XXVII. OBSERVATIONS ON SOME METALLIC COMPOUNDS OF HAEMATOPORPHYRIN.

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INTRODUCTION.

It has long been recognised that haematoporphyrin forms at least three distinct types of compounds. It unites with mineral acids to form crystalline salts, *e.g.* the hydrochloride [Zaleski, 1902], in which the pigment plays the part of a base or cation, secondly it unites with strong bases to form salts, *e.g.* sodium, potassium and ammonium compounds [Nencki and Sieber, 1888], in which the pigment functions as an acid or anion, and thirdly it forms so-called metallic compounds, in which the metals enter into a more or less stable organic combination with the pigment, the metals ceasing to exist as ions. The absorption spectrum of haematoporphyrin in alkaline solution is probably to be regarded as that of the anion and the spectrum of the pigment in acid solution as that of the cation. An absorption spectrum of neutral haematoporphyrin has also been described, which may be that of the non-ionised pigment [Garrod, 1896]. The first two types are readily convertible into one another, while the pigments belonging to the metallic type are comparatively stable substances, their formation being in many cases an irreversible reaction.

The present paper deals mainly with the third class of compounds, being, in the first instance, an investigation of the spectroscopic characters of a number of synthetic compounds of pure haematoporphyrin, prepared by Nencki's method [Nencki and Zaleski, 1900], with various metallic hydroxides. The work of previous observers will be referred to in connection with each pigment.

A diffraction spectroscope was chosen for making the observations, since it yields a normal spectrum and the measurement of wave lengths is therefore greatly facilitated. On the other hand, there are certain disadvantages in the

use of a diffraction spectroscopé as compared with a prismatic one for the observation of absorption spectra. The relatively great dispersion in the red region of the diffraction, as compared with the prismatic, spectrum results in absorption bands in that area being spread over a greater length with a consequent reduction in their intensity. The fact that the sensitivity of the eye for red rays is very low, the region of maximal sensitivity being about 530μ [Ebert, 1888], further increases the difficulty of visual observation in this region. The question of the comparative efficiency of prismatic and diffraction spectra is fully discussed by Kayser [1900], from whom the following quotation is taken.

"Let us consider a prismatic spectrum, which has the same dispersion in the middle of the visible region as a diffraction spectrum, then it will be found that the prismatic spectrum while relatively extended in the blue and violet region is contracted in the red. Since it is just the latter parts which have a low intensity of light, observation in the prismatic spectrum is still possible, when it has ceased to be so in the diffraction spectrum."

Prism spectroscopes are therefore on the whole better adapted for the visual examination of pigments in gradually decreasing molecular concentrations, especially when the pigments show absorption bands in the red region. My original intention was to prepare "absorption curves" or "curves of molecular vibrations" according to Hartley's method [Kayser, 1905; Baly, 1905, p. 410] with the aid of a prism spectroscopé, but delay in obtaining suitable apparatus has compelled me to limit the present observations to solutions of only one or two molar concentrations. Although the observations are therefore incomplete, yet they have the advantage over previous analogous investigations of allowing one to compare similar molecular concentrations of the pigments. I hope shortly to be in a position to supplement the following results by plotting out the absorption curves of the pigments with the aid of a prism spectroscopé provided with means for the rapid and accurate determination of wave lengths.

The instrument used for the following work was a spectrometer provided with a graduated circle reading by opposite verniers to one minute of arc, and a transparent diffraction grating containing 14,500 lines to the inch.

The source of light employed was a small carbon arc lamp, the light being focussed on the slit of the spectroscopé. The plane of the grating was adjusted so as to be perpendicular to the incident light.

The wave lengths (λ) of the margins of the absorption bands are stated in μ or 10^{-7} cm.

Two stock solutions of haematoporphyrin of $M/1000$ concentration were prepared, one an acid and the other an alkaline solution. In calculating the molar concentrations Willstätter's [Willstätter and Stoll, 1913, pp. 44, 406] formula, $C_{33}H_{38}N_4O_6$; M.W. = 586.349, was adopted as a basis.

0.0586 g. of pure haematoporphyrin was dissolved in glacial acetic acid, 1 g. of pure sodium acetate was added, and the solution was made up to 100 cc. with glacial acetic acid (Solution 1). An alkaline solution of the same concentration was made up by dissolving 0.0586 g. of haematoporphyrin in 85 per cent. alcohol containing 10 cc. of $N/10$ caustic potash, the solution being made up to 100 cc. in a standard flask with 85 per cent. alcohol (Solution 2).

The absorption spectra of haematoporphyrin in alkaline and in mineral acid solutions are first recorded for the sake of comparison with their metallic derivatives.

$M/10,000$ haematoporphyrin in alkaline solution prepared by diluting Solution 2 with 85 per cent. alcohol.

Thickness of layer	Band I	Band II	Band III	Band IV
25 mm.	624.5-615.4	575-561	540.3-527.8	508.9-491.4

10 cc. of Solution 1 were made up to 100 cc. with 85 per cent. alcohol containing sulphuric acid (2 cc. of the concentrated acid in 100 cc. alcohol). The resulting solution is therefore $M/10,000$, and shows the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	600.7-589.3	$\overbrace{577.3-560.5-540.3}$
		Faint Dark absorption absorption

I. *The conditions under which the metallic compounds are formed.*

Haematoporphyrin was found to combine with certain metals in the presence of excess of alkali or in acetic acid solution. When the metallic hydroxide was soluble in ammonia, that alkali was used. On the other hand, it is necessary to employ caustic soda or caustic potash as the alkali in the preparation of the lead compound. No combination takes place in the presence of free mineral acids. When salts of the metals with mineral acids are added to solutions of haematoporphyrin in glacial acetic acid, it is therefore necessary to add at least an equivalent amount of sodium acetate.

II. *Compounds of haematoporphyrin with certain metals.*1. *Zinc and Cadmium compounds.*

The absorption spectrum of the zinc compound was first described by MacMunn [1889], later by Garrod [1892], and Hammarsten [1893]. It was prepared by the action of zinc chloride on an ammoniacal solution of haematoporphyrin. A $M/5000$ solution of the zinc compound in ammoniacal alcohol shows the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	591.2-569.2	559.1-527.3
10 mm.	587.4-572.1	551-534

The zinc compound can also be easily prepared as a $M/5000$ solution in glacial acetic acid in the following way. 0.01 g. of zinc acetate is added to 10 cc. of $M/1000$ solution of haematoporphyrin in glacial acetic acid. The solution is then heated for about 20 minutes on the water-bath, cooled, and made up to 50 cc. with glacial acetic acid.

The following was its absorption spectrum:

Thickness of layer	Band I	Band II
10 mm.	585-568	550-532

A $M/5000$ solution of the cadmium compound was prepared in the following way. 10 cc. of the $M/1000$ alkaline solution of haematoporphyrin were mixed with 5 cc. of concentrated ammonia, and 0.2 cc. of $M/1$ cadmium chloride, and the solution made up to 50 cc. with 50 per cent. alcohol. After standing at room temperature for 24 hours, the solution showed the following absorption bands on spectroscopic examination.

Thickness of layer	Band I'	Band II
25 mm.	596.4-578.8	560.5-542.74
10 mm.	Too faint to define	556.2-543.2

The cadmium compound cannot be prepared in solution in glacial acetic acid. Both the zinc and cadmium compounds are unstable substances, being at once converted into acid haematoporphyrin on acidification with hydrochloric or sulphuric acid.

2. *Compounds of haematoporphyrin with nickel, cobalt and iron.*

These compounds are most readily prepared from solutions of haematoporphyrin in glacial acetic acid, but can also be prepared from ammoniacal solutions. The latter two compounds were first prepared by Laidlaw [1904], and the nickel compound by Zaleski [1904].

A $M/5000$ solution of the nickel compound was prepared in the following way. 5 cc. of a $M/1000$ solution of haematoporphyrin in glacial acetic acid

were mixed with 5 cc. of *N* sodium acetate dissolved in glacial acetic acid and 0.005 g. of nickel acetate. The solution was then heated on the water-bath for about half-an-hour, allowed to cool and made up to 25 cc. with glacial acetic acid. The cobalt compound was prepared in a similar way, 0.01 g. of cobaltous chloride being added instead of the nickel salt.

These solutions showed the following absorption bands on spectroscopic examination.

<i>M</i> /5000 NICKEL COMPOUND.		
Thickness of layer	Band I	Band II
10 mm.	558.15-543.7	(522.9-510)?
<i>M</i> /5000 COBALT COMPOUND.		
25 mm.	567.8-552.9	539.4-515.2
10 mm.	564.9-553.3	(531.15-516.15)?

In both cases the first bands are well defined and persist on considerable dilution. The second bands are much fainter and their margins are difficult to define with the diffraction spectroscope. The absorption spectra of both pigments bear a striking resemblance to that of haemochromogen. The addition of mineral acid to solutions of these compounds does not produce any distinct change in their absorption spectra. They differ, therefore, from the zinc and cadmium compounds in being stable in the presence of the mineral acid. The following experiments prove this statement: 25 cc. of *M*/5000 solutions of the pigments were mixed with an equal volume of 85 per cent. alcohol strongly acidified with sulphuric acid (10 cc. of the concentrated acid made up to 100 cc. with 85 per cent. alcohol). The resulting solutions were therefore *M*/10,000.

NICKEL COMPOUND.		
Thickness of layer	Band I	Band II
25 mm.	560.55-542.7	523.9-507.4?
COBALT COMPOUND.		
25 mm.	567.3-553.3	533.6-519.2?

The synthetic compound of haematoporphyrin with iron is somewhat more difficult to prepare. The following method was found to lead to the desired result: 0.03 cc. of *M*/2 ferric chloride solution was placed in a 25 cc. flask, and 10 cc. of glacial acetic acid and a trace of pure iron wire were added to convert the ferric into a ferrous salt. The addition of metallic iron is essential. No interaction was found to take place between haematoporphyrin and iron when present as a ferric salt. On the other hand Willstätter and Stoll [1913, p. 412] have proved that iron in the ferric form can be linked with aetio-porphyrin in the presence of an equivalent amount of sodium acetate. The

solution was then heated to the boiling point on the oil-bath for some minutes, 5 cc. of *M*/1000 haematoporphyrin dissolved in glacial acetic acid were added and the fluid was maintained nearly at the boiling point for fully an hour. Finally the solution was cooled and made up to 25 cc. with glacial acetic acid. An absorption band in the red region of the spectrum closely resembling in position and character the corresponding band of acid haematin can easily be distinguished on examination with a direct vision spectroscop. This band is somewhat difficult to define on examination with the diffraction spectroscop.

M/5000 SYNTHETIC HAEMATIN IN ACID SOLUTION.

Thickness of layer	Band I
25 mm.	633-611-69

A *M*/5000 solution of pure haematin (M.W. 621.14 Willstätter), prepared from crystalline haemin, in glacial acetic acid showed the following absorption band in the red region on examination with the diffraction spectroscop. A layer of 45 mm. was found to be necessary in order to define the limits of the band with the diffraction spectroscop.

Thickness of layer	Band I
45 mm.	647.2-612

Although the fact, that the thicknesses of the layers examined were different for the two pigments, renders comparison difficult, it will be noticed that the absorption band lies farther towards the extreme red end of the spectrum in the case of natural haematin than in that of the synthetic pigment.

Since the absorption bands of reduced haematin in alkaline solution are better defined and more persistent than those of acid haematin, it was considered necessary to compare the reduced forms of the synthetic and natural pigments in alkaline solution.

Synthetic reduced haematin or haemochromogen in alkaline solution was therefore prepared in the following way from haematoporphyrin. 5 cc. of *M*/1000 haematoporphyrin dissolved in glacial acetic acid, 0.03 cc. of *M*/2 solution of ferric chloride and a trace of iron wire were heated in the same way as the previous preparation. The solution was cooled and rendered alkaline by the very gradual addition of concentrated ammonia, the solution being cooled from time to time by immersion in cold water. 0.3 g. of hydrazine hydrochloride was then added and the solution was made up to 25 cc. with 50 per cent. ethyl alcohol. This *M*/5000 solution of reduced synthetic haematin in alkaline solution or haemochromogen showed the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	562.47-556.2-539.4	526.8-506.9
	Faint absorption Dark absorption	
10 mm.	552.9-543.2	524.4-512.8
Band II is much less intense and well defined than Band I.		

For comparison with the synthetic pigment, a $M/5000$ solution of haemochromogen was prepared from pure haematin in the following way: 0.1553 g. of pure haematin (m.w. = 621.14) [Willstätter and Stoll, 1913, p. 35] was placed in a 100 cc. flask, dissolved in 70 per cent. ethyl alcohol containing 2 cc. of N caustic soda, and the solution was made up to 100 cc. with 70 per cent. alcohol. The concentration of this solution was therefore $M/400$. 4 cc. of this solution were placed in a 50 cc. flask, 5 cc. of N caustic potash and 0.5 g. of hydrazine hydrochloride were added. Finally the solution was made up to 50 cc. with N ammonia. This $M/5000$ solution of haemochromogen showed the following absorption bands on spectroscopic examination.

Thickness of layer	Band I	Band II
25 mm.	566.3-542.3	529.7-508.4
10 mm.	557.7-545.15	527.3-513.7

The absorption spectra of natural and synthetic haematin are therefore similar, but not identical.

The similarity of their absorption spectra indicates that the nickel and cobalt compounds are closely related to the reduced form of haematin. They differ from haemochromogen in their stability, being resistant to the action of mineral acids and unaffected by exposure to air. Haemochromogen, on the other hand, is rapidly oxidised to haematin on exposure to air. The stability of the nickel and cobalt compounds is probably due to the fact that both metals form stable divalent compounds; while divalent iron tends to undergo conversion into the trivalent form. Haemochromogen prepared from haematin is so unstable in acid solution, that special precautions have to be taken in its preparation [Milroy, 1905; Dhéré, 1917].

3. *Compound of haematoporphyrin with copper.*

This substance was first prepared and described by Laidlaw [1904], who also proved that it is identical with or very closely allied to the pigment turacin [Church, 1869, 1892]. I think that the pigment prepared from the urine and described by Sallet [1896] as "iron free haemochromogen" was probably also this copper compound. Sallet obtained it by the action of ammonia on urinary haematoporphyrin. Recent work [C. Powell White] indicates that a minute trace of copper is present as a constant constituent

of urine, and it, therefore, seems probable that the pigment was formed by the action of a trace of a copper salt on the ammoniacal solution of haematoporphyrin. I have repeated Sallet's experiment using pure haematoporphyrin without obtaining the "iron free haemochromogen."

The cupric compound can be easily prepared either in ammoniacal solution, or dissolved in glacial acetic acid. A $M/5000$ solution in glacial acetic acid was prepared in the following way. 0.002 g. of cupric acetate was added to 5 cc. of $M/1000$ haematoporphyrin dissolved in glacial acetic acid, 15 cc. of $N/10$ sodium acetate dissolved in glacial acetic acid were added, and the solution heated on the water-bath for about 20 minutes. The solution was then allowed to cool, and made up to 25 cc. with glacial acetic acid. The following absorption spectrum was found on examination.

Thickness of layer	Band I	Band II
25 mm.	587.8-580.2-553.3	539.4-516.6
	Faint Intense absorption absorption	
10 mm.	571-557.7	535-519.5

A $M/10,000$ solution of the copper compound in alcohol containing sulphuric acid of the same concentration as that used in the preparation of the similar solution of the nickel compound gave the following absorption spectrum.

Thickness of layer	Band I	Band II
25 mm.	572.5-553.3	538.4-517.12

Band II is much less intense and persistent than Band I.

The copper compound therefore resembles the nickel and cobalt derivatives in being stable in the presence of free mineral acids.

4. Compound of haematoporphyrin with tin.

Haematoporphyrin also forms a compound with tin which is characterised by two well-defined and persistent absorption bands, and by its great stability in mineral acid solutions.

A $M/5000$ solution of this pigment was prepared in the following way. 0.5 cc. of M stannous chloride was added to 5 cc. of $M/1000$ haematoporphyrin dissolved in glacial acetic acid, 8 cc. of N sodium acetate dissolved in glacial acetic acid were then added, the solution was heated for 20 minutes on the water-bath, then cooled, and made up to 25 cc. with glacial acetic acid. The resulting solution was filtered, and examined spectroscopically.

Thickness of layer .	Band I	Band II
10 mm.	580.7-568.7	547.5-526.8
5 mm.	579.25-569.2	545.6-528.7

A $M/10,000$ solution of the stannous compound in alcohol containing sulphuric acid was prepared in the same way as the corresponding solutions of the other pigments and showed the following absorption spectrum.

Thickness of layer	Band I	Band II
25 mm.	580.7-568.2	547.5-525.4

The stannous compound can not only be prepared synthetically from haematoporphyrin, but also directly from haematin, or from defibrinated blood.

The following method was used for preparing the pigment from pure haematin. 4 cc. of a $M/400$ solution of pure haematin, prepared in the way previously described, were placed in a 50 cc. flask, 20 cc. of glacial acetic acid were added, and the solution was then heated on the water-bath for 20 minutes. 3 cc. of $2M$ stannous chloride were then added and the heating continued for one hour. On examining this solution with a small direct vision spectroscope, the absorption spectrum of acid haematoporphyrin was recognised. 15 cc. of N sodium acetate in glacial acetic acid were added, and the fluid was again heated for half an hour. The cooled solution was then made up to 50 cc. with glacial acetic acid. One portion of the solution was diluted with an equal volume of ethyl alcohol so as to form a $M/10,000$ solution. Another portion was diluted with an equal volume of alcohol containing sulphuric acid of the same concentration as that used in the previous preparations. The filtered solutions showed the following absorption spectra.

Nature of solution	Thickness of layer	Band I	Band II
$M/10,000$ Sn compound in acetic acid and alcohol	25 mm.	586-569.7	548-531.6
$M/10,000$ Sn compound in sulphuric acid and alcohol	25 mm.	587-570	552-530

It will be noticed that the absorption bands are situated nearer the red end of the spectrum than those of the synthetic pigment, and that no marked change is produced in the absorption spectrum by the action of sulphuric acid.

The absorption bands of the stannous compound are very persistent. A $M/250,000$ solution of the synthetic pigment still shows faintly the two characteristic absorption bands examined in a layer of 10 mm. with a direct vision spectroscope. Its absorption bands have a greater power of resisting dilution than those of haemochromogen. The more intense of the two

absorption bands of a $M/100,000$ solution of pure haemochromogen, prepared by the reduction of haematin in alkaline solution with hydrazine hydrate could not be detected in a layer of 10 mm.

Stannous compound as a test for blood pigment.

The formation of the stannous compound can therefore be utilised as a very delicate test for blood pigment. The test may be rapidly carried out in the following way. Three drops of defibrinated ox blood are added to 5 cc. of glacial acetic acid placed in a test tube. The test may also be carried out equally well with much less blood; *e.g.* one drop of blood to 10 cc. of glacial acetic acid. The use of a larger amount of blood has the advantage of allowing one to trace clearly the intermediate stages in the formation of the stannous compound. The solution is then heated to the boiling point. It will be found to show the absorption spectrum of acid haematin. Three drops of $2M$ stannous chloride are added, and the solution is boiled for about one minute. When the precipitate, which forms, has been filtered off or allowed to settle, the absorption bands of acid haematoporphyrin can easily be seen on spectroscopic examination. A small quantity of solid sodium acetate is then added, and the solution again boiled. The fluid which has become brighter red in colour, is cooled and filtered. On spectroscopic examination of the filtrate the two characteristic absorption bands of the stannous derivative will be clearly seen. They closely resemble in position and character the bands of oxyhaemoglobin.

The application of the method to the examination of suspected stains for dried blood is very simple. A portion of the stained tissue is extracted by boiling with a little glacial acetic acid in a small test tube, and the solution of haematin thus obtained is treated in the way already described.

The following simple experiments illustrate the delicacy of the reaction as a test for blood pigment compared with the haemochromogen test.

1 cc. of diluted defibrinated ox blood (1 in 10 dilution in water) was added to 7 cc. of glacial acetic acid, boiled for a few minutes, mixed with four drops of $2M$ stannous chloride and again boiled gently for about five minutes. A small quantity of sodium acetate was added to the warm solution, which was then again boiled, allowed to cool, and finally made up to 10 cc. with glacial acetic acid; 5 cc. of this solution made up to 50 cc. with ethyl alcohol, and filtered, showed very distinctly the two absorption bands of the stannous derivative on spectroscopic examination. 5 cc. of the latter solution were diluted to 25 cc. and again filtered. The final stage of dilution therefore

corresponded to one volume of blood in 5000. When this solution was examined in a layer 25 mm. thick, the two absorption bands of the stannous compound could be seen, although they were faint.

The haemochromogen test was carried out for comparison in the following way.

10 cc. of a 1 in 1000 dilution of defibrinated ox blood were heated with 1 cc. of concentrated ammonia, then mixed with 5 cc. of ammonium sulphide solution, and made up to 20 cc. with water. Spectroscopic examination of this solution which corresponds with one volume of blood in 2000, showed one very faint absorption band. At a dilution of 1 in 5000, no absorption band could be detected in a layer of 25 mm.

A method for increasing the delicacy of the test.

After several other methods had been tried, the following method was found to give the most satisfactory results. Since known molecular concentrations of the various pigments have been dealt with throughout the greater part of this paper, it was considered advisable to work with solutions of oxyhaemoglobin of approximately known molecular concentration. Defibrinated ox blood having a specific gravity of 1050 was used for the test. Abderhalden's [1898] determinations of the iron as ferric oxide in ox blood were taken as a basis for calculation.

100 g. of ox blood contain 0.03805 g. of iron (calculated from Abderhalden's figures for ferric oxide). The ox blood used had a specific gravity of 1050 and 100 cc. of it therefore contained 0.03995 g. of iron; 139.8 cc. of ox blood would consequently contain 0.05584 g. of iron, and would be equivalent to 100 cc. of a $M/100$ solution of iron. 60.2 cc. of water were measured into a 200 cc. flask, and the solution was made up to 200 cc. with blood. The resulting solution is $M/200$ as regards its iron content, and, on the assumption that all the iron is present in combination as haemoglobin, the solution would also be approximately a $M/200$ solution of oxyhaemoglobin. 10 cc. of this solution were made up to 250 cc. with water. The resulting solution of oxyhaemoglobin was therefore $M/5000$. It may be, incidentally, of some interest to give the results of a spectroscopic examination of this solution compared with one of carbonic oxide haemoglobin of the same concentration.

Pigment	Thickness of layer	Band I	Band II
HbO ₂	10 mm.	585 -567	550.7-527
HbCO	10 mm.	582.1-560	548.5-522.9

2.5 cc. of the $M/5000$ solution of oxyhaemoglobin were transferred to a 500 cc. flask, and made up to 500 cc. with water. The resulting solution of

oxyhaemoglobin was therefore $M \times 10^{-6}$. 150 cc. of this solution were measured into a 250 cc. flask, 6.3 g. of pure re-distilled phenol were added, the fluid was gradually heated on the water-bath, and then kept at about 90° for 20 minutes. The solution was then saturated with ammonium sulphate (about 114 g.). Sodium chloride or sodium sulphate may be used instead of ammonium sulphate, but are less effective, the separation of the phenol being slower and apparently less complete, especially in the case of sodium chloride.

The phenol, after being thus "salted out," rose to the surface of the saline solution as a brown coloured layer containing the blood pigment in the form of haematin. Sufficient saturated solution of ammonium sulphate was added to the cooled fluid to bring the phenol solution entirely into the narrow neck of the flask. A small quantity of the phenol solution was transferred by means of a pipette to a test tube, filtered, mixed with half its volume of glacial acetic acid, and heated nearly to the boiling point. 4-6 drops of 2*M* stannous chloride were added and the solution was boiled for about two minutes, a few crystals of sodium acetate were added, and the solution was again boiled, and filtered. On examining the filtrate, placed in a test tube of about 11 mm. diameter, with the aid of a small direct vision spectroscope, the two characteristic absorption bands of the stannous compound were distinctly visible.

It is possible by means of this method to detect the presence of blood pigment in a concentration of $10^{-6} \times M/5$. In the latter case, 200 cc. of a $10^{-6} \times M/5$ solution of oxyhaemoglobin were placed in a 300 cc. flask, and 5.9 g. of phenol were added. The rest of the method was carried out in the same way as for the solution of higher concentration.

I believe that the foregoing test will be found to be the most delicate specific reaction for blood pigment as yet available, the guaiac, benzidine, phenolphthalin, and similar tests not yielding absolutely conclusive results.

Isolation of the stannous compound.

Attempts to isolate the stannous compound in a pure state have not been entirely successful. The following is a description of the method used in the preparation of the pigment. 10 g. of stannous chloride, 10 cc. of concentrated hydrochloric acid, and about 2 g. of pure finely granulated tin were heated with 100 cc. of glacial acetic acid in a round bottomed flask of one litre capacity until the greater part of the tin had dissolved. 400 cc. of glacial acetic acid and 25 g. of anhydrous sodium acetate were then added. While this solution was kept at 90° , 100 cc. of defibrinated ox blood were gradually

added. The flask containing the solution was then closed with a rubber stopper provided with a glass tube and stopcock. The solution was allowed to cool to about 80°, the flask was evacuated, care being taken that none of the fluid was lost by frothing, the glass tap was turned so as to cut off the interior of the flask, and the flask together with the glass tap was detached from the aspirating pump. The object of heating the fluid *in vacuo* was to hinder re-oxidation of the reduced haematin by the oxygen of the air. The evacuated flask was then heated on the water-bath to about 80° for 45 minutes. The solution acquired a bright red colour. A small sample of the fluid after dilution with glacial acetic acid showed on spectroscopic examination the two characteristic absorption bands of the stannous compound.

On standing for 24 hours, a large proportion of the pigment was found to have separated out mainly in the amorphous form, but partly as small rhombic crystals of red colour. At first, it was hoped that the pigment might be separated from the mother liquid as in Schälfejeff's [1885] method for the preparation of haemin; but this proved to be impossible as the pigment settles very slowly from the solution. It also proved to be impossible to separate the pigment by means of the centrifuge. The jelly-like products of the action of the acetic acid on the proteins of the blood also render filtration impossible.

The following method was therefore adopted for freeing the pigment from the excess of stannous chloride, and from part of the proteins of the blood. The solution and suspension of the pigment in glacial acetic acid was concentrated nearly to dryness by distilling off the glacial acetic acid *in vacuo* on the water-bath. The residue was washed several times with water, saturated with potassium carbonate and extracted with ethyl alcohol. The alkaline alcoholic solution containing the greater part of the pigment was separated from the aqueous saline layer, filtered, and transferred to a distillation flask. The alcohol was then distilled off under reduced pressure. The pigment residue was dissolved in water, sufficient alkali being present to bring the greater part of it into solution, the liquid was then filtered, and the pigment was precipitated from the filtrate by the addition of dilute acetic acid. This precipitate was filtered off, washed with water, and dissolved in dilute sodium carbonate solution. The alkaline solution was then filtered, and the pigment again precipitated from the filtrate by the addition of dilute acetic acid. This precipitate was finally collected on filter paper, washed with water and dried *in vacuo* over sulphuric acid.

The pigment obtained in this way is insoluble in neutral acetone, ether,

ethyl and amyl alcohols and chloroform. It dissolves in ether, ethyl and amyl alcohols, and chloroform acidified with acetic acid; but only dilute solutions can be thus obtained. It is practically insoluble in acetone containing about 2 per cent. of acetic acid; but dissolves slightly in acetone acidified with hydrochloric or trichloroacetic acid. It is only slightly soluble in pyridine, and in mixtures of pyridine with ethyl alcohol, but is more readily soluble in ammoniacal alcohol. It is practically insoluble in aniline; but dissolves readily in phenol. The pigment is insoluble in dilute aqueous mineral and organic acids, but is readily soluble in dilute aqueous solutions of the fixed caustic alkalies, in ammonia, and in dilute sodium or potassium carbonate solutions.

Further experiments are being made with the object of improving the method of preparation; but are at present too incomplete to justify description.

Determinations of the ash yielded by the pigment after purification by separation from saturated solutions in acid acetone and ammoniacal alcohol have not yielded constant percentages. The quantities of pigment dealt with were small varying from 0.14 up to 0.2972 g., and little stress can therefore be laid on the results. The solubility of the pigment in the above organic solvents is so slight that relatively large quantities of the solvents must be used in the preparation of small amounts of the pigment. One result of these estimations may prove of some significance, namely the fact that the percentage of stannic oxide yielded by the pigment was never higher than half that, which one would anticipate on the hypothesis that in the formation of the pigment one atom of tin replaces one atom of iron in the haematin molecule.

The stannous pigment is so resistant to the action of mineral acids that it may be dissolved in concentrated sulphuric acid without undergoing any distinct change in its absorption spectrum. The results of spectroscopic examination of solutions of the pigment of unknown strength in concentrated sulphuric acid are given in the following columns:

Pigment dissolved in H_2SO_4	Thickness of layer	Band I	Band II
Solution (1)	20 mm.	592.1-562.4	551.4-519
Solution (2) (more dilute)	20 mm.	581.1-568.7	542.7-527.8

This pigment is therefore even more resistant to the action of mineral acids than haematin.

5. *Compound of haematoporphyrin with lead.*

This compound is only formed in alkaline solution, being decomposed by acetic as well as by mineral acids. It was prepared in the following way.

10 cc. of *M*/1000 alkaline solution of haematoporphyrin, placed in a 50 cc. flask, were mixed with 25 cc. *N*/10 caustic soda and 0.3 cc. of *N*/2 solution of lead acetate. The solution was then heated for a few minutes to the boiling point, cooled and made up to 50 cc. with *N*/10 caustic soda. The following is the absorption spectrum of the compound:

Thickness of layer	Band
10 mm.	600-578
25 mm.	600-570

The solution has a greenish brown colour, and shows only one band in the visible spectrum.

The above mentioned metals are the only ones which have been found to form compounds with haematoporphyrin with the exception of manganese [Zaleski, 1904]. No spectroscopic evidence was obtained of the formation of metallic compounds with aluminium, uranium, silver, or magnesium in either acetic acid or alkaline solutions. The pigment, which I described in an earlier paper as resulting from the reduction of haematin with aluminium in acetic acid solution, owed its formation to the presence of a trace of tin in the aluminium powder [1901]. It is not formed when pure aluminium is used.

A SIMPLE METHOD FOR PREPARING METALLIC COMPOUNDS OF HAEMATOPORPHYRIN.

It is necessary to use pure haematoporphyrin for the preparation of metallic derivatives of known concentration; but, as a considerable amount of labour is involved in their preparation, the following simple method has proved of some value in facilitating the preparation of these pigments, and thus rendering them more accessible for observation. The proteins of defibrinated blood were precipitated with alcohol, dried and finely powdered. The precipitate, while still moist with alcohol, may also be used. 10 g. or somewhat less of the powder were then extracted with about 100 cc. of 85 per cent. ethyl alcohol acidified with sulphuric acid (2 cc. of the concentrated acid in each 100 cc. of alcohol). The dark brown solution of haematin thus obtained was filtered, a trace of aluminium powder was added to the filtrate, and the fluid was heated to the boiling point on the water-bath for at most three minutes. The solution, which acquired a bright red colour, was then filtered. Aluminium was selected as a reducing agent instead of zinc, since it does not form a compound with haematoporphyrin. On more prolonged heating the haematoporphyrin undergoes reduction, the solution becomes brown in colour and shows an absorption band in a similar position to that

of urobilin. It is therefore necessary to avoid more prolonged heating. A small portion of the filtrate was diluted with ethyl alcohol, filtered, and examined spectroscopically. It gave the absorption spectrum of haematoporphyrin in acid solution.

The greater part of the filtrate was then divided into two portions.

One of these was rendered alkaline with ammonia, and the precipitate of aluminium hydroxide, which formed, was filtered off. The alkaline filtrate, which on spectroscopic examination showed the bands of haematoporphyrin in alkaline solution, was divided into three portions. To one of these a trace of a copper salt, to another a zinc salt, and to a third a cadmium salt was added. The solutions were then heated on the water-bath for a few minutes, allowed to cool, diluted with alcohol, filtered, and examined with a spectroscope. They show the absorption spectra of the metallic compounds. The copper compound is formed very rapidly, the zinc and cadmium compounds more slowly. In the case of the latter two compounds it is advisable to allow their solutions to stand for some hours before diluting them with alcohol and examining them with the spectroscope.

Excess of finely powdered anhydrous potassium carbonate was gradually added to the other half of the acid solution. After each addition of potassium carbonate the evolution of carbon dioxide should be allowed to cease before more of the alkali is added. The fluid was then heated on the water-bath until the alcoholic solution became brown and reacted alkaline to litmus paper. After the alcoholic solution had been allowed to cool, it was separated from the aqueous saline layer, and filtered. A small portion after dilution with alcohol was found to give the absorption spectrum of alkaline haematoporphyrin.

Preparation of the lead compound. A considerable excess of *N* caustic soda was added to a small portion of the alcoholic solution, a few drops of a solution of lead hydroxide in caustic alkali were added, and the fluid was heated on the water-bath for a few minutes. The solution acquired a greenish tint and showed the absorption spectrum of the lead compound.

Preparation of the other metallic compounds. The remainder of the alkaline alcoholic solution was rendered acid by the addition of about one-fourth its volume of glacial acetic acid. Traces of zinc, copper and nickel acetates, cobaltous chloride, and stannous chloride were added to five separate portions of the acid solution. When chlorides or sulphates of the metals are added it is advisable also to add a little sodium acetate. The solutions were then heated on the water-bath for about 20 minutes, diluted with ethyl alcohol

or glacial acetic acid, filtered, and examined with the spectroscope. They showed the absorption spectra of the corresponding metallic derivatives.

Preparation of synthetic haematin in acid solution. A portion of the above mentioned acid solution was further diluted with twice or thrice its volume of glacial acetic acid, a trace of ferric chloride and a trace of iron wire were added, the solution was heated to the boiling point and kept in the water-bath for at least 30 minutes. On spectroscopic examination it was found to show the absorption spectrum of synthetic haematin in acid solution.

COMPARATIVE RESISTANCE TO THE EFFECT OF DILUTION SHOWN BY THE
ABSORPTION BANDS OF THE PIGMENTS.

Since the observations already made with the aid of the diffraction spectroscope only refer to solutions of one or two molar concentrations, and therefore leave the question of the relative intensity of the absorption bands practically untouched, it was considered essential to supplement them by determining approximately the concentrations at which the various absorption bands are just visible.

For this purpose, solutions of known molecular concentration were examined in layers of gradually diminished thickness until the absorption bands ceased to be visible. The solutions investigated were placed in a Baly's [1905, p. 413] adjustable cell, and examined with the aid of a prism spectroscope. The pigments were examined in the same order as those already investigated by means of the diffraction spectroscope. The wave lengths of the limits of certain additional absorption bands which became manifest in the red region of the spectrum are also recorded. The absorption bands previously defined are numbered in the same way as in the earlier part of this paper.

A $M/10,000$ alkaline solution of haematoporphyrin examined in a layer 37 mm. thick shows two bands in the red region in addition to those already described, (1) having the limits 680-665 and (2) having the limits 650-640. Six absorption bands are therefore visible at this concentration. When a $M/100,000$ alkaline solution of haematoporphyrin is examined, each of these bands just remains visible, when the thickness of the layer traversed by the light is not reduced beyond the limits stated in mm. given in the following columns:

$M/100,000$ ALKALINE SOLUTION OF HAEMATOPORPHYRIN.

Band (1)	Band (2)	Band 1	Band 2	Band 3	Band 4
120 mm.	55 mm.	37 mm.	22 mm.	15 mm.	15 mm.

The two most persistent absorption bands are therefore 3 and 4.

A $M/100,000$ solution of haematoporphyrin in alcohol acidified with sulphuric acid gave the following results:

Band 1	Band 2
13 mm.	8 mm.

Both bands of the pigment in acid solution are therefore more persistent than those of the pigment dissolved in alkali.

1. *Zinc and Cadmium compounds.* A $M/100,000$ solution of the zinc compound dissolved in alcohol acidified with acetic acid gave the following results:

Band 1	Band 2
11 mm.	11 mm.

The two bands are therefore equally persistent.

A $M/5000$ ammoniacal solution of the cadmium compound examined in a layer of 25 mm. shows two faint bands in the red region in addition to those already described. These bands have the following positions: (1) 645 and (2) 630–612. Their power of resisting dilution was not determined. When the solution was allowed to stand for some hours, bands (1) and (2) disappear. They were, therefore, apparently due to the presence of some unchanged haematoporphyrin.

A $M/50,000$ solution of the cadmium compound gave the following results:

Band 1	Band 2
32 mm.	30 mm.

2. *Nickel, Cobalt and Iron compounds.* $M/100,000$ solutions of the nickel and cobalt compounds gave the following results:

Pigment	Band 1	Band 2
Nickel compound	12 mm.	26 mm.
Cobalt compound	11 mm.	15 mm.

It will be noticed that in both cases bands 2 are less persistent than 1; but in the case of the cobalt compound they are of more nearly equal intensity.

The results for an alkaline solution of haemochromogen prepared from pure haematin by reduction with hydrazine hydrate are given below for comparison:

Pigment	Band 1	Band 2
$M/100,000$ haemochromogen	12 mm.	55 mm.

The difference in intensity of the two bands is more marked in this case than in those of the nickel and cobalt compounds.

Iron compounds. The characteristic absorption band in the red region given by solutions of haematin, both natural and synthetic, is much less

persistent than the absorption bands of the foregoing pigments. The solutions examined were $M/25,000$; but the results are stated for $M/100,000$ concentration to secure uniformity.

Pigment	Band
Synthetic haematin	132 mm.
Natural haematin	160 mm.

The absorption band in the red of natural haematin is therefore less persistent than that of synthetic haematin. Both pigments were dissolved in alcohol acidified with acetic acid, the acid concentration being the same in both cases.

3. *Copper compound.* A $M/100,000$ solution of the copper compound dissolved in alcohol acidified with acetic acid gave the following results:

Band 1	Band 2
6 mm.	22 mm.

It will be noticed than Band 1 is more persistent than the absorption bands of any of the pigments hitherto examined. The absorption spectrum resembles those of the nickel and cobalt compounds and of haemochromogen in the marked difference in relative intensity of the bands.

4. *Stannous compound.* A preliminary examination of a $M/100,000$ solution of the stannous compound of haematoporphyrin dissolved in alcohol acidified with acetic acid proved that further dilution was necessary. A $M/500,000$ solution was therefore examined, and gave the following results:

Band 1	Band 2
10 mm.	11 mm.

The two bands therefore appear to offer practically the same resistance to dilution. The absorption bands have also a greater intensity than those of any of the other metallic compounds of haematoporphyrin.

When higher concentrations of the stannous compound of haematoporphyrin are examined with a prism spectroscope, an additional band is seen in the red region of the spectrum.

The following is the absorption spectrum of a $M/5000$ solution examined in a layer of 25 mm.

Band (1)	Band 1	Band 2
638-620	598-563	560-517

When a $M/25,000$ solution of the stannous compound is examined in a layer 10 mm. thick, Band (1) can just be detected. It ceases to be visible when the thickness of layer is reduced beyond this amount.

The foregoing results are obviously incomplete not only as regards the limitations of the methods employed, to which reference has already been made in the introduction; but also because the observations are limited to the visible spectrum.

The great stability of the stannous compound gives rise to the view, that it would be interesting to find out what metabolic changes, if any, it undergoes within the animal body after subcutaneous or intravenous injection. The great persistence of the bands in its absorption spectrum would facilitate its recognition in the organism after injection. Further, in view of the marked optical sensitising action which haematoporphyrin exercises on the animal organism [Pfeiffer, 1911, and others] the question arises whether the stannous compound or other stable metallic derivatives of haematoporphyrin have a similar action.

At a later date, I hope to carry out work on this subject along these lines.

SUMMARY.

1. The methods of preparation and the absorption spectra of the compounds of haematoporphyrin with zinc, cadmium, nickel, cobalt, iron, copper, tin and lead have been described.

2. The resistance of the pigments to the action of mineral acids has been determined.

3. A method for the direct preparation of the stannous compound from blood or haematin has been described.

4. A delicate test, based on the preparation of the stannous compound has been devised for the detection of traces of blood pigment.

5. A simple and rapid method for the preparation of metallic compounds of haematoporphyrin has been given.

6. The resistance of the absorption bands of the pigments to dilution has been approximately determined.

In conclusion, I should like to express my deep indebtedness to Professor W. B. Morton for lending me two spectrometers for use with grating and prism.

REFERENCES.

- Abderhalden (1898). *Zeitsch. physiol. Chem.* 25, 67.
Baly (1905). *Spectroscopy*.
Church (1869). *Proc. Roy. Soc.* 17, 436.
— (1892). *Chem. News*, 65, 218.
Dhérs (1917). *Compt. Rend. Soc. Biol.* 79, 1087.
Ebert (1888). *Wiedemann's Ann.* 53, 136.
Garrod (1892). *J. Physiol.* 13, 613.
— (1896). *J. Physiol.* 17, 350.
Hammarsten (1893). *Skand. Arch.* 3, 329.
Kayser (1900). *Handbuch der Spectroscopie*, 1, 448.
— (1905). *Handbuch der Spectroscopie*, 3, 149, 248.
Laidlaw (1904). *J. Physiol.* 31, 464.
MacMunn (1889). *J. Physiol.* 10, 71.
Milroy (1901). *J. Physiol.* 27, *Proc.* xiv.
— (1905). *J. Physiol.* 32, *Proc.* xii.
Nenoki and Sieber (1888). *Arch. exp. Path. Pharm.* 24, 437.
— and Zaleski (1900). *Zeitsch. physiol. Chem.* 30, 384.
Pfeiffer (1911). *Abderhalden's Handbuch der Biochem. Arbeitsmethoden*, 5, 562.
Saillet (1896). *Revue de Méd.* 16, 548.
Schalfejeff (1885). *Ber. Ref.* 18, 232.
White. Unpublished observations.
Willstätter and Stoll (1913). *Untersuch. über Chlorophyll*.
Zaleski (1902). *Zeitsch. physiol. Chem.* 37, 59.
— (1904). *Zeitsch. physiol. Chem.* 4, 131.