LI. HAEMOGLOBIN IN RELATION TO OTHER METALLO-HAEMATOPORPHYRINS

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(Received December 18th, 1924.)

THE object of this communication is to describe some observations which seem to throw some light on the union of the pigment to the protein in haemoglobin. The absorption spectra of different derivatives of haematoporphyrin are compared with those of haemoglobin, oxyhaemoglobin and haemochromogen. Then follow some experiments on the action of alkali on haemoglobin showing the formation of a pigment still containing the protein, but having the spectrum and properties of haemochromogen; on removal of the alkali the haemoglobin is regenerated. Finally some experiments on haemochromogen and on its ester are described showing their similarity in properties and the independence of the presence of water as a solvent in the formation of the compound of the ester with carbon monoxide.

Many of the metallic derivatives of haematoporphyrin have been described and the close resemblance in spectrum between the zinc compound and oxyhaemoglobin has long been known [Schultz, 1904]. The tin compound was prepared by Milroy [1909] and is noteworthy for its great stability to acids, and for the sharpness of its absorption bands which far exceeds that of the other metallic compounds. In the present communication an aluminium compound is described which closely resembles the tin compound. The fact that the cobalt compound of haematoporphyrin having a two-banded spectrum can be reduced to a one-banded form was discovered by Laidlaw [1904]; it was found that the reduced cobalt compound was similar to the nickel compound and under the conditions here described actually showed two bands. The variety of properties and spectra shown by metallic derivatives of haematoporphyrin seems to deserve a collected description and a plate of spectra has been included.

It is apparent that three groups typified by acid haematoporphyrin, oxyhaemoglobin and haemochromogen, are present. They are independent of the valency of the metal in combination, as can be seen by noting the difference between silver and sodium, and the similarity between zinc, aluminium and tin. This could be explained most simply by supposing the spectrum to be an indication of the co-ordination number of the metal, with reference to the

pigment. Starting with the above assumption an explanation of the differences in spectrum between haemochromogen, oxyhaemoglobin and haemoglobin, can be reached. Zinc and magnesium are given a co-ordination number of 4, nickel and copper 6. Hydrogen and the alkali metals are supposed to exhibit but a slight tendency to complex salt formation and form a group apart. It will be noted that the α band becomes less intense as we pass from sodium to potassium and haemoglobin itself can be regarded as the limit to this series. This would mean that in haemoglobin the iron is only attached to the pigment by its two normal valencies. When alkali acts on haemoglobin and the spectrum and properties of haemochromogen appear the iron is completely attached to the pigment and has a co-ordination number of 6. When the alkali is removed the spectrum and characteristic properties of haemoglobin are recovered; this seems to show a definite action of the globin on the iron atom, which is temporarily destroyed by the action of alkali. In oxyhaemoglobin, according to the spectrum, the iron would have a co-ordination number of 4 with reference to the pigment. If the oxygen molecule is combined with the iron atom, which has a co-ordination number of 6, no direct action of the globin on the iron atom could occur. This conclusion is supported by the fact that oxyhaemoglobin is not acted on by alkali under conditions which cause the formation of haemochromogen from haemoglobin. The fact that haemochromogen and its ester form carbon monoxide compounds leads to the supposition that there are two forms of haemochromogen, a form in which the iron is wholly combined with the pigment and which is oxidised to haematin, and a form similar to haemoglobin, which yields the carbon monoxide compound, and from which the iron is readily split off by acid. In "ordinary" haemochromogen, if there is an equilibrium between the two forms, it is easily seen that the action of oxygen must produce, in the end, nothing but haematin. In haemoglobin there can be under ordinary conditions no possibility of the oxidisable form of haemochromogen being produced. Haemochromogen, apart from globin, cannot, so far as is known, be made to show a haemoglobin spectrum; the action of globin is apparently specific. Conversion of the globin into metaprotein can readily take place without the pigment being split off; the compound (kathaemoglobin) on reduction has only the properties of haemochromogen as regards oxygen.

These assumptions can be represented graphically as follows:

1. METALLIC DERIVATIVES OF HAEMATOPORPHYRIN.

Preparation of haematoporphyrin. The haematoporphyrin used in the following experiments was prepared by the method of Nencki. After solution of recrystallised haemin in hydrobromic and acetic acids and hydrolysis of the addition product the pigment was precipitated from solution by adding concentrated sodium acetate solution containing about 10 % of tartrate. The pigment could then be readily filtered off and after washing with distilled water all the iron was found in the filtrate. The washed precipitate was dissolved in $N/20$ hydrochloric acid and reprecipitated with the minimum amount of Rochelle salt. This method of separating the iron was found preferable to the co-precipitation of the iron and pigment with sodium acetate, and separation from the gelatinous ferric hydroxide by solution in caustic soda.

Haematoporphyrin itself does not correspond with the iron-free pigment of haemoglobin; the relation of haematoporphyrin to the true iron-free pigment has been elucidated by H. Fischer and Müller [1925]. The difference in the position of the absorption bands between haemochromogen and the artificial compound from haematoporphyrin [Laidlaw, 1904] is very clearly shown in reference to the plate. The type of spectrum remains the same however; and when a qualitative study is being made haematoporphyrin serves for the purpose.

The introduction of metals. There are many methods of introducing a foreign metallic atom into the iron-free blood pigment, and different metals vary as to their ease of substitution. Of all the metals tried zinc was found to give the haematoporphyrin derivative most easily [Schultz, 1904]. The most general methods are: (1) to act on haematoporphyrin in glacial acetic acid with the acetate of the metal at 80° ; (2) to heat the pigment to 80° for 5–10 minutes with an ammoniacal solution of a metallic salt. By method (1) manganese, iron (ferric), cobalt, nickel, copper, zinc and tin could be substituted; by method (2) iron (ferrous), cobalt, copper and lead. Magnesium can be introduced into haematoporphyrin ester by acting on it with the Grignard reagent, or the iron can be replaced directly by magnesium if haematin ester is used.

Aluminium. As the iron is trivalent in haematin and no other compound of the pigment containing a trivalent element was available, attempts were made to prepare a chromium or aluminium compound. All methods on similar lines to those adopted in the preparation of the other pigments failed and at present a chromium compound has not been produced. It was found however that by treating haematoporphyrin with pyridine saturated with anhydrous aluminium chloride at 60° a new pigment containing aluminium was produced. An examination of its properties shows that it must be regarded as the normal compound of the pigment with aluminium. It has a brilliant magenta colour and closely resembles the magnesium compound in its spectrum and shows a similar dusky yellow fluorescence. It is very soluble in both water and organic solvents, but the solution on standing gradually decomposes

with the production of brown resinous material. Like the tin compound described by Milroy [1909], its most remarkable property is its stability to acids. The metal is removed from the magnesium compound by the weakest acids, but the aluminium compound can withstand the action of concentrated sulphuric acid, and of hydrobromic in acetic acid. The solution in concentrated sulphuric acid has a blue colour which only shows a broad region of absorption in the red, but on dilution with water the original colour and absorption spectrum return. The tin compound, like acid haematoporphyrin, dissolves in concentrated sulphuric acid without change of spectrum. When arsenic trichloride is substituted for aluminium chloride under the above conditions a solution of a deep yellowish-brown colour is produced. The spectrum resembles that of alkaline haematin; on pouring into alcohol a solution of acid haematoporphyrin is at once obtained showing that no permanent alteration in the pigment has taken place.

Alkali metals. Acid haematoporphyrin itself must be regarded as the hydrogen compound corresponding to the other metallic derivatives showing the characteristic two-banded spectrum. This is justified by an examination of the sodium and potassium compounds. The alkali metals can be introduced into haematoporphyrin by shaking a solution of the pigment in dry pyridine containing 5 % of methyl alcohol with an excess of powdered sodium or potassium hydroxide; the red-brown colour of the haematoporphyrin changes to a bright magenta in the case of the sodium and to red-purple in the case of the potassium. Traces of water or even alcohol added to the solution at once remove the alkali metal yielding ordinary alkaline haematoporphyrin.

By shaking a solution of alkaline haematoporphyrin with silver oxide or carbonate preferably in the presence of alcohol, silver is introduced and the solution shows a spectrum similar to that of the copper compound. Thus the univalent silver atom when introduced shows no similarity with the alkali metals. The stability of the silver compound to light and most reagents is of interest. Hydrochloric acid only slowly liberates haematoporphyrin, silver chloride being precipitated.

Cobalt. Of all the metallic derivatives of haematoporphyrin examined only those with iron and cobalt show a reduction and oxidation effect. Laidlaw [1904] found that the ordinary two-banded cobalt derivative was reduced to a one-banded form. By acting on a solution of the cobalt derivative in caustic soda with hydrazine hydrate it was reduced to a form showing two bands, one much stronger than the other and similar to those of the nickel compound. Thus this reduced form of the cobalt haematoporphyrin is probably the normal cobaltous compound corresponding to that of nickel. Oxidation to the original two-banded form took place with great ease, and the reduction could only be made to occur in strongly alkaline solution. By acting on the reduced form with nitric oxide a spectrum similar to that of the oxidised form was produced which did not change when treated with the reducing agent. As Laidlaw showed, there was no reaction with carbon monoxide. The oxidised form of the

cobalt derivative shows little resemblance to oxyhaemoglobin. The substance is unaffected by hydrochloric acid and organic solvents, and the oxygen is not removed by a vacuum and only by the action of a powerful reducing agent. It seems clear that it involves a change from the cobaltous to cobaltic state and possibly more nearly resembles the oxidation of haemochromogen to haematin.

Collective properties of these compounds. When the absorption spectra of a series of metallic derivatives of haematoporphyrin are examined, those whose solutions have a red or purple colour can be placed in three groups which do not depend on the valency of the metal. For the type of each group we can take (1) acid haematoporphyrin, (2) oxyhaemoglobin and (3) haemochromogen. In group (1) the α band is less developed than the β band and the colour is usually purplish red or purple. In group (3) the α band is more strongly developed than the β band and the colour is usually scarlet red. Group (2) is intermediate in character. The main properties of metallic derivatives of haematoporphyrin are summarised in the following table. The figures refer to the above types of spectrum, whilst the letter H indicates a type of haematin spectrum. The property of dissolving in acetate buffer shows that the normal solubilities of the pigment are strongly influenced by combination with the metal. The ease with which the metal is removed by different acids is indicated by a positive or a negative sign.

Metal	Type of spectrum	Soluble in acetate buffer	Metal removed by			
			Water	Dilute acetic acid	Hydrochloric acid	Conc. sulphuric acid
Na	1	.	+	.	.	.
K	1	.	+	.	.	.
Ag	3	-	-	-	+	.
Mg	2	-	-	+	.	.
Zn	2	-	-	-	+	.
Al	2	+	-	-	-	-
Mn	H	+	-	-	-	+
Fe ^{••}	3	-	-	-	±	.
Fe ^{•••}	H	-	-	-	-	+
Co ^{••}	3	.	-	.	.	.
Co ^{•••}	2	+	-	-	-	+
Ni	3	-	-	-	-	+
Cu	3	-	-	-	-	+
As	H	.	+	.	.	.
Sn	2	+	-	.	-	-
Pb	H	.	-	+	.	.

2. THE ACTION OF STRONG ALKALI ON HAEMOGLOBIN.

In some unpublished observations J. S. Haldane found that when haemochromogen was prepared by adding caustic soda to reduced blood the mixture would, on standing, revert to haemoglobin. Experiments were tried in imitation of this, using different concentrations of alkali, and ferrous sodium tartrate as a reducing agent. In $N/2$ alkali the reduced haemoglobin shows a strong haemochromogen spectrum but the solution on dilution with water

(being kept reduced) departs widely from Beer's absorption law, equivalent thicknesses, showing less of the haemochromogen spectrum as dilution proceeds until finally the absorption due to reduced haemoglobin becomes dominant. This clearly shows the reversible nature of the action of alkali.

When the solution of reduced haemoglobin in $N/2$ alkali is oxidised haematin results together with oxyhaemoglobin. It was found that, though the solution showed the haemochromogen bands only, about 50 % of the haemoglobin remained undecomposed. If, however, the solution in $N/2$ alkali was warmed it became of a lighter scarlet colour, and showed no alteration of absorption spectrum on dilution. When oxidised it yielded haematin alone—whether diluted or undiluted—showing a complete and irreversible decomposition into haemochromogen. Experiments were then tried using stronger solutions of alkali and all the solutions were cooled. As the experiments are only concerned with the *reversible* action of alkali, precautions had to be taken to avoid irreversible haemochromogen formation taking place. It was not found possible under the conditions used completely to eliminate this action, but it could be reduced to a low enough level not to interfere with qualitative experiments. It was found that minute traces of almost any foreign organic material—cork, wax, filter paper, or organic solvents in the soda solution—would cause an almost instantaneous irreversible haemochromogen formation, especially when the concentration of alkali added was high. In the absence of such impurities no such rapid action took place. Similarly hydrosulphite could not be used as a reducing agent; fortunately alkaline ferrous tartrate did not prove injurious even in a large excess.

The experiments were of the following type—a 1 % solution of haemoglobin either from washed corpuscles or defibrinated blood was reduced and cooled; to it was added a cooled solution of 40 % sodium hydroxide containing a trace of the reducing agent (ferrous tartrate) until a definite concentration of sodium hydroxide was reached; the mixture was then examined. At -5° conversion into haemochromogen was not complete until the solution contained 20 % of sodium hydroxide. Such a solution had, when reversible, a scarlet colour, more brilliant than when the solution was irreversible; no difference has been detected up to the present in the bands, and on oxidation both are completely converted into alkaline haematin. The solution in both cases seems to contain ordinary haemochromogen. The change from reversibility to irreversibility must be due to a change in the protein—as is evidenced by a turbidity which often shows itself. To test the mixtures for reversibility they were diluted with an ice-cold solution of an ammonium salt (either sulphate or chloride) to which a little Stoke's reagent had been added. The soda is thereby replaced by ammonia which has comparatively little action on haemoglobin. Under these conditions the scarlet colour of the haemochromogen changed to the purple of haemoglobin, and the change was usually almost complete in 5 minutes. If irreversible haemochromogen formation had taken place the solution, instead of being clear, was turbid and remained

scarlet indefinitely. The oxyhaemoglobin obtained after the alkali treatment had an absorption spectrum identical with that of the untreated substance, no shift in the bands being observable.

When the amount of alkali was increased so that there was 30 % in solution the pigment was completely precipitated as a scarlet flocculent mass, still showing the haemochromogen spectrum. The mixture could be converted into a clear solution of haemoglobin on treatment with an ammonium salt. If, however, it was allowed to warm to room temperature the precipitate swelled up slightly and became of a paler colour; it was then insoluble in the solution of an ammonium salt and gave no haemoglobin. When a solution of haemoglobin from twice washed corpuscles was used for the experiment the precipitate had a decidedly crystalline appearance and was intensely scarlet. A small quantity was filtered on asbestos in a Gooch crucible surrounded by an ice and salt jacket and washed twice with 40 % sodium hydroxide. The washings were colourless. On treating the residue with dilute ice-cold ammonium sulphate solution it partially dissolved forming a clear solution of haemoglobin. The insoluble portion consisted of coagulated protein and haemochromogen. From the homogeneous appearance of the precipitate obtained by the action of 30 % sodium hydroxide and from the fact that it will yield haemoglobin after washing with 40 % sodium hydroxide, it is concluded that it is a compound of haemochromogen and globin, probably of a colloidal nature. In solution in 20 % sodium hydroxide the haemochromogen cannot be precipitated in the presence of a calcium salt, whilst in the absence of globin no haemochromogen can remain in solution.

Thus, whilst the *specific* action of globin on haemochromogen is temporarily inhibited by alkali, the protein itself is capable of resisting the reagent. It now became important to examine the behaviour of oxyhaemoglobin and carboxyhaemoglobin under the same conditions. It is usually believed that oxyhaemoglobin yields haematin with moderate concentrations of alkali, but under the above conditions this is found not to be the case. Oxyhaemoglobin remained unaltered in colour and absorption spectrum in solutions of sodium hydroxide of concentrations up to 30 % and precipitation of the pigment did not occur. Carboxyhaemoglobin behaved similarly. When the solution of oxyhaemoglobin containing alkali was submitted to a vacuum both haematin and haemochromogen were formed, showing that the oxygen was still dissociable and that the haemoglobin, presumably formed, suffered decomposition by the alkali into haemochromogen which then became partially oxidised.

3. HAEMOCHROMOGEN IN PYRIDINE.

A solution of haemochromogen in dry pyridine can readily be prepared. Whereas haemin dissolves in the moist solvent with a red-brown colour (giving the characteristic haematin-pyridine spectrum), in the dry solvent a brown solution is obtained having the absorption spectrum of acid haematin. This

brown solution is completely reduced to haemochromogen on shaking for 1 minute with metallic mercury. Haemin thus resembles ferric chloride in the ease with which a chlorine atom is removed when dissolved in a dry organic solvent, the iron being reduced to the ferrous state. If haemin is added to dry piperidine, haemochromogen is at once produced without the addition of mercury.

The solution of haemochromogen in dry pyridine was bright scarlet and stable in air, not forming haematin as in the case of the aqueous solutions. Dry oxygen was passed through this solution at room temperature for 1 hour, and no change was observed either in the colour of the solution or in its absorption spectrum. The resistance to oxidation was diminished as increasing amounts of water were added. If an equal volume of distilled water was added the haemochromogen was completely converted into haematin. Coal gas passed through the solution produced no change in the absorption spectrum. Thus both the oxidation of haemochromogen to haematin and the formation of the carbon monoxide compound are prevented by pyridine, and if there is an oxygen compound analogous to that with carbon monoxide it might be thought to play a part in the oxidation of haemochromogen to haematin.

4. METHYL ESTER OF HAEMOCHROMOGEN.

Amorphous haematin ester was obtained by washing an ethereal solution of haematin, methylated with dimethyl sulphate, with sodium carbonate solution; after drying with anhydrous sodium sulphate the solid ester was obtained by distilling off the ether. The dried product was dissolved in absolute alcohol and treated with the minimum quantity of hydrazine hydrate in an atmosphere of coal gas. Immediate reduction and rapid conversion of the haemochromogen ester into the carbon monoxide compound took place. The solution was warmed to 60° and diluted with water until precipitation of the pigment commenced. After cooling the solid was filtered off in an atmosphere of coal gas and washed with distilled water saturated with coal gas and containing a trace of hydrazine hydrate. It was dried in coal gas, all contact with air being avoided until the moisture had all been removed. The compound consisted of a pure Bordeaux-red powder, made up of minute hexagonal plates. It is soluble in all the usual organic solvents forming brilliant carmine red solutions which decompose in a few seconds, forming dull-brown solutions of haematin ester. If the solvent is carefully dried the action is retarded, but oxygen and moisture from the air rapidly produce oxidation. The absorption spectrum agrees closely with that of carbon monoxide haemoglobin. When the carbon monoxide ester of haemochromogen was dried over P_2O_5 in coal gas and dissolved in dry xylene, a stable carmine solution was obtained if air was excluded. But on passing dry oxygen the solution at once turned brown and was found to contain haematin ester. Even when great precautions were taken to avoid the presence of moisture haematin was produced by the action

of oxygen. The solid ester compound could not be kept; even over P_2O_5 it was gradually converted into a black powder.

It was found that pyridine not only prevents the formation of the carbon monoxide compound of haemochromogen but also causes the decomposition of that substance. When a little of the dry carbon monoxide compound was added to dry pyridine, the substance dissolved with vigorous effervescence forming a scarlet solution having the absorption spectrum of haemochromogen.

A concentrated solution of oxyhaemoglobin was prepared by centrifuging blood and laking the corpuscles with ether. One drop of this was added to 2 cc. of dry pyridine in a test-tube. Protein was precipitated and in the precipitate bubbles of gas were entangled. On standing 1 minute the clear liquid was scarlet red and showed the absorption spectrum of haemochromogen. Methaemoglobin prepared with ferricyanide and dialysed did not produce a trace of haemochromogen when added to pyridine but simply the brown-red solution of haematin.

SUMMARY.

1. Metallic derivatives of haematoporphyrin showing two-banded spectra can be divided into three classes which are independent of the valency of the metal.
2. Haemoglobin is completely changed to a substance having the spectrum and properties of haemochromogen by high concentrations of alkali. In the cold the action is reversible.
3. Oxyhaemoglobin and carbon monoxide haemoglobin are unaltered by high concentrations of alkali in the cold.
4. Haemochromogen in dry pyridine is not acted on by free oxygen, neither is a carbon monoxide compound formed.
5. Haemochromogen ester in organic solvents shows the characteristic reactions of the free substance.
6. Pyridine causes the decomposition of the carbon monoxide compound into free haemochromogen ester. Pyridine forms haemochromogen from oxyhaemoglobin as well as from haemoglobin.

I wish to thank Sir F. G. Hopkins for his kind encouragement and advice, and Dr Haldane for his private communication. I desire also to express my gratitude to the Department of Scientific and Industrial Research for grants held during the period of this research.

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