (to avoid entangling air) into a parallel-sided white glass vessel with a stopper. An ordinary white flat-sided bottle will do quite well both for the large demonstration and work with the pocket spectroscope.

An ordinary Haematinometer is quite out of the question when acid of such strength as this is to come in contact with the small amount of cement which holds its several glass sides together. For liquids not so excessively acid as Haematoporphyrin a special form of Haematinometer, (constructed by Messrs Baird & Tatlock, scientific instrument makers, Glasgow) having a lining of indiarubber all round the ends & bottom of it, does very well indeed. But even it fails to be reliable when the concentrated sulphuric acid touches the indiarubber owing to the formation of H_2S from the action of the acid upon the sulphur particles always present in indiarubber. A solid glass vessel must therefore be used & one whose sides are not cemented together but which is blown out of one piece of glass. The instrument makers maintain that the sides of a Haematinometer must be made of window-glass not of blown glass, but I find that bottle-glass of the best manufacture in no way interferes with the production of spectrum and absorption-bands in a large number of very different pigments which I examined in flat sided bottles.
If it is found that the Haematoporphyrin made as above is too concentrated & dark for the power of the illuminant used, it may be diluted, so as to make it more transparent, with concentrated sulphuric acid but not by water, as in the dilute acid there is a turbidity at once produced by ferrous sulphate formed from the iron of the decomposed Haemoglobin, Haematoporphyrin being iron-free Haemoglobin.

The Halitus sanguinis.

The odour of the animal (in the above cases sheep) was very distinct when the blood was heated with glacial acetic acid & sulphuric acid; especially strong was the odour with the latter acid, being free from the "acetous" smell of the former.

II Notes on Met-Haemoglobin.

Acid Met-Haemoglobin may be obtained by merely allowing
1 solution a ·4 or a ·5 % of HbO_2 to remain corked up in a bottle
till it becomes of a rusty-brown colour, gives an acid reaction,
and shows a faint band in the red better seen by sun-
light than by lamp- or gas- light. The change from
HbO_2 occurs at least within two weeks, showing that
dilute solutions of HbO_2, in no way antiseptic, do not
become reduced to Haemoglobin at first.
If this weak solution of acid Met-Haemoglobin be reduced
with NH_4HO it passes through the condition of HbO_2 before
becoming Hb. This fact no doubt has given rise to the idea
that Met-Haemoglobin is "hyperoxygenated" from of HbO_2.

Met-Haemoglobin as made by Potassium Permanganate.
The following method is rather striking —
add one small crystal of the salt to 20cc of a ·5% solu-
tion of HbO_2, and with the spectroscope watch the ~~change~~ vanishing
of the two bands of HbO_2 into the one in the red of the
Met-Haemoglobin. The change is very rapid, and at the above
dilution the two bands between D & E are very well seen, but
if any more salt be added the five bands of the Potass:
Permanganate appear in the green.
It is said that when the band in the red is most distinct the
bands in the green are least so, and vice versa: this I
have found; and have also noticed that if one dilutes the
pigment with water with the view of bringing out the bands
in the green, the band in the red is very apt to fade away,
and the five bands of Potass: Permang: (some uncombined)
appear & persist in the green.
In short, unless proportions are carefully attended to, this
salt is not nearly so reliable for preparing Met-Haemoglobin
as is Potassium Ferricyanide, and not so suitable for

large demonstration purposes. Failure may result either from using blood too much diluted or from adding, even to the proper strength of blood, too much Potassium Permanganate.
I have quite failed to make acid Met-Haemo: from the above solution by direct acidification as is recommended by some authors. If to 30 c c of the above neutral Solution of Met-Haemoglobin be added 6 m of dilute acetic acid a very poor specimen of acid Haematin is produced.

Met-Haemoglobin made by Potass: Nitrite is not suitable for use in large quantities, as it gives a very poorly marked band in the red when 1 part of a 10% solution is added to 8 parts of defibrinated blood diluted one half.

Met-Haemoglobin made by Nitrite of amyl (Jaquet's method). This cannot be formed in the cold nor unless a pretty strong solution of defibrinated blood is used. The "chocolate coloured blood" will not appear in dilute solutions. It is quite unsuitable for large demonstration, but on a small scale the proportions are a few minims of the Nitrite to a test-tube full of blood.

Met-Haemoglobin made with Potass: chlorate is the least satisfactory of all. A higher temperature is required and for a longer time than with any other reagent. As the solution of HbO_2 must be pretty strong, one is thus very apt to coagulate the Proteids and so render it opaque.

The Spectroscopy of Met-Haemoglobin.

It is being asserted, and with increasing emphasis, that it is only the band in the Red that is characteristic of Met-Haemoglobin, at any rate in Neutral or acid solutions, and as these are much the commonest, the following remarks apply exclusively to such.

as to the preeminent importance of the band in the Red, I fully agree with Hewgies ("On Met-Haemoglobin." Journal of Physiology Vol: XVII no: 6, 1895, p 413) finding, as I do, that the concentration of the pigment has much to do with its spectroscopic appearances. I have already alluded to the disappearance of the band in the Red in very dilute solutions, but it is quite stable when compared with the two central bands found between D & E.

Thus if one oxygenate by aeration a neutral solution of Met-Haemo: made with Potass: Ferricyanide, the band in the Red persists, though it may become somewhat fainter, while in place of the two between D & E a very dense broad band appears similar to that yielded by a solution of HbO_2 greater than 1%. I am inclined to think this band is not due to HbO_2, seeing that, if the solution be now ~~further~~ diluted one half (with cold de-oxygenated water) the spectrum as obtained above persists, and on further dilution the broad dark band becomes very faint, but is not resolved into two bands.

On extreme dilution the band in the Red still persists (at least in Met-Haemo: made with Pot: Ferric:), while the broad dark band vanishes amidst a general dimness in the Yellow-Green region.

But further, it is quite common in urine and pathological fluids to find Met-Haemoglobin complicated by the presence of either HbO_2 or Hb. Without having recourse to the measurements of wave-lengths (a process not commending itself to the ordinary practitioner) one could not eliminate (by the spectroscope alone) the one pigment from the other by merely looking at the bands between D & E, unless the following characteristic were present. —

Given that the band in the Red is faint, whereas the two between D & E are fairly distinct, is the pigment present Oxy-Haemoglobin or Met-Haemo:?

If the band near E (the right-hand one) be the darker of the two, the pigment is almost certainly Met-Haemo:, whereas

if the left hand band be the darker, it is oxy-Haemoglobin.
If from other data it be known that the solution is dilute, and there be no doubt of the presence of a faint band in the Red, the pigment is met-Haemoglobin conforming to the rule that its bands between D & E are, in dilute solutions, distinct when the band in the red is not. Neutral or acid met-Haemoglobin often requires to be distinguished from acid-Haematin because its characteristic band is also in the red and its other three bands are only brought out by special reagents (§ the 2g.). without recourse to measuring wave-lengths the differences are —
(1) In acid-Haematin the band in the red is farther from the blue
(2) It is usually the darker (blacker or denser) band.
(3) It is usually the broader band (always in strong solutions)
(4) In acid-Haematin there is almost always a larger region of green yellow-green visible and unfailingly in strong solutions.
(5) The band in the Red in met-Haemo: disappears on reduction with NH_4HS, whereas that in acid-Haematin does not.

There is no pigment per se "Reduced met-Haemoglobin" analogous to "Reduced alkali-Haematin", because on reducing neutral, acid or alkali-met-Haemoglobin you obtain Haemoglobin as an end-product which, on being oxidised, yields ordinary Oxy-Haemoglobin not met-Haemoglobin. Haemochromogen however on being oxidised yields alkali-Haematin which can thus be reconstituted after reduction: met-Haemoglobin cannot be reconstituted after reduction.

III On Sulph-Haemoglobin and the occasional appearance of a band in the Red in the Spectrum of Reduced-Haemoglobin.

The pigment named by Lankester "Sulph-Haemoglobin" (Journal of anatomy and Physiology 1869 p 119) can be very readily obtained by passing a stream of freshly prepared H_2S gas through 100 cc of fresh half-diluted defibrinated blood for 15 minutes. During the first 5 minutes no visible change comes over the colour of the blood, but after that it assumes the familiar purple hue of reduced Hb, and at the end of the time it has become of a dull black colour with a suspicion of green by reflected light, but in a thin layer, with transmitted light, it presents a very dark claret tint.

Its spectrum is best described by saying that it is that of Reduced-Haemoglobin with the addition of a thin but distinct band in the Red at λ 610 it a little to the left of the D line but nearer it than the band in the red of the Haemo: and, a fortiori, still nearer it than the band in Red of Acid-Haematin.

I had repeatedly observed while preparing solutions of Haemoglobin, that if I used freshly made, warm NH_4HS I very often got a spectrum indeed as of Haemoglobin so far as the one broad band between D & b was concerned, but possessing in addition a band at the right-hand extremity of the red: in some cases it scarcely amounted to a band, being perhaps more correctly described as a dark line of demarcation where the orange ended and the red began. If, however, I took NH_4HS which had been prepared for some time (½ years) in which it was to be presumed no free sulphur particles were present, as it had been more than once filtered, the Reduced Haemoglobin made thereby never had the extra band in the Red. There was little doubt that the sulphur was the active agent in producing this band, although at first I was led to think it was a band of alkaline Met-Haemo: which exists in the Red, that pigment being inadvertently produced by the alkali of the NH_4HS.

Believing that I was misled on this point, I will not quote a number of experiments I made with the view of testing this idea. What undoubtedly happens is that the H_2S first reduces the HbO_2, and then the sulphur asserts itself and the extra band appears.

How it might be required to distinguish this pigment from others with similarly situated bands.

In the first place, oxidation of sulph-Haemoglobin causes the single broad band to break up into two as of HbO_2, while the narrow band in the red persists: this would distinguish it from acid-Haematoporphyrin which also has a narrow band to the left of D.

Sulphuric acid decomposes Sulph-Haems: forming acid-Haematin: it of course does not destroy acid-Haematoporphyrin. Further acid-Haematopor: allows no blue-green to pass, whereas o Hb, unless very concentrated, does.

The band in red of acid-Haematopor: is darker and broader, and in this pigment there is some intra-linear green: there is none in the case of Sulph-Haemoglobin.

One may urge that there can only be a spectroscopic confusion between Hb (which shows the sulphur band) and acid-Haematoporphyrin, as the former would not be nearly so acid as the latter or even alkaline: this is only true of them when made experimentally: Hb can exist in solutions not alkaline, and acid Haematopor: in those not more than faintly acid.

The extra band in Hb is not due to the mechanical presence of NH_4HS, as that solution gives no such appearance before the spectroscope: it merely cuts off the blue end.

(Cf Mc Munn "The Spectroscope in Medicine" pp 81-84).

IV On the action of Hydrochloric acid upon Haemoglobin

I thought it would be interesting and perchance important to investigate experimentally the effect of the action of HCl upon blood-pigment, seeing that this is the acid free in the stomach and the chief cause of any change in blood-pigment to be examined in cases of P.S. Haematemesis.
(A clinical case is alluded to later).
Accordingly I made concentrated HCl replace Glacial Acetic Acid in the preparation of Acid-Haematin, and obtained, as might be expected, a pigment whose spectrum differed in no particular from that of acetic-acid-Haematin. Whereas when I used a 2% solution of HCl (8cc first to 12cc of half-diluted defibrinated blood) a mixture of acid het-Haemoglobin & some unaltered HbO_2 was obtained. That it was het-Haemo: & not Acid-Haematin was proved by the spectrum of Hb alone being yielded after reduction. Hoppe's corroborative as to the difference of the pigment obtained when strong and when weak acid is used cf "Hoppe" on the action of certain acids on blood-pigment" (Journal of Physiology Vol. XVII No 6 1895.); and Mr. Hamm I fully bear out in his remarks on p: 99 "Spectroscopic in Medicine" apropos of Methaemoglobin.

V On the effect of a rise of Temperature upon HbO_2.

Professor Mc Kendrick was anxious that I would make observations upon the effect (if any) of an application of heat to blood-pigment. The temperature of an asbestos-covered water-bath containing several specimens of a 5% solution of HbO_2 from defibrinated sheep's blood was steadily and continuously raised from 10° C.
The tubes were very rapidly scanned from time to time with a Browning's spectroscope.

Up to 40°C there was no change in the spectroscopic appearance or, visibly, in the chemical state.
Between 40°C and 55°C there was slight turbidity, but no change in the two absorption-bands.
From 60°C to 65°C there was an increase in the turbidity of the solution with therefore opacity to light and an increasing absorption in the extra-linear green region which showed itself as an apparent extension of the β band towards the blue.
At 67°C the specimens had become very turbid from a precipitate of Proteids, although even now with a strong light and by the most careful centring the two bands could still be discerned amid a general haziness.
When boiled the solution became perfectly opaque, the Haemoglobin being decomposed into Haematin and the Proteid globin: spectroscopic examination was now impossible.
The pigment which had been precipitated was not retained upon a filter-paper.
A rise of temperature therefore has no effect upon the characteristic spectroscopic appearances of Oxyhaemoglobin, the changes that occur being all concomitants of the inevitable chemical and physical alterations in the fluid heated.

VI On the spectroscopic recognition of blood stains in situ.

Blood-stains upon a pocket-handkerchief, examined before they have had time to dry, yield the spectrum of HbO_2.
Transmitted light is of course used, and the fibres of the cloth do not interfere with the passage of the light to the extent of preventing a spectrum; the porosity is such that enough light for this purpose is allowed to pass.
Blood-stains that have dried, but are examined, say, within one hour yield the absorption-bands of Oxyhaemoglobin.
Old blood-stains when dry and brown do not yield any absorption-bands

either of Haematin or Haemoglobin. This blood was not defibrinated.
Filter-paper stained by defibrinated blood viewed by transmitted light gives the two absorption-bands of oxyhaemo: up to 14 days even when the stain is not wetted with water.
These stains appear of a dull brown by reflected light, but of a very fine crimson colour with transmitted light.
As long as four months after the stains were made, they gave absorption-bands: when dry the spectrum resembled that of HbO_2 of greater amount than 1%, but I am not disposed to lay much stress on this appearance; when, however, the stains were wet with water, the absorption-bands of acid or neutral Met Haemo: were very distinctly given.

VII On the spectroscopic examination of the circulating Blood.

It is possible, and not very troublesome, to obtain the spectrum of blood-pigment while the blood is circulating in the living vessels. One must however be prepared to see in many cases the one band and not the two bands of HbO_2, whenever the blood is interposed in layers of considerable thickness. If I hold the hand before in front of a powerful light, and the spectroscope be directed to the crimson glow between the vascular edges of the fingers, we can see the single band of oxyhaemoglobin as one would in a thick layer or a concentrated solution. Light reflected from the surface of the living nail yields also the same spectrum, as does light which has passed through the human Pinna (of the ear) or has been reflected from its surface.
Seeing that the two bands were not obtained in any of these cases it seemed useless to compress the part hoping to press the blood in sitû: I however, did so in the case of the finger, but could not say that I detected any change in the darkness of the single band such as might have been expected in

if when we remember that the band of Reduced Haemoglobin is always less dark than the darker of the two HbO_2 Haemoglobin bands. If, however, we fix the translucent ear of an albino rabbit between a powerful light and the spectroscope, the blood-sheet is sufficiently thin to give the two bands of oxyhaemoglobin. When one widely constricts the vessels by pinching the base of the ear between finger and thumb, one can actually watch the two bands in the course of 30" gradually fuse into the one of Reduced-Haemoglobin.

The absorption-bands of oxyhaemo: can also be seen with a white rabbit by means of the light reflected from the interior of its eye. One has, of course, to hold the spectroscope quite close to the cornea and catch the red glare or "reflex".

Suppose now that the web of a frog (prepared as for showing the circulation in the web under the microscope) be kept moist and interposed between a source of light and the spectroscope, one familiar with the spectroscope has no difficulty in seeing the two bands of HbO_2.

I made such a preparation, and was able to demonstrate the spectrum to a class of senior students at intervals during two hours. If now the entire leg be constricted by a tight ligature it is found impossible to reduce the oxyhaemo: after such a ligature had been applied for three hours, the two bands of oxyhaemo: were still visible.

I regard this as an additional and most striking proof of the great difference in energy between the reducing (deoxidising) power of living mammalian ("warm-blooded") tissue on the one hand and that of living amphibian ("cold-blood") tissue on the other. It corroborates all we know as to the profoundly less active metabolism in such a creature as a frog compared with a rabbit. It is not that the frog's Haemoglobin was reduced and then filled by "interstitial" oxygen; the oxidative powers of its tissues were too quiet to effect the Reduction.

tissue distal to the ligature

VIII On the Guiac-Reaction and the Haemoglobin derivatives

It struck me that a systematic investigation as to the behaviour of the various Haemoglobin-derivatives in presence of Tincture of Guiac and Ozone (Ether) would be a point of considerable interest in the light of the explanation of that reaction viz that the blood-pigment, by reason of its affinity for oxygen, deprives the Ozone (Ether) of oxygen and conveys it to the Guiac which in a few seconds becomes blue. (A solution, even if very weak, of oxyhaemo: gives this reaction at once (see later on for statistical details of this).

(2) Haemoglobin, reduced by NH_4HS, as might have been expected, gave no blue colour.

(3) Haemochromogen gave no blue colour: this also is interesting as it is reduced (de-oxydised) alkali-Haematin.

(4) Acid-Haematoporphyrin gave no blue colour.

(5) Neutral met-Haemoglobin (made with Potassic Ferricyanide) gave the blue colour, as also, and even faster, did

(6) alkaline-met Haemoglobin.

(7) acid-Haematin and alkali-Haematin both gave the blue colour frankly.

(8) Carbon-monoxide-Haemoglobin also yielded the colour.

Thus the advantage of the Spectroscopic over purely chemical methods of research into the presence of Haemoglobin or its derivatives in fluids is established, for, if the Guiac Reaction be yielded, we are only able to say (from it alone) that one or more of the following may be present viz oxyhaemoglobin, Co-Hb, met-Haemoglobin, acid-&alkali-Haematin; whereas we can assert that the fluid does not contain fully reduced Haemoglobin, Haemochromogen or acid Haematoporphyrin. The fact that Co-Hb yields this reaction is, I think, very interesting, indicating, as it probably does, that all the oxygen of the Haemoglobin has not been replaced by the Carbon monoxide gas (CO)

IX The Spectra of pigments whose absorption-bands closely resemble those of Haemoglobin or its derivatives.

(1) Carmine. Waller (Human Physiology. p 40. Second Edition) says "The discovery of a two-banded spectrum" is "not proof positive that Haemoglobin is the colouring agent, for carmine gives a very similar spectrum." Dr Waller admits that they could only be confused upon hurried examination.

A solution of Borax-carmine was placed in a standard Haematinometer and diluted till it was of the same transparency as a ·3% sol. of HbO_2.

(a) The absorption-spectrum of carmine does in general resemble that of HbO_2; but the two bands, whose centres coincide with those of the bands of Haemoglobin, have their edges on both sides more hazy and ill-defined than is the case with the blood bands.

(b) The right-hand band is slightly, but distinctly, darker in tint than the left band, this is the reverse of what holds good with regard to the bands of Oxyhaemoglobin: this fact is, I think, a very important differentia.

(c) At the particular concentration mentioned above, all the blue light passes through in the case of carmine, this is in marked contrast with Oxyhaemoglobin in which no blue passes; while in the green and green-blue regions there is less absorption with carmine than there is with HbO_2.

(d) The left margin of the left band in the case of carmine is hazy: this causes a dimness in the orange-yellow region due to the extension of absorption towards the red, whereas in the case of Oxyhaemoglobin the corresponding band has its left margin peculiarly sharply demarcated so that more orange is visible with blood-pigment than with carmine.

Turning to very concentrated solutions, I find that the red gets through in both carmine and HbO_2, while in the former only does any violet light pass.

Further, in these strong solutions a narrow zone of green light at the D line appears in the case of carmine only.
The inter-linear green light is in all strengths of carmine characteristically deficient, while in the weaker solutions of HbO_2 it is as markedly present.

II. Haematoxylin.
The spectrum of alum-Haematoxylin is remarkably similar to that of Reduced Haemoglobin. Viewing a solution of Haemoglobin reduced by NH_4HS by transmitted light, the Haematoxylin appears purplish while the Haemoglobin has a more reddish tint.
(a) In the spectrum of Haematoxylin all the blue rays are present; this is notably not so with Reduced-Haemoglobin.
(b) The spectra resemble each other by reason of the broad band which overlaps the D line, in the case of Haematoxylin this band extends much farther into the green on the right cutting out all light here, whereas Haemoglobin (unless exceedingly concentrated) allows some green light to pass.
(c) The left extremity of the peat band extends farther into the orange than is the case with Haemoglobin, so that there is less orange light visible in the case of Haematoxylin.
(d) One would, of course, never find the band of sulph-Haemoglobin in the Red by any possibility in Haematoxylin: one may in Reduced-Haemoglobin.

III. Magenta. The aniline dye soluble in water was examined. In concentrated solution the spectrum is very like that of HbO or of HbO_2 of strength greater than 1%. On dilution the single broad band obscuring the orange and yellow region gives way to two bands remarkably similar to those of Haemochromogen. The right-hand band, in the case of Magenta, is however fainter, broader and more to the right in the green than the corresponding band of Haemochromogen, while the left band is rather broader than its homologue of the blood-pigment.

IV Saffranine, soluble in water, was also examined. In strong solution the absorption-spectrum of this pigment very closely resembles that of Reduced Haemoglobin, except that the one large band does not approach the D line so closely as in the case of Hb: in Saffranine no blue light is transmitted. Upon diluting one notices that all the blue light gets through, and the single band locates itself much more to the right so as to be very nearly in the same position as that of Urobilin from which it only differs in not being so ill-defined towards its left extremity.

V Roseine. This also in strong water solution very closely resembles Reduced Haemoglobin; on dilution, however, some blue appears and two bands which remind one of Haemochromogen. These latter are hardly bands: the appearance is more accurately described by saying that there is one very broad absorption-area whose darkest portion is in the position of the left band of Haemochromogen, the whole of the green being invaded by a considerable amount of absorption.
Further, the left-hand margin of the area of absorption is far more hazy than the corresponding situation in the case of Haemochromogen.

VI Fuchsine. The absorption-spectrum of this pigment, soluble in water, resembles that of Roseine exceedingly closely, both as regards the changes in it consequent upon dilution of the pigment, and its possessing the same ill-defined left extremity. The pigment behaves, just as Saffranine does, with reflected and transmitted light; after dilution there is much spectral similarity to Haemochromogen. There is, as before, an area of dimness extending over the region occupied by the Haemochromogen bands, but in which we can distinguish an intensification of absorption answering to the left hand band and a less dense portion answering to the band near D; between these extends the general haziness which

is responsible for the absence of definition at the extreme left.
VII Eosine. In dilute solution this pigment gives one band in the green at b very similar to the fourth and most distinct band of alkaline-Haematoporphyrin.
VIII Red writing ink (not "Copying" ink).
An undiluted specimen cuts off all light from orange to violet and therefore resembles a solution of HbO_2 Strength greater than 1%. diluted with an equal volume of water a little green to the left of the absorption-area is transmitted. A still more dilute solution resembles Bilirubin spectroscopically, except that the left margin of the band is more hazy than is the case with the Bile-pigment.
IX Tinctura Coeci. For an account of this pigment see Appendix I.

X. On the detectibility of Blood and of Oxyhaemoglobin in Urine.

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(1) By the guiac test.
I thought it well to ascertain the minimal quantity of Blood and of the Pigment per cent of Urine which could, with absolute certainty, be discovered by means of this largely used test. Tincture of guiac and ozone Ether were used, and added to the blood-containing urine in the order mentioned. Undiluted defibrinated blood was dropped into the urine beginning with 5m in 25cc of the urine. This was at once detected by the guiac, the blue coloration being frankly given. Proceeding, I found 5m in 50cc, 5m in 100cc, 5m in 200cc all gave the blue colour readily in descending degrees of distinctness.
5m in 300cc, 5m in 400cc, 5m in 500cc, 5m in 600cc, 5m in 700 cc all yielded the colour, the last three giving the blue only in the uppermost layer of Resin, not in the supernatant Ether.
After careful trials I concluded that 5m in 750cc was the last point of dilution at which an observer could with certainty say (1) "I see a blue colour here": ten minutes were allowed before the colour was declared to be present or not.

5 minims of Blood in 750cc of Urine represents
·04% of Blood; taking Blood as a 14% solution of Oxyhaemoglobin this amount corresponds to a percentage of ·0056 of the Pigment itself.

II <u>The minimal quantity detectible by the Spectroscope.</u>
I noticed that, for the purpose of calculation, it was of no moment whether one used urine or water; the latter was used in view of the extensive dilution required.
I began with 5cc of defibrinated blood in 800 cc of water, this was seen to give the two bands quite unmistakeably. At a dilution of ·625cc in 1100 cc both bands could have been detected by any one a little familiar with the spectroscope. at ·625 in 1300 cc, however, the two bands could only be seen by very careful observation and by the artifice of looking at them obliquely and making the flame jump suddenly up and down. Nevertheless a spectroscopist would have no difficulty in seeing the two bands, while at ·3125cc in 1300 cc the α band was visible enough, but the β band could only be seen by looking obliquely: at ·3125 in 1500 cc the β band had vanished and the α band was just visible; at this dilution therefore we had minimal spectroscopic detectibility.
This point represents a Percentage of <u>·02 of Blood</u> in urine now taking blood as a 14% solution of Haemoglobin, this is equivalent to <u>·0028% of Oxyhaemoglobin</u> in urine (a figure very nearly equal to Hoppe's for minimal visibility of oxyhaemoglobin viz ·003%)
Compared with the guiac Test, the spectroscope is thus seen to detect half as small a quantity of Blood viz ·02 instead of ·04% as stated in H.U.O2, ·0028% can be detected by the spectroscope while by the chemical method ·0056% can be ascertained. Thus the spectroscope is twice as delicate a method as the guiac: it would detect a loss of ·3cc of blood per the urine per 24 hours.

XI On the spectra of Urobilin and Bilirubin, and the spectroscopic examination of Urine in cases of Choluria.

A specimen of normal urine, normal at any rate as to colour, when examined by the spectroscope cannot be said to give any true absorption-band, although there is present upon the most careful scanning a slight haziness at the right extremity of the green region in the position of the Urobilin band as it appears in the published charts.
An alcoholic solution of pure Urobilin of such concentration as to have the same amber tint as normal urine yields exactly the same spectroscopic appearance as urine viz a faint haziness towards the right of the green — an absorption scarcely amounting to more than a diminution (apparently) of illumination.
Normal urine, evaporated to one-fifth of its volume, gave typically the band of Urobilin as a broad absorption-area in the green and green-blue regions. One must wait after the evaporation for the deposit of pink urates to occur and be filtered off.
From examination of a very large number of specimens of normal urine I would say that the band of Urobilin is never defined on its left margin with the definiteness of the drawings in the chart of spectra in the Text books.
I mention this point, not at all with the view of criticising classic work, but to draw attention to the fact of the obvious indefiniteness of the left margin of the band of Urobilin as one of the distinguishing characteristics between it and the band of Bilirubin, which has (as we shall see presently) a comparatively clearly demarcated left extremity. The observation of the edge of these bands is the more important from the fact that their centres are similarly situated viz midway between b and F lines

thus affording us no help in distinguishing them.
A further point of difference is this, that Bilirubin in urine allows no blue light to pass, whereas Urobilin gives access to some of these rays on the right hand side of the band. Fresh ox's bile was added to urine to the extent of $\frac{1}{10}^{th}$ its bulk, but, as viewed by the colour, the Bilirubin was not present to anything like the extent to which it may be in many cases of human Choluria. The spectrum of this artificial bile-containing urine confirmed my observations upon Choluria (as given below) in that (1) the left margin of the band of Bilirubin is very much more sharply defined than that of Urobilin, and (2) vastly more blue twilight light is cut out in the case of Bilirubin than in Urobilin.
Further, I am able to state in general terms that a little Bilirubin in urine gives a band of an intensity or depth which could only be given by Urobilin alone in highly concentrated urine; and in Choluria there is a band present of such darkness that were it due to Urinary pigment only, the urine would require to have been artificially evaporated. In other words, given a choluric and a normal urine of the same depth of colour to the eye (it usually yellowish or brownish) and it will be found that the choluric gives the darker absorption-band, conversely, given two urines which yield equally dark absorption-bands, one choluric the other without Bile that specimen, which to the eye seems the more deeply pigmented will be the urine without Bile-pigment ie ~~the normal~~ concentrated urine, for if it had Bile as the cause of its excessive pigmentation or coloration it would yield a band of such intensity as could only be yielded by artificially concentrated pure urine and never by urine naturally pigmented. Put briefly, Urine with a little Bile has many of the spectroscopic features of very concentrated (natural) Bile-less urine, while with much Bile it has the spectroscopic characters which could only be given by artificially concentrated urine

The following examination of a specimen of urine illustrates certain of these points. Accompanying the specimen was this description in the words of the House-Physician in charge of the case, "Man aged 52. Has malignant disease in abdomen with secondary deposits in liver. No Jaundice as yet. Blood in urine sometimes; no Bile with ordinary tests." Eight days after I received the first specimen of urine, the following intimation was kindly made to me. "Result of Post Mortem: A large cancer was found in liver, nothing in kidneys, and a small ulcerated cancer in bladder." The latter could evidently account for the occasional Haematuria.

When the urine reached me it appeared by transmitted light to be a yellowish-green colour, but was by no means opaque, at the same time it was slightly turbid. It was acid in reaction, and did not give Gmelin's reaction. Placed before the Spectroscope there was no blood-pigment to be observed, but in the green-blue region there was a broad band which after the most careful study could only be assigned to the presence of Bilirubin for the following reasons —

(1.) The depth or intensity of the band was such that it could only have been given by a urine 4 or 5 times more concentrated than this, the specific gravity of which was not notably high. On the ground of colour alone, there was not nearly enough of the amber-coloured Urobilin to give so dark a band.

(2) The band observed here had a comparatively definite left margin, very different from the hazy one of Urobilin even when giving a band of depth equal to that presented here.

(3) I diluted a specimen of Urobilin to the limit of the suspected Bile-containing Urine and found that it gave only a faint hazy band as different as possible from the sharply-defined dark band present in the urine in this case.

(4) The amount of Blue light cut out here corresponded much more with the known spectrum of Bilirubin than with that of Urobilin (& this urine showed, in addition to the band of Bilirubin, the absorption in the green-blue region which Urobilin by itself does not possess. This latter characteristic persisted even after the increased translucency due to diluting the urine one-half.

I was favoured with another specimen of urine which bore on this topic. It was merely described as from "Cancer of liver." The urine, when I got it, gave an alkaline reaction and had a peculiar, "sweet," treacle-like smell: it yielded Gmelin's reaction frankly. The absorption-spectrum of it was that of Bilirubin. From a careful study of this urine by the spectroscope I find that besides the greater degree of definition on the left border of the Bilirubin band, when compared with the band of Urobilin, the centre or darkest zone of the Bilirubin band is slightly nearer the left or closer to the b line. I think we are justified in finding —

(1) That Bile-pigment can be spectroscopically detected in urine even when present in such small amount as to make Gmelin's Reaction negative or ambiguous (that Pettenkofer's should, in many of these cases, be "negative" is not at all surprising as it is a test for bile-salts (acids not pigment))

(2) That in ordinary, normally-coloured "amber" urine the band of Urobilin is not characteristically or evidently present (either with a pocket-spectroscope or a large instrument) until the urine has been evaporated to about $\frac{1}{5}$ of its volume.

(3) When urine has been diluted to such a point that the band of Urobilin may be said to be about to vanish, Bili-rubin in urine, of at least the same degree of dilution, can still form an absorption-band.

(4) It is especially noticeable in weak solutions that Urobilin permits distinctly more blue light to pass than does Bili-rubin.

(5) In urines in which Bilirubin is present to such an amount as to cut out all the Blue end of the spectrum, it will be found that Bile-less urines of the same or even greater depth of colour (as judged by the eye) will allow some Blue light to pass.
(6) The degree of darkness or intensity of the centre of the band of Urobilin (as seen in a fairly concentrated urine) is about the same depth as that of a rather dilute solution of Bilirubin.
(7) Besides that the left margin of the Bilirubin band is more clearly demarcated than that of the Urobilin, the absolute extension of the hazy region of absorption due to the latter pigment is much more to the left, crossing, in fact, the F line, so that, in short, while Urobilin shows more Blue, Bilirubin shows more Green.

In any actual clinical case, even if the colour and concentration of a bile-less urine approached that of a bile-containing urine, so as make the degree of darkness of the centres of the two bands under discussion equal, the presence or absence of Bile could be ascertained by one or more of the above-given differentiae.

In connection with this subject I quite agree with Mr Thomas (p 160 top) as to Nitric or other acid intensifying the depth of the Urobilin band, although I have been unable to observe "the spectrum of Gmelin's reaction" in bilious urine and the "shading at D" as mentioned on p. 163. I find, however, that Nitric acid intensifies the band of Bilirubin and if one commence with a specimen of normal urine and one from cholaemia of equal tint to the eye, that the acid will deepen the tint of the band due to Bilirubin much more rapidly (using equal amounts of acid) than it does in the case of Urobilin.

Of course it may be pointed out that in cholaemia we are dealing with a case of superimposed spectra (that of Bilirubin on Urobilin), but as a matter of fact I have found in cholaemia that the normal urinary pigment was diminished, the bilious taking its place rather than co-existing with it; and to this, I believe, is due the fact that

in bilious urine ~~there~~ there is a conspicuous absence of the haziness in green about the D line characteristic of Urobilin.
Another interesting specimen I received was a urine from a case merely described as "Cancer of liver and stomach".
It was turbid, of acid reaction and did not yield Gmelin's test. On being filtered there was obtained a very pale fluid which gave the absorption band of Bilirubin. The centre of the band in this case (as I found by actual comparison) was as dark as would have been yielded by a distinctly deep amber coloured bile-less urine. It was impossible that the band here seen could have been due to Urobilin, as the amount of that pigment present was less than the amount in twice its volume of diluted normal urine.
I may here conclude the account of urines sent me during the study of Bilirubin detectible in urine by the spectroscope, with the notice of a "negative" case. The specimen was from a case of nodular Cancer of liver, nodules perceptible on deep respiration", and it neither gave Gmelin's reaction nor the band of Bilirubin. The urine was acid, very transparent and pale.

XII

In connection with Cholæmia, I thought it well to apply the spectroscope to the problem of distinguishing Carbolic acid from Bile in urine.
This may appear rather superfluous seeing that the chemical tests are so efficient, still the spectroscopic method may be resorted to on occasions where chemical reagents are not obtainable.
In order to obtain the dark colour when mixed with urine, I had to use not the crystals of the pure acid (which give a colourless solution) but the dark liquid sold by druggists for disinfectant purposes.
This will not mix (become soluble) in water, but the slightly turbid suspension of it (at a tint as nearly as possible equal to that of the dark olive-green urines of Carbolic-acid-poisoning) cut out all the light to the right of the D line. To obtain a clear transparent fluid for the dilute solutions, I had to dilute with methylated spirit, and found that these strengths cut out much of the orange,

dimmed the entire green, and absorbed the green-blue junction, very much after the fashion of Urobilin itself.
Some violet rays are however permitted to pass, which does not occur with either Urobilin or Bilirubin; the amount of dimming in the green is, moreover, more excessive than in the case of those pigments (more nearly equal to that in the pigment of "Pink Urates" (Thudicum's Uroerythrine).
Thus the fact of absorption in the orange region, coupled with a dimming of the entire green, are the facts differentiating Carbolic acid in urine from either Bile in urine or that fluid itself in a state of high concentration.

XIII The spectroscopic Examination of certain Specimens of Pathological urines.

Case I. Note accompanying the specimen — "The urine for several days has no blood and then it suddenly appears. Diagnosis: Encephaloid carcinoma of the left kidney in a man." The urine was turbid and opaque, of faintly acid reaction, and contains several large reddish masses resembling loose blood-clots which had sunk to the foot of the glass. The microscope showed a large number of red corpuscles; the urine gave the guiac reaction. Examined without being filtered the spectrum resembled that of HbO_2 in Percentage greater than 1, but in addition a narrow band in the position of that of acid-hæmoglobin was visible in the Red.
After filtration, the band in the Red disappeared, while the two bands of oxyhæmoglobin were seen: on diluting the specimen with half its volume of water, the same changes occured. We had here evidently two superimposed spectra, one of oxyhæmoglobin, the other of some pigment having a band in the Red. Was this latter acid-Hæmatin or acid-hæmoglobin?

On Reduction with NH_4HS the band in the Red disappeared and the single band of Haemoglobin appeared; such behaviour is like met-Haemoglobin and unlike acid-Haematin. The fact of the band disappearing on dilution renders it further probable it was methaemoglobin, and not acid-Haematin, we had here, as it is well known the band in the Red of the former pigment is worst seen in dilute solutions. Of course the other bands of the met-Haemoglobin would of necessity be obscured by the superimposed spectrum of the HbO_2.

Landois and Stirling (Third Edition p 413) state that met-Haemoglobin is precipitated by "Lead acetate." I applied this reagent in the hope that it would throw down only the met-Haemoglobin, but I found that the addition of $\frac{1}{5}$th of the volume of "neutral Plumbic acetate" precipitated both pigments. On the other hand, with the same salt in the same proportions, I quite failed to precipitate any of the artificially-prepared neutral met-Haemoglobin (with Potassium Ferricyanide).

I found it was possible to imitate artificially the above mixture by taking defibrinated blood diluted with an equal volume of water and adding to that twice its volume of .2% HCl, when these two superimposed spectra were obtained. Dilution and Reduction produced the same results as in the pathological urine.

In all probability this was a case of Haemoglobinuric Haematuria, the met-Haemoglobin not being excreted from the blood but making its appearance in the acid urine either during the time it lay in the bladder or after being voided.

I believe the weak acid or exposure is quite sufficient to account in such cases for the presence of met-Haemoglobin, so that we are by no means driven to disguise the grave and rare condition of met-Haemoglobinuria.

Case II. This was merely described as "Blood in urine". The urine was very pale, of neutral reaction, and had at the bottom of the vessel three or four stringy bright red clots in which all the pigment resided, as there was no pigmentation of the supernatant fluid.
The fluid yielded no absorption-spectrum, but light reflected from the sides of the clots yielded the two bands of HbO_2, while there was no sign of Met-Haemoglobin. The absence of this pigment was probably due to the absence of acidity.

Case III. Urine from a case of "acute Nephritis". This gave acid reaction and the picric Test. Examined by the spectroscope it showed the two bands of Oxy-haemoglobin. This urine had presumably not remained long in the bladder, else some Met-Haemoglobin would have been produced.

Case IV. I was fortunate in being given some matter from a case of Haematemesis. The physician in charge of it reported it as merely vomited, and was of opinion that "it could not have been long in the stomach". The material was of a soft, jelly-like consistence, of a very dark-red colour not at all of "coffee-ground" description, was unmixed with food, gave a faintly acid reaction and emitted that peculiar pickly acid smell of gastric juice & peptone produced by it! A smear of the substance examined between two glass slides showed the two bands of HbO_2, but after 18 hours the band in the red of Met-Haemoglobin was quite distinct. This latter observation shows that, as surmised, the acid juice had not been long enough in contact with the blood to produce Met-Haemoglobin a pigment produced by weak acid as well as by exposure to air. Of this I met with many instances.

I was at this time very interested in the alleged appearance of Haematoporphyrin in the Urine of patients suffering from Addison's disease, acute Rheumatism, Pneumonia and Sulphonal-poisoning. (For this last cf Oswald in "Journal of Mental Science" Jan: 1895).

Case V. This was the urine from a case of "suspected addison's disease" in a woman in whom there was the general debility, vomiting and "some pigmentation". The urine was pale and acid in reaction: it certainly could not be described as a pigmented urine, and did not give the characteristic spectrum of Haematoporphyrin. In bright sunlight I could just detect a faint broad band about the position of the right-hand band of the pigment. Concentration of the urine led to no more definite results.

Case VI. This was also one of "suspected addison's disease in a woman". She was reported to have "great debility, and pigmentation where the clothes pressed on the loins and also in the axillae, on the neck, arms and hands; there were no buccal patches". The urine gave no spectrum of Haematoporphyrin, not even when concentrated, although sunlight revealed a band similar to that seen in Case V.

Case VII. Urine from "Unequivocal Addison's disease". This was acid in reaction: it gave no evidence of the pigment sought for. It was not a pigmented but a pale urine.

Case VIII. Urine from a case of Pneumonia. The spectrum of Oxyhaemoglobin (about .008%) was present. No Haematoporphyrin could be discovered.

Case IX. I had the rather rare opportunity of examining the urine in a case of Melanuria from "melanotic sarcoma". Examined without any dilution, the brownish-black fluid cut out all light except in the Red, and the spectrum therefore strongly resembled that of HbO_2 greater than 1%.

Diluted with one half its volume of water, a band appeared very nearly in the position of that of ~~Hæm~~ Bilirubin although its left margin was less definite than in Cholæmia. On further dilution, the spectrum became indistinguishable from that of Urobilin, so that the spectroscope cannot be relied upon in cases of Melanuria.
As to the effects of Reagents: NH_4HS produced no alteration: glacial acetic acid deepened the tint of the band in the green-blue region and caused the area of absorption to advance as a haziness to the left hand side. It also increased the depth of the band.

XIV Blood in acid and in alkaline urine.

With the view of studying the effect (if any) of the Reaction of urine upon blood, I compared the spectroscopic appearances of equal volumes of alkaline urine and acid urine to which an equal quantity of defibrinated blood had been added 36 hours previously.
When examined, both presented what would be called in clinical language a "smoky appearance".
The alkaline urine was obtained by adding a few drops of NH_4HO to neutral urine, whereas the acid urine was so naturally. In the alkaline urine the bands, although hazy as to their outlines, were still those of HbO_2, while in the acid the bands were on the point of fusing into one, but there was not the slightest trace of Acid-Hæmatin.
In twelve hours more, the bands in the acid urine had completely fused into the one of Reduced-Hæmoglobin; while those in the alkaline were still those of Oxy-Hæmoglobin, no alkaline-Hæmatin being discernable.
From another experiment, I found that blood allowed to remain in contact with urine for a very long time [illegible]

two months - until the alkaline fermentation is thoroughly established and maintained, did become alkaline-Haematin. These facts are interesting in the light of the observation that acid-Haematin and alkali-Haematin are very rare in blood-containing urines, HbO_2 and met-Haemoglobin being the forms of the pigment usually met with.
The latter indeed, and not acid-Haematin, is the pigment produced by the action of dilute acids as opposed to strong. My own observations on this point fully corroborate Hoppe's ("Journal of Physiology" Vol XVII no: 6. 1895.) who finds that general acids in weak solution convert HbO_2 first into met-Haemoglobin and thence into Haematin.
In fact we might summarise thus —
Weak acetic acid yields met-Haemoglobin, strong acid-Haematin,
Weak Sulphuric " " " " " Acid-Haematoporphyrin,
Weak Hydrochloric " " " " " Acid-Haematin,
when acting on Oxy-Haemoglobin.
Now although in the artificially acidified urine I did not get met-Haemoglobin, I did find it in the acid urine from the case of bleeding ulcer of the urinary bladder, and I never got acid-Haematin in either naturally or artificially acid urine. It is most probable that the formation of met-Haemoglobin by slightly acid urine in the body, is due (in part at least) to the relatively high temperature favouring the change: in the above experiment the temperature was not raised above that of the room (15°C).

XV On Carbon-dioxide-Haemoglobin as a distinct Pigment.

Halliburton (p 81 "Essentials of chemical Physiology") quoting C. Bohr on this subject says — "Bohr has recently advanced the theory that oxyhaemoglobin forms a compound with CO_2. He considers the union is a dissociable one ..... If this is really the case, HbO_2 appears to be not only an oxygen-carrier but a Carbon-dioxide carrier .... Bohr does not consider that the gas is united to the HbO_2 in the same way as oxygen is. Perhaps it may be united to the Proteid globin rather than to the Pigment. The subject cannot yet be considered settled. CO_2-HbO, if it does exist, shows no spectroscopic differences from Reduced Haemoglobin".

Thinking that in the light of the above state of matters any definite research would be a contribution to the subject, I made the following experiments.

I first examined spectroscopically a thin film of defibrinated blood which had been shaken up in a vessel containing pure Carbon-dioxide gas (made in the usual way by HCl on marble) as is done frequently when the Lecturer on "Respiration" wishes to show how CO_2 in blood gives it the purple, "claret" or "venous" hue.

I was a little surprised to find the spectrum of this was that of oxy-haemoglobin, two bands being distinctly present.

I next took a solution of oxyhaemoglobin (1 c sheep's defibrinated blood diluted with water to about the concentration of 1% of oxyhaemoglobin) and allowed a vigorous stream of CO_2 gas to pass through it. at the end of 2½ hours, the gas having bubbled through the solution all the time, the pigment I was still unreduced, a smear of it examined in situ on per without admitting the air, showed the two

bands of Oxyhaemoglobin, and the colour of the fluid, although of a slightly darker red, had by no means changed to the well-known "venous" purple-hue.
Under the microscope not a single corpuscle could be found in this solution, the red discs, having imbibed water and their pigment been dissolved out, had ultimately burst or become too transparent to be visible.
The undiluted defibrinated blood, however, was crammed with red corpuscles.
[I noticed that the addition of a few drops of strong HCl to the solution through which the CO_2 had been passing reduced it at once into so stable a form of Reduced-Haemoglobin that subsequent vigorous aëration failed to oxidise it. Within 24 hrs this pigment had become acid-Haematin.]
In marked contrast to the behaviour with CO_2 of a dilute solution of Oxyhaemoglobin is that of defibrinated blood through which the gas is passed.
It was a matter of no little difficulty to examine this blood by the spectroscope without re-oxygenating it in the act. If required to be in a sufficiently thin layer, and yet all the methods of obtaining it so, involved manipulation of it in the air. The following device succeeded —
The stream of gas was made to ascend through the column of blood in a Haematinometer, the upper part of which was placed before a bright light. The free surface of the blood frothed as the gas left it, and by directing the spectroscope to the thin films of blood constituting the bubbles I saw at once the single broad band of Reduced-Haemoglobin. A very beautiful phenomenon was then seen, for as each bubble burst on the free surface of the blood into the air, the pigment was immediately oxidized, and one could see the single band fade insensibly, but with great rapidity, into the two of HbO_2.
In fact we had side by side a bubble (not yet burst) of

the Reduced pigment and one (just burst) of the Oxidised. By using great dilution I could obtain a film (between two glass slides) of this Reduced Pigment (Carbon dioxide-Haemoglobin) but the rapidity with which it became oxidised was very remarkable.

If Carbon-dioxide-Haemoglobin be a separate pigment it is a compound of the greatest possible degree of instability, being oxidised with extreme facility.

Compared with Reduced-Haemoglobin, prepared by NH_4HS, the CO_2-HbO is very much less stable, or otherwise stated, the CO_2-HbO can be much more readily oxidised than the NH_4HS-HbO.

In short the combination of CO_2 with the blood-corpuscle is a very loose one, the gas being very easily displaced by oxygen.

But there is a further change; whereas the pigment Reduced by NH_4HS after being oxidised returns to its reduced state (the single band appearing again), the pigment reduced by CO_2 on being oxidised remains as HbO_2 with its two bands. The latter has been permanently broken up as a combination, whereas the Reduced Pigment made by NH_4HS is far more stable and can return to and remain in the reduced condition for a considerable time. The importance in the living animal of the great chemical instability of CO_2-HbO needs only to be mentioned, as the rapidity of this dissociation is one of the most necessary processes in Respiration. Further, the union of CO_2 with the corpuscle is a vastly looser one than that of oxygen with the pigment — a point of much physiological significance seeing that the corpuscle must carry the oxygen to the inmost cells of all tissues, while the blood coming from the tissues must be able to part with its CO_2 at the earliest possible opportunity.

With what element of the corpuscle does the CO_2 unite? Seeing that the CO_2 could not displace the oxygen from its union with the Haemoglobin when that pigment was in solution in the water (the red corpuscles having disintegrated) but did readily effect the dissociation so long as the pigment was in organic union with the Proteid Globin of the red corpuscles, we must conclude that the physiological integrity of the red discs is a sine qua non for the combination of Carbonic acid gas with one or other of the constituents.
Now it will not unite with Haemoglobin in solution, but it will Reduce Haemoglobin when in the corpuscles, or at any rate displace Oxygen from the pigment while still within the corpuscles, so that we are justified in believing with Bohr that the CO_2 unites with the Proteid element of the corpuscle.
My experiments tend to substantiate the views of Bohr as quoted above by Halliburton.
We have an explanation, I think, of the extreme dissociability of CO_2-Hbo if we conceive the union of the CO_2 as not so much a chemical combination as a physical, interstitial or intermolecular existence in the Globulin Globin; whereas the union of the oxygen with the Pigment-molecules is (to a certain extent at least) a chemical combination, this gas being actually attracted by the Haemoglobin, the energy of this union being sufficient to displace the much more loosely fixed CO_2. This "oxygen" is, of course, the movable Oxygen of Respiratory function, not that which is an integral part of the Pigment and exists, even in one-banded "Reduced" Haemoglobin. (Hb O)

XVI. On the Reducibility, Resistance to decomposition and Permanence of Haemoglobin, with some reference to the relative Permanence of certain of its derivatives.

Through the kindness of Professor M^c^Kendrick I was able to examine on (18/2/95) certain solutions of Haemoglobin which were hermetically sealed up by him in the summer of 1888.
The specimens, aqueous solutions of blood-pigment, were contained in test-tubes and were of such dilution (circa 25% of HbO_2) that I could examine them spectroscopically in situ, none of them was, luckily, turbid, although in several a deposit or precipitate has fallen to the foot of the tube.
They had been immersed for seven years.
Number I had been originally a solution of Oxyhaemoglobin from the dog, in which a small piece of the eye had been placed with the view of testing, the reducing-power, if any, of that organ. The colour of the fluid was a pale red-brown; a copious red precipitate had ensued. The spectroscope revealed the single band of Haemoglobin.
Number two had been originally a similarly dilute solution of Oxyhaemoglobin into which a small portion of cerebral cortex had been introduced. The colour resembled that of (I), although a little paler, there was also some red precipitate: its spectrum showed the two bands of HbO_2.
Number Three had been a solution of HbO_2 into which a small piece of skin had been introduced. In colour and precipitate it resembled no: 2. It showed the two bands of HbO_2.
Number Four was a dilute solution of HbO_2 without any foreign material introduced. The colour was a fine violet-red, there was no precipitate in this case: the spectroscope showed Reduced-Haemoglobin.
Number Five, like 4, had nothing put into it.

Its appearance as to colour was identical with four: it had a slight reddish precipitate. The spectrum was that of Reduced-Haemoglobin.

Number Six was a glycerine extract of a piece of fresh muscle. It yielded the spectrum of Reduced Haemoglobin.

Number Seven was a dilute solution of Oxyhaemoglobin with a piece of skin inclosed in it. The colour was a rusty-yellowish-brown and there was a copious precipitate. The spectroscope showed the two bands of Oxyhaemoglobin.

Number Eight. A small quantity of Oxyhaemoglobin from living muscle. The colour was violet-crimson, there was a copious precipitate. Spectroscopically it was Reduced Haemoglobin.

Number Nine was a similar preparation to six. It was crimson in colour: its spectrum was that of Reduced-Haemoglobin.

Of the nine solutions originally consisting of Oxyhaemoglobin, six in the course of seven years had become reduced to Haemoglobin, while three remained unreduced.

Into some of them, pieces of tissue had been introduced with the view of testing the action, if any, of these tissues upon the blood-pigment, with the rather curious result that the two solutions containing no foreign material should have become reduced, while the three which had (viz one cerebrum and two skin) should have remained spectroscopically unaltered.

In none of these tubes was there any putrefaction, they behaved as antiseptic, if not aseptic, solutions, but originally, in the very nature of things, it was impossible to sterilize the solutions (as the heat necessary to do so would have destroyed the Haemoglobin), the only germicidal process performed being the heating of the air above the liquids whilst the tubes were being fused.

I do not think these observations are sufficiently extensive to justify one reducing anything dogmatically as to the relative reducing-powers of the tissues placed in the tubes.

but this much is clear, that Oxyhaemoglobin in aqueous solution is capable of remaining (provided putrefaction be prevented) as such spectroscopically for a period of at least seven years (summer 1895).

XVII Putrefaction and Haemoglobin.

Any specimen of defibrinated blood (of sheep's or ox's) corked up in a bottle with some air between the surface of the blood and the stopper, loses, in about 24 hours, its deep crimson colour and becomes of a "claret" hue both in mass and in thin layer: in a few hours more the spectroscope shows that the pigment is now Reduced-Haemoglobin. In order to examine it spectroscopically one must have it somehow in a thin layer; but the making of this layer ipso facto introduces the liability to re-oxidation through aëration. For instance, opening the cork and pouring a little of the blood upon a glass plate (even though this is instantly covered by another plate), causing the blood to smear the inside of its own bottle, diluting some of it with water, (whether boiled or not) all introduce a certain amount of air to the blood, and with it the possibility of oxygenation. One can, however, with some care observe this: allow blood to remain in a haemat*in*ometer for two or three weeks by which time putrefaction will be thoroughly established, then, placing a bright light behind the glass, tilt it quickly but gently so that a thin film of the blood is thrown up on one side and at that moment observe the spectrum. It is seen to be that of Reduced-Haemoglobin which almost at once is oxygenated by the air acting on the film, and one can see the single band giving place to the two.

The immediate influence from this is rather interesting, for, in the first place we see that this Reduced or Putrefaction-Haemoglobin (as it may be called) is very unstable, but possesses a great affinity for oxygen of which, it would seem, the substances in the decomposing blood have robbed it.
It is still chemically and spectroscopically Haemoglobin; although reduced, its chemical integrity has not been compromised, for it can unite, on the first occasion, with any available oxygen forming HbO_2 apparently as perfect as ever. although the "blood" is decomposing, the Pigment into corpuscles (or dissolved out of them) is not decomposed, is not chemically disintegrated, for as long as it can unite with oxygen and yield the spectrum of HbO_2 we must not use the word "decomposed" of it.
The pigment is intact but reduced in and by a putrefying fluid. Blood examined after 5 months was stinking and of a brown colour and alkaline. It yielded the spectrum of Haemochromogen, so that it must have passed through the stage of alkali-Haematin. The Haemochromogen was oxidised with great ease.
We have hitherto alluded only to putrefying blood: quite otherwise is the behaviour of weak watery solutions (3%) of Haemoglobin. Observations were made from day to day on a test-tube full of such a solution. On the 4 or 5 day this loses its bright appearance (being Oxy-haemoglobin to begin with), becomes slightly turbid and of a pale, pasty-brown colour. Its reaction is neutral or faintly acid, while it yields the spectrum of Met-Haemoglobin.
Thus within the first week Putrefaction is not the order of things in dilute solutions, but a change in the direction of the oxygen's being more firmly fixed to the pigment, as it is supposed to be in Met-Haemoglobin.
This solution was made on 30/1/95 and by the 4/2 had become Met-Haemoglobin, in which state it remained until the 9/2 when there was no putrid smell.

By the 18/2 it had a slight bad odour and was reduced to Haemoglobin, in which state it remained until at least 4/3 when it was found to have lost the rusty brown colour and become of a dull pink hue rather similar to what it had as HbO_2. This dilute Putrefaction-Haemoglobin was very easily oxidised by agitation with air.
A larger quantity (150cc) of the same strength of HbO_2 became met-Haemoglobin about the fourth day and ~~became~~ continued as such for 18 days with progressive deepening of the brown tint. Within 14 days it was quite turbid!
Its remaining longer as met-Haemoglobin is evidently due merely to the larger quantity of pigment to be altered and the larger volume of oxygen (in the superincumbent air) available to do it. On the 23/2 it was once more HbO_2, in which condition it remained till 15/3.
But this latter HbO_2 was very clearly much more dilute than originally, which leads me to believe that the met-Haemoglobin was mainly decomposed into precipitable Haematin, a little deoxyhaemoglobin being re-formed.
In solutions which show no precipitate, we have either Reduced Haemoglobin formed (as in the test-tube solutions of Professor McKendrick's own experiments) or we have the Reduced Pigment slowly converted into alkali-Haematin by the alkalinity of the decomposing Blood and that reduced finally to Haemochromogen, as in the putrefying sheep's blood above described. This blood, 5 months old, showed under the microscope distorted, shrivelled corpuscles.
Of course, as McKendrick's test-tube preparations show, we can have dilute solutions of deoxyhaemoglobin resisting putrefaction for years. Wherever the Pigment splits up with the precipitation of Haematin and its precipitation, clearly ipso facto the spectroscopic method cannot be applied.

XVIII Thymol as an antiseptic in solutions of Haemoglobin and its derivatives.

I made a number of observations upon whether Thymol would exert a preservative action on these solutions, and if so, whether it left them so unaltered that they would be available for demonstration purposes after a lapse of weeks.
I added powdered Thymol to solutions of —
dilute Oxyhaemoglobin, Reduced Haemoglobin, Carbon-monoxide Haemoglobin, Neutral and Alkaline met-Haemoglobin, alkali-Haematin, Haemochromogen, acid-Haematin, and acid-Haematoporphyrin, with the result that after 8 days there was spectroscopically no change except that alkali- had become Neutral met-Haemoglobin, but already there was a certain amount of pigment precipitated in HbO_2, Co-Hbo, and Neutral met-Haemoglobin.
after 18 days the HbO_2 and Neutral met-Haemoglobin were all precipitated, the acid Haematin was turbid, and the band in the Red therefore much obscured; the Haemochromogen was of a bright green colour and its spectrum had an additional band in the Red corresponding in the position of its centre with that of alkaline met Haemoglobin; while the solutions of Haemoglobin, COHbo, alkaline met Haemoglobin, alkaline-Haematin, and acid-Haematoporphyrin remained spectroscopically in statu quo.
after one month the Co-Hbo had been precipitated in addition, the Hbo had become Haemochromogen (it had been made with NH_4HS) & the Haematoporphyrin was too opaque for examination. Those that still remained unaltered were just those which, as we shall see a little later on, do not of themselves tend to decompose, at any rate for some considerable time; I therefore do not attach any value to Thymol or any similar substance as a preservative of such solutions against chemical changes. Of course any acid such as Carbolic would be clearly quite inadmissible.

XIX The Permanence of Carbon-monoxide-Haemoglobin.

The same behaviour of small as contrasted with large quantities of CO-HbO may be observed as in the case of HbO_2. A large quantity bottled on 30/1/95 showed its two bands quite characteristically on 23/2, and as late as 15/3 was still in status quo; whereas a small quantity corked in a test-tube on 30/1 had become Reduced-Haemoglobin by 18/2, and smelt putrid with a mingled odour of coal-gas: on 4/3 it was still HbO. Neither the large nor the small quantity was ever agitated with air. In other words a solution (about 250 cc) of Carbon-monoxide-Haemoglobin in which putrefaction was allowed to proceed remained spectroscopically the same pigment for six weeks.
There is in these observations justification for the statement of the medical jurists that CO-HbO "resists putrefaction for a long time." This is true provided large quantities of the pigment are alluded to, and the time is a matter of weeks, after which reduction to Reduced-Haemoglobin occurs.
After five months the larger solution yielded the spectrum of Alkaline Haematin giving a slightly alkaline reaction. The solution was of a pale smoky-brown colour and slightly turbid.
I next tested the power of CO-HbO to resist reduction by the ordinary chemical method of using NH_4HS.
Some freshly-prepared pigment was accordingly mixed with an $\frac{1}{8}$th pt. volume of fresh NH_4HS on 19/2, and on 15/3, or in 28 days, was still CO-HbO with its two bands clearly defined; whereas some ten days' old pigment, mixed with $\frac{1}{8}$th pt. volume of NH_4HS, became decomposed in 28 days to Haemochromogen.
The former solution as late as on 17/5/95 was still spectroscopically unimpaired, it after three months'

contact with a reducing agent the Co-HbO was not decomposed. The power therefore which Co-HbO possesses of resisting decomposition in the presence of putrefaction or of a chemical reducing agent is very great and considerably greater than that of HbO_2 in the same circumstances. Co-HbO is an exceedingly stable compound.

XVII (continued) On the Permanence in air of the other Haemoglobin derivatives.

Reduced-Haemoglobin. This pigment, reduced by NH_4HS and kept well corked and not agitated with air, remains spectroscopically in statu quo for at least five months. In some specimens there is a partial conversion into "sulph Haemoglobin" (Lankester) whose affinities have been already discussed. After five months, dilution of the HbO with water oxygenated it to HbO_2, while agitation with air failed to do so. The pigment still smelt strongly of NH_4HS, and there was nothing to indicate it had entered on any phase of putrefactive decomposition.
After six months this pigment had become Haemochromogen.
Neutral Met-Haemoglobin. This pigment made with Ferricyanide of Potassium remained spectroscopically characteristic for 17 days, after which time the band in the Red became progressively fainter until at the end of 4 weeks the pigment had become Reduced Haemoglobin with a certain amount of precipitate in the now deoxidised fluid. The addition of water aërated it to HbO_2.
After five months it was still HbO without having lost the power of being oxidised.

Alkaline Met-Haemoglobin. For 17 days this was in statu quo in all respects, after one month the faint band to the left of D had disappeared, but there was no chemical or apparent physical change: after 5 months it had become alkaline-Haematin.

Acid Haematin at the end of 17 days was spectroscopically intact though a precipitate had fallen: the band in the Red persisted for 5 months.
Alkaline Haematin remained for 5 months without any kind of change.
Acid-Haematoporphyrin. This was in no respect altered after 5 months.
Haemochromogen, however, underwent a very striking change. The pigment had been made by precipitating alkaline-Haematin with $NH_4 \cdot HS$, and both the solution which had the Thymol and that which had not, became by the 17th day of a beautiful clear green colour. The change of colour from a deep portwine tint to a light green was very surprising, the reaction of course was still alkaline, and there was no precipitate or turbidity. The spectrum showed, in addition to the two well-known bands in the green, a broad area of absorption in the Red region extending from the C line to very near the D line. After one month, the two bands in the green had given place to a single broad band extending from the b line as its extreme left limit and cutting out all the blue end of the spectrum, while the band in the Red had become a definite line identical in position with that of Met-Haemoglobin. This state of matters persisted for four months: the reaction was still alkaline, but the solution had no smell of H_2S mixed with that of ammonia.

In connection with the permanence of Oxyhaemoglobin I should mention a preparation (by Hessiens successeurs of Landrin of Paris) of the pigment for administration in Chlorosis. The substance is put up in dry amorphous masses, having no crystalline structure under the microscope, readily soluble in cold water. The solution gives the two bands of Oxyhaemoglobin. As the specimen has been at least four years in the Physiological Laboratory it is evident that in certain forms Oxyhaemoglobin, guarded from putrefaction, can persist as such indefinitely.

We might therefore construct a table of these pigments arranged in the order of their powers to resist decomposition induced by Putrefaction.

A { Weak aqueous solutions of Oxyhaemoglobin; / Certain preparations of HbO2 in an anhydrous state. } Putrefaction being excluded.

B { alkaline Haematin. / acid-Haematoporphyrin.

C { acid-Haematin. / Carbon-monoxide-Haemoglobin. / Sulph-Haemoglobin. / Reduced-Haemoglobin.

D alkaline Met-Haemoglobin.

E acid or neutral Met-Haemoglobin.

F Haemochromogen.

G Oxyhaemoglobin in presence of Putrefaction.

From which classification it would appear that when protected from Putrefaction Oxyhaemoglobin is the most stable pigment, and, when exposed to it, one of the most unstable.

XX The Spectroscopy of freshly drawn blood.

Without doubt the most satisfactory spectroscopic examination of the blood is in situ in the living animal, and cases in which this was possible, owing to the transparency of the tissue illuminated, have been already alluded to; but in the vast majority of cases one has to be content with examining the blood the instant it is shed.
The very great haste is in order to prevent oxidation.
One rather rough-and-ready, though, I believe, efficient method of examining the blood as from the Jugular vein, is to lay the animal with its head & neck projecting slightly over the table and having the vein dissected out for a short distance on the side which is lowermost. You have previously cleaned two thin plates of window-glass of equal area: one of these you hold below the neck to receive the blood which pushes from the vein the instant it is cut across by a sweep of the assistant's knife. This lower plate you hold in your left hand, and as soon as the blood has flowed over it, you apply the other plate with your disengaged right hand very quickly over the blood-smeared plate, and then instantly pick up the pocket-spectroscope (previously focussed as to the Fraunhofer lines) & look at the film between the two plates. This is always thin enough to admit enough light to display the bands, and if the blood have been at all abundant you include no air nor admit it afterwards.
For small quantities from small animals like mice birds &c 2 microscopic slides serve very well: an assistant is by no means absolutely necessary.
My experiments were made upon animals which had just died i.e. Respiration had ceased before the vessel was opened, so that I was examining the spectrum of blood

possibly pass in so short a time altered from its state as circulating venous blood in a living vein.
Mr Mann believes that the blood in the right side of the heart and in the veins is reduced as soon as the animal has ceased to breathe, but that the haemoglobin of the blood in the left side of the heart and aorta does not become reduced for some time after death". I cannot do much more at present than put my observations on record although they do not all seem to be in agreement.
(1) Blood was examined in the above mentioned rapid manner from the External Jugular Vein of a kitten chloroformed till visible Respiration had ceased.
The observation was undertaken the instant the last Inspiratory spasm was made, and I obtained a spectrum with the two bands of Oxyhaemoglobin.
If a reliable observation, this is entirely against the view that Chloroform-Narcosis is a partial asphyxia and that death from Chloroform is an Asphyxia.
I, however, was never able to repeat this observation.
(2) The blood from the Internal Jugular of a dog chloroformed till Respiration ceased, yielded the spectrum of Reduced-Haemoglobin very rapidly changing on oxygenation to HbO2.
(3) a month-old rabbit was chloroformed and the blood from the Right Ventricle as well as from the Internal Jugular was examined. Each gave the single-banded spectrum of the Reduced pigment, rapidly yielding two bands on the admission of the smallest quantity of air.
(4) A large dog was chloroformed, and immediately on the cessation of Respiration blood from the Internal Jugular was examined and found to exhibit Reduced-Haemoglobin, while blood from the Left Ventricle examined afterwards yielded Oxyhaemoglobin.

Mr. Mann in his work already alluded to, found the same appearances in blood from chloroform-narcosis ff 72 & 73.
On the other hand I have been unable to corroborate his statement that "in death from asphyxia the blood, arterial as well as venous, immediately after death, gives the spectrum of reduced Haemoglobin". (4) I examined the blood from the neck of a mouse just previously drowned, and obtained the spectrum of Oxyhaemoglobin — a result which, upon reflection, may be homologated with Mr. Mann's statement if we take into account the fact that the mouse was debilitated from starvation before death. The reducing-power of the tissues is said to be diminished after starvation: reckoning with this fact, certainly explains the appearance.
Having mentioned the reducing-power of the tissues, I might here record an observation made with the view of ascertaining some notion of that capacity after death. (5) A cat was chloroformed, and after lying dead for 2 hours, by which time Rigor Mortis was well established, the blood of the Internal Jugular was examined, which yielded the bands of HbO_2. This was entirely contrary to what I anticipated and to what I observed on several later occasions.
Being enabled to examine upon two different occasions the blood from a dog killed by a poisonous dose of inhaled Hydrocyanic acid (about a 5% solution), I may here briefly record the appearances. (6) An adult Irish Terrier being poisoned by inhalation of Prussic acid, died in the characteristic manner, and within five minutes its Internal Jugular was opened. The spectrum of the blood examined between two plates was indistinguishable from that of Oxyhaemoglobin, the blood as it lay round the animal had a very bright cherry-red tint quite unlike that of "venous" blood, and I think, did not clot so quickly as normal blood does.

(7) A Scotch Terrier was similarly killed: the blood from the Left Ventricle as well as that from the internal jugular showed a 2-banded Spectrum indistinguishable from that of HbO_2. I find the Spectrum in complete agreement with the one on these points of p 89 of "The Spectroscope in Medicine". (8) Blood from the Left Ventricle of a Cat just killed (by a blow on the head) showed the two bands of HbO_2, the colour being scarlet, while blood from the Right Ventricle, whose colour was "purple", gave the single band of HbO capable of being oxidised on the admission of the smallest possible quantity of air. In the case of a cat similarly killed which had been dead 18 hours, blood from the Left Ventricle of a dark "maroon" colour gave the one band of HbO but was easily oxidised, blood from the Right Auricle also gave one band but was not so easily oxidised.

XXI

Some observations upon the crystallization of the Oxyhaemoglobin of the Rat.

These microscopical observations were undertaken with the view of determining how long it was possible to keep intact crystals of Haemoglobin mounted in gum-dammar, and of throwing any light upon the subject of the polymorphism of these crystals as worked at by Professor Halliburton whose results are given in a paper "on the Haemoglobin crystals of Rodents' blood" (Quarterly Journal of microscopical Science. August 1888).

On the 2nd of February the blood of a rat was defibrinated, one large drop of this serum was by means of a needle mixed with an equal volume of gum-dammar: This is slide A.

Preparation B was made in the same manner.

Preparation C consisted of a drop of the defibrinated blood mixed with an equal volume of water and then mounted in dammar; Prep: D was merely a drop of the defibrinated blood mixed with a drop of water, no dammar being used at all.

I may at once remark that unless the blood be defibrinated

no crystallization will take place; in other words those crystals which form so readily in serum will not form in plasma. Crystals of one kind or another formed in all four preparations, and the slides placed in the microspectroscope gave the two bands of Oxyhaemoglobin. In D the gradual formation of prismatic crystals could be strikingly observed under the High Power. They seemed to grow by insensible accretion from some invisible menstruum very slowly but steadily. Irregular, mostly four-sided, prismatic crystals, some of which were very small, were produced in vast numbers, amongst them were some well developed finely-rayed stars. These stars were six-rayed, the rays diverging from the centre at an equal angle $60°\left(\frac{360°}{6}\right)$; many of the rays had subsidiary spicules so that certain stars looked quite feathery. A curious feature to be noticed was that about 50% of these stellate acicular crystals were colourless, while some of the stars looked pinkish by transmitted light, an equal number were quite colourless, like crystals of some colourless salt.

In every other respect they were identical with crystals which were HbO_2, but "colourless Haemoglobin" seems absurd. Were they globin only? If so, globin can crystallise in very mathematical forms. Haemoglobin is soluble, while Haematin is insoluble in cold water. The Haematin therefore could not have been removed in order to leave a crystalline or other form of globin. I noticed that the coloured crystals lay in the centre of a zone of colourless, around which the water was stained. There was a larger proportion of colourless crystals in this prep: D than in any other: it had most water and less viscosity than any of the others. At present I cannot come to an adequate explanation of this phenomenon.

In Preparation C no crystals ever formed, but here the red corpuscles remained longest recognisable and intact; after the lapse of one month they seemed to have undergone very little change and to have become, in a fashion, "fixed".

Preparations A and B, though identical in their method of being made, exhibited some curious differences in the details of their crystals. Taking Preparation A, which had the most varieties, it was found that within an hour or two there was produced a very large number of rosettes whose periphery might be described as bounded by an imaginary equilateral hexagon. These rosettes were dark coloured collections of acicular or clavate crystals radiating from a centre, and could not be described as plates.

(2) The second type was also an equilateral hexagon as to its imaginary boundary-lines, but its interior was composed of a multitude of spheroidal or globular particles arranged in radiating rows. These crystalline collections, which were also of a dark red colour, were not plates either, they measured .002 mm from side to side & breadth between two parallel sides.

(3) There were also crystalline masses of acicular crystals whose periphery was hexagonal, but equilateral only as regards pairs of opposite sides. greatest distance between 2 parallel sides .004 mm, smallest .002 mm. In none of these forms (1, 2 & 3) was there any real crystalline periphery, they were depositions of clubs, globules or needles with hexagonal dispositions as to their boundary-lines and, though in one plane, were not laminae.

(4) There were, however, some real laminae both of hexagonal and polygonal form: mixed with some rhombohedral prisms.

(5) There was finally a large number of very well developed six-rayed stars with regular central angles (60°) resembling in some respects those in Prep: C but not having the same abundant development of secondary rays. The rays were large bold rhombohedral prisms some so narrow as to be almost needles. These stars were all in one part of the preparation towards the edge, occupying a district by themselves. Length of ray or radius .0025 mm. All in preps 4 were perfect after 16 days, showing no splitting: the plates were the more perfect the nearer they were to the edge of the cover-glass. It was also to be noticed that

the smaller the crystal of whatever kind, the nearer it was to the edge of the Preparation.

Turning finally to Preparation B, we find that no crystal here when newly formed showed any sign of radial or other splitting, & we have none of these composite crystals or crystal-groups.

(1) Most noticeable of the crystalline forms was a very large number of very perfect, thin, regularly hexagonal plates, of patently pure homogeneous, exhibiting no striation, radial splitting or cracking of any kind for at least 16 days. In the same part of the field as these were a number of plates irregularly hexagonal, though some were pentagons and other rectilinear figures. All these laminae were in a district by themselves, were deeply coloured although the Dammar surrounding them was also stained. After 16 days they had not cracked, the edges merely having an "eaten away" look.

(2) There was, in the next place, a very large number of irregular rhombroidal prisms differing in size from ·0005mm to ·002mm. In places these were lying quite separate from one another, while elsewhere they were piled up on one another so as to give rise here & there to a star-like form, but inasmuch as the "rays" were not in one plane they could not be said to be true stars, none of which occurred in B. In some places the individual crystals composing the pile were true plates, and as each was a little smaller than the one below it the entire pile was pyramidal.

I noticed that the most perfect crystals in B — and this is especially true of the hexagons — were formed where the Dammar was clearest of dissolved pigment.

Comparing the Water and Dammar Preparations.

The Crystals, on the whole, were larger in Dammar.

There was a much larger percentage of very small & medium sized crystals in water than in Dammar.

There were no hexagons in water.

The crystals in water degenerated much faster than those in dammar.

My observations tend therefore to corroborate those of Halliburton cf pages 190 + 191 of his paper above alluded to viz —

(1) It is possible to obtain hexagonal crystals from the blood of the common rat, the animal does not thus enjoy the undivided honour of its oxyhaemoglobin crystallizing in hexagonal prisms.

(2) As to the size of these hexagons (·002—·003 mm in diam) I quite agree with Halliburton: some of mine were as small as ·001 mm in breadth between two parallel sides.

(3) I further concur with him in finding that these hexagons are formed near the periphery of the preparation, in other words where the drying is going on most rapidly; the rapidity of loss of moisture from the dammar being evidently a factor in determining the form & size of the crystal

(4) I likewise find that many of the hexagons in splitting up, do so radially into needle-like portions.

These preparations were carefully scrutinised from time to time; when after one month it became apparent that in Prep: A the very fine stars had in many cases become disintegrated by the rays becoming separated at the centre, thus transforming the star into clusters of rhomboidal prisms. Some of the perfect hexagons had broken up into an irregular mosaic of polygonal areas. Many of the regular hexagons, many of the stars, all the smaller irregular hexagons and the other plate-crystals were intact. After three months everything in Prep: A was exactly as I found it two months previously (such disintegration as had occurred has not proceeded beyond a certain stage.

In Prep: B after the lapse of one month, many of the hexagons had become metamorphosed into feathery stars, the centre of the star being the centre of the antecedent hexagon.

After three months' interval several of the hexagonal plates

had degenerated into hexagonal areas of spheroidal granules resembling very closely a set of crystalline groups which were formed in Phys: A in this manner de novo.

From the foregoing observations I think there is evidence that there are two fundamental crystal-forms of the pat's Oxyhaemoglobin viz the elongated rhomboidal prism and the thin flat plate. The elongated prism may not always be perfectly formed but deviate from type, so that when tapering at both ends it is acicular and when at one end, clavate, from both of which varieties hexagonal crystals may be formed by the very perfect lateral coalescence of such rod-like elements in apposition in one plane. I have observed, it seems clear, a stage preceding that of perfect lateral coalescence on the one hand, and that of incipient disintegration into these prisms (clubs or wedges) on the other.

[The granule cannot be called a "crystalline form" in itself, yet it is striking that certain hexagonal crystals appear to be the limit or ultimate coalescence-form of a large number of these granules deposited in one plane in such a manner that the periphery of the figure is a regular hexagon.]

The other type is the thin flat plate of which the hexagon is merely a symmetrical variety. These plates may be 3, 4, 5, 6 or many sided, but of whatever shape, they are formed de novo each as a single thin, uniform apparently homogeneous crystal. Not being coalescence-forms of any of the prism-type, we are quite prepared to find that on disintegrating, they do not break up into radially-disposed crystal-elements, but, on drying, crack irregularly into a mosaic of small polygons.

It appears possible to preserve in Canada Balsam certain crystals of the Haemoglobin of the rat for a period of at least six months, and at the end of that time to be able to demonstrate their characteristic form.

One of the factors determining the size, degree of retention of pigment, resistance to degeneration, and, possibly, forms

of crystal appears to be rapidity of passage of moisture from the blood mixed with the dammar, or, in otherwords, the proportion of water of crystallisation seems to determine several of the characteristics of a blood-crystal: with this conclusion Professor Halliburton is in complete agreement.

Appendix I

Pigments whose spectra resemble Haemoglobin (HbO_2)

IX Solution of Cochineal (Tinctura Coccei)

To the naked eye this liquid is of a different hue from Oxy-haemoglobin, being of a violet-red.

The differences between their spectra are such that, in dilute solution at any rate, the substances could not be confused.

These points of difference may be thus stated —

(1) Both the bands of Coccus are broader than the corresponding ones of HbO_2.

(2) They thus invade more of the green region which is conspicuously absent on this account and for following reasons,

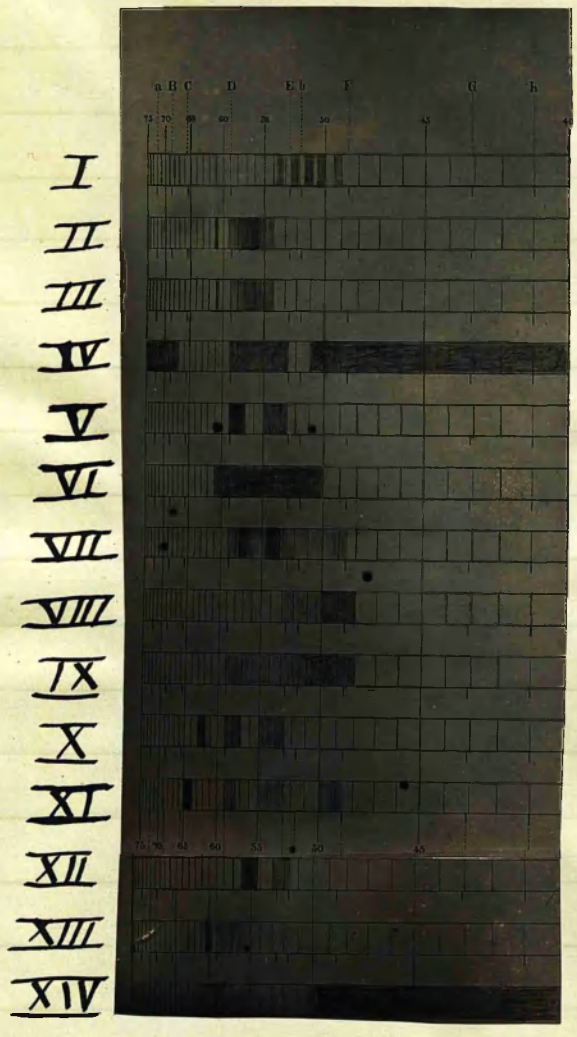
(3) The bands are not well-defined on their internal or external margins, thus rendering them hazy on the latter account, and abolishing the infra-linear green region on the former account.

(4) Compared with the Haemoglobin bands, there is not that difference in depth between them that exists between the blood-bands.

(5) The bands of the Cochineal compared with each other are equally broad, or if there is any difference between them — seeing that their edges are so difficult of definition — it is the left hand that is the broader — the opposite of what holds good for Oxyhaemoglobin.

Chart of Fourteen Spectra
alluded to in the Thesis –

a Photograph of drawings from Nature.



Names of the Spectra
figured in the chart
on the opposite page.

I Spectrum of a dilute solution of Potassium Permanganate

II Spectrum of HbO showing the adventitious band in the Red
III Spectrum of Sulph-Haemoglobin
IV. Spectrum of HbO_2 (oxyhaemoglobin) of strength at least greater than 1%. This is the only spectrum in the chart which shows the general absorption at both ends of the spectrum common to all these pigments.
V Spectrum of Oxyhaemoglobin: strength of solution about .35%.
VI Spectrum of dilute solution of Alum-Haematoxylin.
VII Spectrum of dilute solution of Borax-Carmine.
VIII Spectrum of Bilirubin.
IX Spectrum of Urobilin.
X Spectrum of acid-Met-Haemoglobin.
XI Spectrum of acid-Haematin ("four-banded").
XII Spectrum of Haemochromogen.
XIII Spectrum of acid-Haematoporphyrin.
XIV Spectrum of alkali-Haematin.

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