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The Reaction of Haemoglobin and some of its Derivatives with *p*-Iodophenylhydroxylamine and *p*-Iodonitrosobenzene

By H. JACKSON and R. THOMPSON
The Christie Hospital and Holt Radium Institute, Manchester 20

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Evidence has recently been presented that *p*-iodophenylhydroxylamine ($I.C_6H_4.NHOH$) and *p*-iodonitrosobenzene ($I.C_6H_4.NO$) are produced during the metabolism of *p*-iodophenylurethane and *p*-iodoaniline in the rat (Crick & Jackson, 1952, 1953). These metabolites combine firmly with haemoglobin so that erythrocytes become effectively labelled when radioactive iodo-compounds are used. Concurrently, methaemoglobinaemia develops but disappears in the course of an hour or two without loss of radioactivity from the cells.

Phenylhydroxylamine ($C_6H_5.NHOH$) and nitrosobenzene ($C_6H_5.NO$) are known to induce methaemoglobin formation in red cells, and combination occurs between haemoglobin and nitrosobenzene (Heubner, Meier & Rhode, 1923; Jung, 1940). The reactions of haemoglobin and some of its derivatives with phenylhydroxylamine and nitrosobenzene have been studied spectroscopically by Keilin & Hartree (1943) who concluded that the complexes formed were unstable and easily dissociated.

Radio-isotope work described in this paper using *p*[^{131}I]-iodophenylhydroxylamine and *p*[^{131}I]-iodonitrosobenzene has shown that stable complexes are formed with oxyhaemoglobin, carboxyhaemoglobin and methaemoglobin, and that reaction may occur without apparent change in the absorption spectrum of the pigment. It seems likely that similar interpretations may apply to the reactions with phenylhydroxylamine and nitrosobenzene.

These reactions may be of importance in relation to the known toxic effects of aromatic nitro- and amino-compounds used in industry and medicine, for such substances may be metabolized to hydroxylamino- and nitroso-compounds which could be responsible for the observed toxic effects.

EXPERIMENTAL

The preparation of *p*[^{131}I]-iodophenylhydroxylamine (IPhNHOH) and *p*[^{131}I]-iodonitrosobenzene (IPhNO) has been described elsewhere (Crick & Jackson, 1953).

Crystalline rat oxyhaemoglobin. A modification of the method of Drabkin (1946) was used. The stroma from washed, haemolysed cells (1 vol. of cells to 10 vol. of water) was first removed by filtration through filter paper (Whatman, no. 30, double) and through a Seitz filter. Crystallization was induced by addition of solid $(NH_4)_2SO_4$ to opalescence, followed by refrigeration, and the product further purified by recrystallization three times from phosphate buffer (Sørensen, 0·067 M, pH 6·6).

Alkaline haematin. This was prepared by the method of Anson & Mirsky (1929–30) and twice recrystallized. For use it was dissolved in a mixture of acetone (1 vol.) and 0·5 M-K_2HPO_4 (3 vol.).

Reactions with IPhNHOH *and* IPhNO. Both IPhNHOH and IPhNO are virtually insoluble in water, and for use were dissolved in a minimal amount of absolute ethanol. The hydroxylamine is unstable in solution, especially in aqueous alcohol, and must be freshly prepared; the pure nitroso-compound is more stable. The technique used in studying their reactions with the pigments is illustrated by the following typical example:

A solution of oxyhaemoglobin in phosphate buffer (10 ml. of solution containing 580 mg. of pigment) was treated with a solution of IPhNHOH (25 mg. in 0·5 ml. of ethanol) and the mixture shaken for a few minutes. The violet solution was centrifuged to remove a small amount of debris and any excess reagent, and the supernatant treated with solid $(NH_4)_2SO_4$ added in small portions, to cause partial separation of the pigment. The latter was removed by centrifuging, dissolved in phosphate buffer (pH 6·6) and again partially precipitated. The separated pigment was again removed and dissolved in a suitable volume of the same buffer. From the radioactivity present in the solution (75 000 counts/ml.) and the specific activity of the IPhNHOH (104 counts/μg.), the amount of this latter substance associated with the pigment

was calculated (721 μg./ml.). Since the Fe content of the solution (89 μg./ml.) was known the combining ratio of the pigment and IPhNHOH (Fe:IPhNHOH) could be calculated (89/56:721/235 in the example, or 1:1·94). The process of solution in buffer and partial precipitation with $(NH_4)_2SO_4$ was repeated as necessary to a constant combining ratio.

Estimation of radioactivity was made by the liquid counting technique, whilst Fe was determined in samples ashed in a Pt basin using thiolacetic acid (Jackson, Klein & Wilkinson, 1935). A quartz spectrophotometer (Unicam) was used for spectrophotometric measurements; solutions for these were adjusted so that the concentration of iron was always 400 μg./100 ml.

RESULTS

The reaction of oxyhaemoglobin with p-*iodophenylhydroxylamine and* p-*iodonitrosobenzene*

Except where specifically stated, IPhNHOH and IPhNO labelled with radioactive iodine were used in the reactions described below. Addition of one molecular equivalent of either IPhNHOH or IPhNO to a solution of crystalline oxyhaemoglobin caused the rapid formation of spectroscopically similar violet-red pigments, I (Fig. 1), with combining ratio 1:1 (Table 1). This material did not react further with added IPhNO, but rapidly combined with IPhNHOH (1 mole equivalent) to form a more intensely violet pigment, II (Fig. 1 and Table 1). The absorption spectrum of this substance soon changed on standing, owing to the formation of a third compound, III (Fig. 1), without loss of combined radioactive material (Table 1). This substance underwent a final slow transformation to a brown pigment, IV (Fig. 1), spectroscopically similar to methaemoglobin, with the loss of one half of the associated radioactivity; the final combining ratio was thus 1:1 (Table 1). This material, IV, remained stable for some weeks at least without change in the combining ratio.

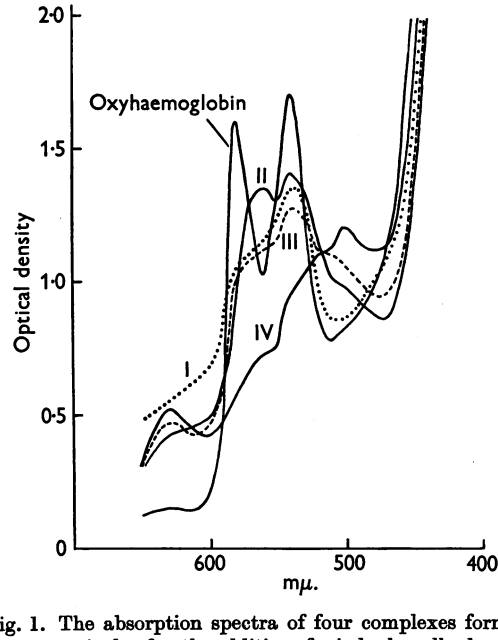


Fig. 1. The absorption spectra of four complexes formed consecutively after the addition of *p*-iodophenylhydroxylamine to a solution of crystalline rat oxyhaemoglobin.

Excess of IPhNHOH added to oxyhaemoglobin solution yielded compound II directly; excess of IPhNO gave I only. The latter substance slowly changed on standing into methaemoglobin-like pigment without loss of radioactivity, so this end product is presumably IV. Addition of an excess of IPhNHOH to substances III and IV caused their conversion back into II (Fig. 2), changes which must be due to the reducing power of the hydroxylamine.

Two experiments were set up as follows: (*a*) oxyhaemoglobin solution (1 mole equivalent) +IPhNHOH, radioactive (1 mole equivalent); (*b*) oxyhaemoglobin solution (1 mole equivalent) +IPhNHOH, inactive (1 mole equivalent). Compound I was formed in each instance, radioactive in (*a*) and inactive in (*b*). To the product from reaction (*a*) another molecular equivalent of inactive IPhNHOH was added, and a similar amount of radioactive IPhNHOH to (*b*). The reactions were allowed to proceed via the complex III to the final stage (compound IV) and the methaemoglobin-like pigment separated in each case. That from reaction (*a*) proved to be radioactive (combining ratio, 1:1·2), whilst (*b*) furnished pigment free from radioactivity. The relative amounts of radioactivity, associated with comparable solutions of the two pigments were 33 700 counts/ml. for (*a*) and 320 counts/ml. for (*b*). These two experiments clearly establish that the IPhNHOH combining in the reaction I → II (Fig. 2) was subsequently lost in the conversion of the pigment III → IV (Fig. 2); also that adsorption of radioactive material during the separation of the pigment complexes was not likely to be a complicating factor in these reactions in general.

Compounds I, II and III were easily reduced by dithionite to pigments spectroscopically similar to haemoglobin (V and VII, Fig. 2), which could then be oxygenated to red pigments (VI and VIII, Fig. 2) with the absorption maxima of oxyhaemoglobin (Fig. 3 and Table 1), all without change in the amount of associated radioactive material. The red, oxygenated pigment solutions slowly oxidized in air (as does oxyhaemoglobin itself) to the brown pigment IV (combining ratio, 1:1).

Since the oxygenated pigment, VIII (Fig. 2), was believed to contain only —NHOH groups, the following experiment was undertaken to find out whether the IPhNHOH molecules were attached to

spatially equivalent positions in the haemoglobin molecule. A solution of oxyhaemoglobin was treated with a slight excess of inactive IPhNO (1·1 mole equivalents), and to the violet product (I) a further molecular equivalent of radioactive IPhNHOH was added. The pigment (II) so formed was then reduced with dithionite, oxygenated, and allowed to stand until conversion into IV was complete. The brown pigment was separated in the usual manner and was found to be free from radioactivity. If the IPhNHOH molecules were attached in a symmetrical manner the loss of only one half of the radioactive molecules would be expected in the change VIII → IV (Fig. 2). As all the radioactive molecules were lost, it may be concluded that the attached IPhNHOH molecules in compound VIII were differently situated.

Reactions with carbon monoxide

The reactions of the various pigment complexes referred to above with carbon monoxide were also examined. Compounds I, II and III reacted directly with the gas, forming carboxyhaemoglobin-like pigments (X, XII and XIII, Fig. 4; see also Fig. 5) without change in the combining ratio, Fe/radioactive iodine (Table 1). The complex XIII, unlike the others, possessed an absorption maximum at 630 mμ. in addition to those characteristic of carboxyhaemoglobin. This absorption maximum was also present in the parent compound III. Both oxygenated pigments VI and VIII also combined readily with carbon monoxide, forming derivatives spectroscopically similar to carboxyhaemoglobin without loss of radioactive material (XI and IX,

Table 1. *Derivatives of haemoglobin combining with* p-*iodophenylhydroxylamine and* p-*iodonitrosobenzene*

For explanation of Table see text. No estimations were carried out on compounds V and VII, but their combining ratios may be inferred, since the ratios of the antecedent complexes (I and III) were the same as those of succeeding derivatives (VI and VIII respectively).

Compound	Suggested formula	Combining ratio (molecules I-compound/atom Fe)	Absorption maxima (mμ.)
I	Hb.IPhNO	0·86 1·1 1·0 1·1 1·2	— 538
II	Hb(IPhNO)(IPhNHOH)	2·0 1·8 1·8	560 538
III	Hb(IPhNO)(IPhNHOH) (ferric)	2·1 2·0 2·0 1·8	630 538
IV	MHb.IPhNO (ferric)	0·89 1·0 1·0 1·2	630 500
V	Hb.IPhNHOH	—	— 560
VI	HbO_2.IPhNHOH	1·0 1·2	580 540
VII	Hb.$(IPhNHOH)_2$	—	— 560
VIII	$HbO_2(IPhNHOH)_2$	1·8 1·9 1·8	577 539
IX	COHb.$(IPhNHOH)_2$	1·8 1·8 1·9	570 538
X	COHb.IPhNO	0·83 0·94	570 538
XI	COHb.IPhNHOH	1·1 1·2	570 538
XII	COHb(IPhNO)(IPhNHOH)	1·8 1·8	570 539
XIII	COHb(IPhNO)(IPhNHOH) (ferric)	1·8 —	630 570 540
	HbO_2	—	577 539
	COHb	—	570 538
	Haematin.IPhNO	1·1 0·87 1·3 0·83	

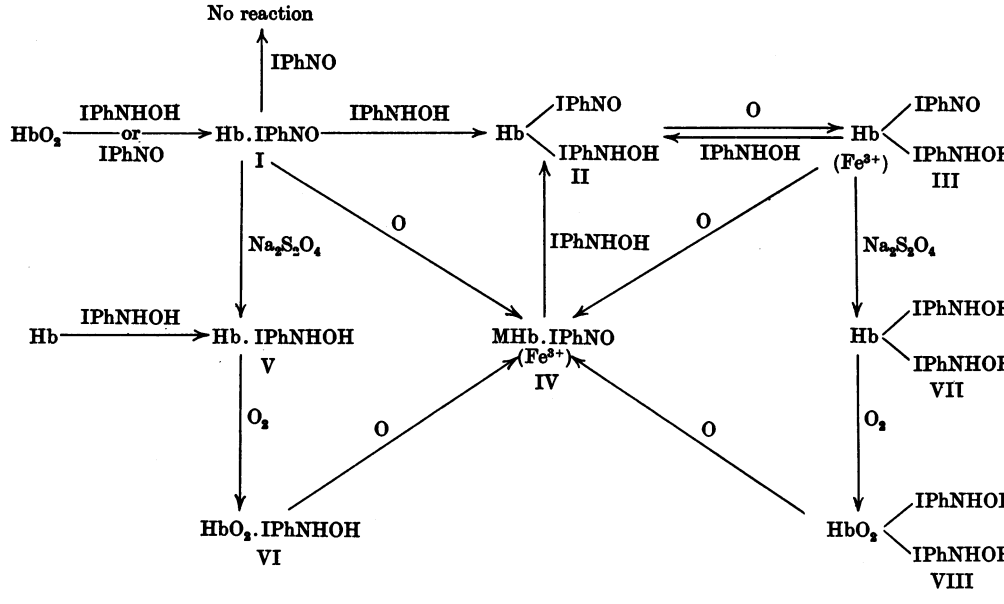


Fig. 2. Proposed scheme for the reactions of oxyhaemoglobin and haemoglobin with *p*-iodophenylhydroxylamine (IPhNHOH) and *p*-iodonitrosobenzene (IPhNO). Except in the complexes III and IV, the iron atom is considered to be Fe^{2+}.

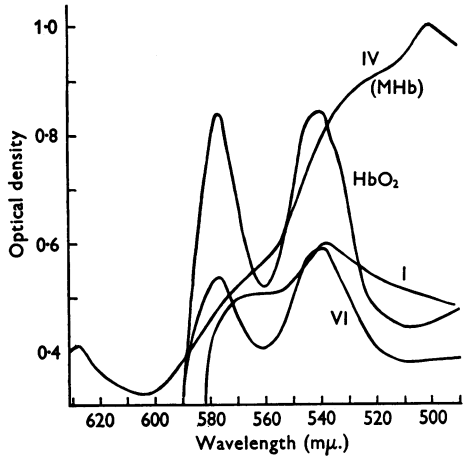


Fig. 3. The absorption spectrum of oxyhaemoglobin compared with HbO_2.IPhNHOH (VI) formed by reduction and oxygenation of Hb.IPhNO (I). Both complexes slowly oxidize to MHb.IPhNO (IV), the absorption curve for which is presented with a modified ordinate.

Fig. 4 and Table 1). The compounds IX and XIII separated in crystalline form, although no serious attempt was made to prepare the derivatives in this state.

Carboxyhaemoglobin itself reacted directly with IPhNHOH or IPhNO without change in absorption spectrum (IX and X, Fig. 4 and Table 1) so that no reaction would be suspected in the absence of a radioactive-tracer technique. Two molecular equivalents of IPhNHOH per atom of iron combined, but only one of IPhNO (Table 1). Quantitatively these reactions resemble those between oxyhaemoglobin and the two iodo-compounds, but oxidation of IPhNHOH to IPhNO could not occur in the reaction of the hydroxylamine with carboxyhaemoglobin, since an atmosphere of carbon monoxide was maintained throughout. IPhNHOH can therefore react directly with haemoglobin and prior or simultaneous oxidation to the nitroso-compound need not occur. Confirmation of this was sought in the following manner. A solution of oxyhaemoglobin was completely reduced with excess dithionite and IPhNHOH (1 mole equivalent) added. When the mixture was oxygenated the colour soon changed to bright red and spectroscopic examination of the solution gave the sharp absorption bands of oxyhaemoglobin. The separated pigment proved to be radioactive with a combining ratio of 1:1 (VI, Fig. 2). If reaction between the haemoglobin and IPhNHOH had not occurred before oxygenation, then the formation of the violet-red pigment I would be expected.

Reaction of haematin with p-*iodophenylhydroxylamine and* p-*iodonitrosobenzene*

Removal of the globin from the pigment complex III by means of acetone–hydrochloric acid mixture as specified by Anson & Mirsky (1929–30) was not

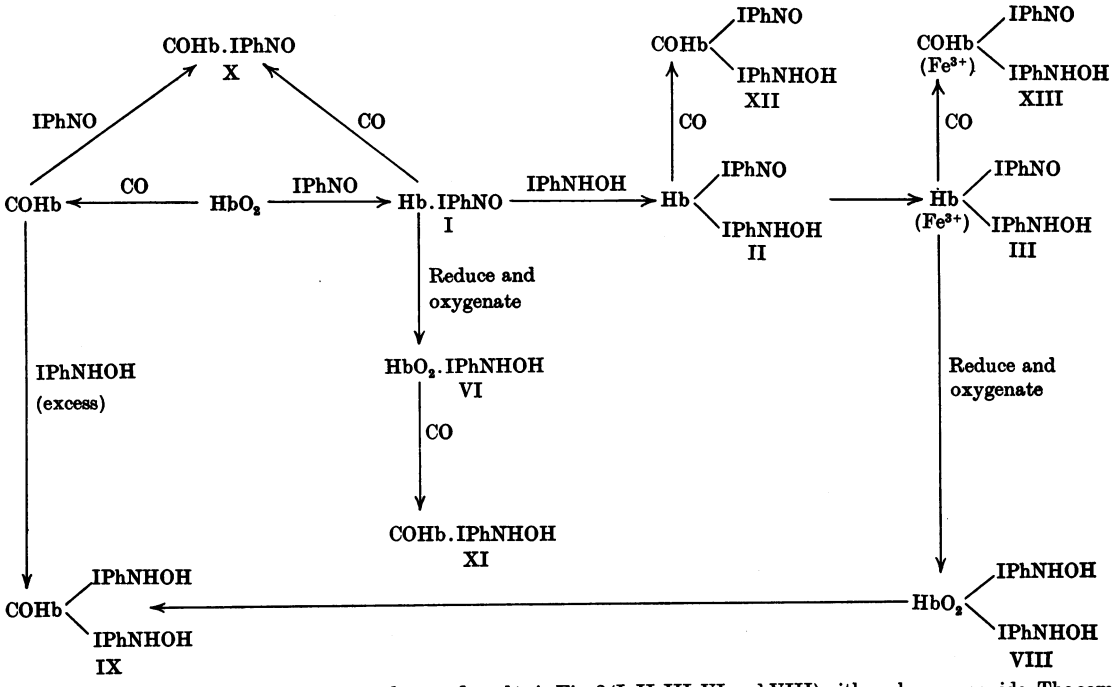


Fig. 4. Reactions of various pigment complexes referred to in Fig. 2 (I, II, III, VI and VIII) with carbon monoxide. The combination of carboxyhaemoglobin with IPhNHOH and IPhNO produces two complexes which fit into this scheme (IX and X).

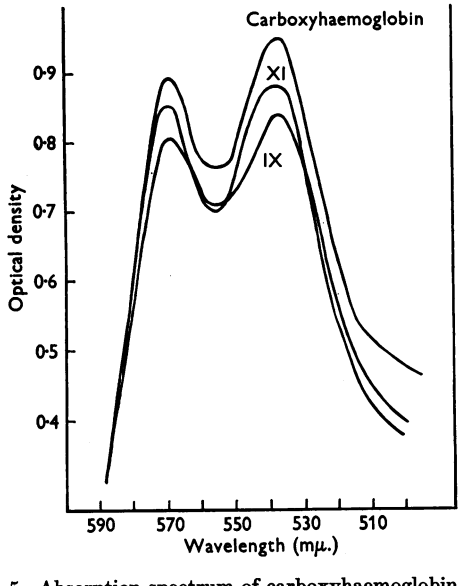


Fig. 5. Absorption spectrum of carboxyhaemoglobin compared with those of the complexes COHb.$(IPhNHOH)_2$ and COHb.IPhNHOH (IX and XI respectively). All the carboxy-pigments showed similar spectra (see Table 1), but the compound XIII showed an additional maximum at 630 mμ.

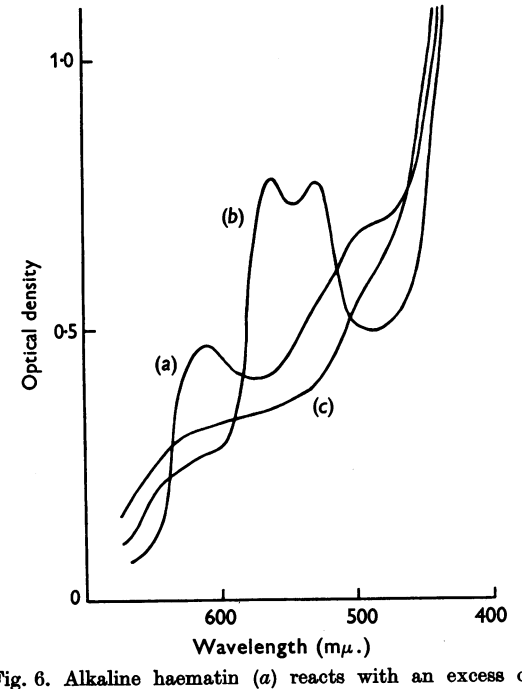


Fig. 6. Alkaline haematin (*a*) reacts with an excess of iodophenylhydroxylamine with the immediate formation of a bright violet-red pigment (*b*). This quickly changes on oxygenation to a greenish yellow material (*c*), with a combining ratio of Fe:IPhNHOH of 1:1. Iodonitrosobenzene added to alkaline haematin causes no visible colour change and the substance (*c*) is formed directly.

accompanied by any significant loss of radioactive material; 92% remained associated with the pigment in the protein-free solution. Alkaline haematin in acetone–phosphate buffer solution (pH 9·0) reacted rapidly with IPhNHOH and IPhNO. When IPhNHOH was added to the greenish yellow haematin solution the colour changed immediately to a bright red-violet with absorption maxima at 560 and 530 mμ. (Fig. 6). This compound was very unstable and the solution rapidly became greenish yellow again, especially on oxygenation. The final pigment was not haematin according to spectroscopic data and was found to retain one molecular equivalent of radioactive material (Table 1). No obvious change in colour occurred when IPhNO was added to a solution of alkaline haematin, but the separated product was similar spectroscopically and in its content of radioactive material to that from the reaction of alkaline haematin and IPhNHOH (Fig. 6 and Table 1).

Failure of either IPhNHOH or IPhNO to react with protoporphyrin 7-methyl ester suggested that the presence of iron was necessary for combination to occur.

DISCUSSION

The results presented show that oxyhaemoglobin, carboxyhaemoglobin and alkaline haematin react directly with *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene, and the questions arise as to the nature of the complexes and the mechanism by which they are formed. Keilin & Hartree (1943) studied the reactions of oxyhaemoglobin and alkaline haematin with phenylhydroxylamine and nitrosobenzene using a spectroscopic technique. They concluded that the violet pigment formed from PhNHOH and oxyhaemoglobin was very unstable, being decomposed in air into free methaemoglobin, oxidized by ferricyanide to methaemoglobin, reduced by hydrosulphite to haemoglobin and converted by carbon monoxide into carboxyhaemoglobin. The violet haematin complex produced by the reaction of alkaline haematin and PhNHOH underwent a similar series of changes, liberating haematin, haem, or forming carbon monoxide haem as the case may be. Some modification of their conclusions concerning the nature and stability of the complexes is suggested by the results of the present work with the labelled IPhNHOH.

An important conclusion which emerges is that the presence of combined IPhNHOH or IPhNO in the pigment molecule may be without effect on the absorption spectrum. Thus, spectroscopic data may neither indicate if reaction between the haemoglobin pigment and these aromatic molecules has occurred, nor show if the complexes formed have been dissociated when they are subjected to some simple chemical procedure (e.g. oxygenation, reduction, oxidation, etc.) which causes a change in absorption spectrum. According to Keilin & Hartree, the formation of violet pigment from oxyhaemoglobin and PhNHOH was preceded by the appearance of methaemoglobin. We have not observed the prior formation of this brown pigment when IPhNHOH was added to solutions of crystalline rat oxyhaemoglobin. It appears to us that the initial formation of methaemoglobin occurred in red cells or with less-purified pigment solutions, when it is apparently catalysed by the presence of the hydroxylamine (Crick & Jackson, 1953).

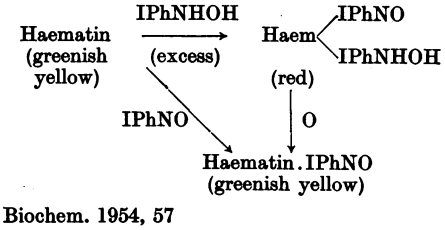
Whilst we have no information on the nature of the union with IPhNHOH or IPhNO in these pigment complexes, sufficient data have been accumulated to enable a scheme of reactions to be suggested (Figs. 2, 4). Oxyhaemoglobin reacts stoicheiometrically with one molecular equivalent of either IPhNHOH or IPhNO to form the same pigment complex (I). During the reaction with the former substance, the hydroxylamino group is oxidized to nitroso, although this does not mean—as was previously thought—that the haemoglobin molecule cannot react directly with the aryl hydroxylamine (see below). Reaction of IPhNO with oxyhaemoglobin is accompanied by evolution of all the bound oxygen; this has previously been found to occur when nitrosobenzene was used (Heubner *et al.* 1923; Jung, 1940). The fact that compound I does not react further with IPhNO but rapidly takes up an additional molecule of IPhNHOH suggests the formulation II, the iron atom remaining in the ferrous state. Both these complexes (I and II) are reduced by dithionite yielding a haemoglobin spectrum, so that this change can only represent a reduction of nitroso- to hydroxylamino-groups with the formation of compounds V and VII. The presence of combined IPhNHOH apparently does not interfere with subsequent oxygenation of these haemoglobins, for red pigments are then formed (VI and VIII) spectroscopically similar to oxyhaemoglobin (Fig. 3 and Table 1). The process of oxygenation does not cause oxidation of the unstable —NHOH groups to —NO, i.e. there is no reverse change back to compound I which would be expected if this happened. The complex II is unstable and soon changes into a third substance, III, without loss of the combined iodo-component, and this change appears to be due to the formation of a ferric pigment. This is supported by the appearance of an absorption band with maximum at 630 mμ., the observation that this pigment (III) is converted back into II by addition of IPhNHOH (obviously a reduction) and the knowledge that only one molecule of IPhNO can be attached to haemoglobin. The slow conversion of III on standing, into a methaemoglobin-like pigment could then be due

to the gradual oxidation of the —NHOH to —NO, leading to the loss of one of the two molecules of IPhNO. Preparation of compound III with one of the iodine components labelled, followed by its conversion into IV, demonstrated that the IPhNHOH molecule is specifically lost in this change. The substance VIII, when similarly labelled, also lost the corresponding molecule of IPhNHOH on oxidation to IV. These results suggest that the two molecules of combined iodo-compound are not symmetrically disposed in the haemoglobin molecule. The complex I is a ferrous pigment, for it slowly oxidizes to the methaemoglobin pigment IV without loss of combined IPhNO (Fig. 2). Addition of excess of IPhNHOH to IV converts it back into II, the hydroxylamine causing a reduction of ferric to ferrous in this reaction.

Pigments spectroscopically similar to carboxyhaemoglobin are formed by the action of carbon monoxide on the substances I, II and III without loss of combined radioactive material (Figs. 5, 4). The fact that IPhNO also reacts directly with carboxyhaemoglobin without causing a change in absorption spectrum, while it releases all the oxygen from oxyhaemoglobin in the formation of compound I, suggests that the presence of combined IPhNO lowers the affinity of haemoglobin for oxygen. Carbon monoxide, with its greater affinity for the pigment, is still able to combine, and is not displaced when IPhNO reacts with carboxyhaemoglobin.

IPhNHOH also reacts directly with carboxyhaemoglobin under conditions which preclude its oxidation to the nitroso compound; no alteration in absorption spectrum accompanies this process. Haemoglobin must combine similarly with the hydroxylamine in the presence of dithionite, for subsequent oxygenation yields an oxyhaemoglobin-like complex (VI, Fig. 2) and not compound I. For this reason it appears that oxyhaemoglobin converts added IPhNHOH into IPhNO before the formation of a pigment complex. It is likely that PhNHOH also combines directly with haemoglobin, for evidence to the contrary was based on the absence of spectroscopic change when it was added to reduced haemoglobin (Keilin & Hartree, 1943).

PhNHOH and IPhNHOH added to alkaline haematin produce the same sequence of colour changes. Since IPhNO forms directly the same end product as the iodophenylhydroxylamine, the reactions may be represented as follows:



SUMMARY

1. The reactions of haemoglobin and some of its derivatives with ^{131}I-labelled *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene have been followed spectroscopically and also by determining the combining ratio of the radioactive substance per atom of iron.

2. It has been shown that spectroscopic data alone are inadequate in the study of these reactions, for combination between pigment and iodo-compound may occur without accompanying change in absorption spectrum. Also, alteration in the absorption spectrum of a formed complex to that of a known blood pigment may be induced without loss of the combined radioactive material.

3. Iodophenylhydroxylamine combines directly with haemoglobin and carboxyhaemoglobin under conditions which preclude its oxidation to the nitroso compound.

4. A series of reactions follows the addition of the iodophenylhydroxylamine to oxyhaemoglobin. Three intermediates have been recognized and the final product is spectroscopically similar to methaemoglobin but retains one molecular equivalent of the iodo-compound. The interrelationships of these substances have been examined.

5. Carboxyhaemoglobin combines directly with iodophenylhydroxylamine or iodonitrosobenzene without loss of carbon monoxide or change in absorption spectrum. When oxyhaemoglobin reacts with iodonitrosobenzene, the oxygen is quantitatively displaced. It is suggested that the difference in behaviour between oxyhaemoglobin and carboxyhaemoglobin is due to the combined iodonitrosobenzene reducing the affinity of the pigment for oxygen; the much greater affinity of carbon monoxide, however, permits the retention of the latter.

6. It appears probable that phenylhydroxylamine and nitrosobenzene behave in a manner similar to their iodo-derivatives, so that the complexes formed with haemoglobin and its derivatives are not necessarily dissociated in subsequent reactions, as has been previously supposed.

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