LXXXVII. THE REDUCTION OF HAEMATIN AND METHAEMOGLOBIN.

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THE question of the oxygen content of methaemoglobin relative to haemoglobin has long interested physiologists. The recent work of Keilin [1926] has brought haemochromogen into prominence as a type of important biological substances. The great ease with which haematin is reduced in comparison with protoporphyrin has led to the assumption that in the reduction of the former to reduced haematin the essential change is that of the valency of the iron. This would involve one equivalent of hydrogen per atom of iron. Küster [1910] suggested that methaemoglobin was a compound of haematin in which the iron was in the ferric state. In oxyhaemoglobin, haemoglobin, haemochromogen and reduced haematin the iron might therefore be held to be in the ferrous state. If this is the case, the term "oxygen content" of methaemoglobin and haematin has little meaning, as is pointed out by Conant and Scott [1926]. Determinations of the equivalents of hydrogen necessary for the reduction of haematin have failed to confirm this view. The data and conclusions of many workers are summarised by Nicloux and Roche [1926]. As a result of their own experiments these workers concluded that the reduction of methaemoglobin required two equivalents of hydrogen. Later, Roche [1926] contended that haematin needs four equivalents for its reduction. Their work has been discussed by Conant and Scott [1926].

Conant [1923] had previously titrated electrometrically both methaemoglobin and haematin and found one equivalent of hydrogen necessary for the former but two for the latter substance. He, however, considered the method unsuitable for haematin.

Haurowitz [1927] had reduced haematin with hydrazine hydrate and by measuring the nitrogen evolved found one equivalent for the reduction. It must however be taken for granted that the volume of nitrogen measured corresponds with the whole of the hydrogen used in reducing the pigment, for which the available evidence is satisfactory. For the reduction of haematin to reduced haematin, hydrazine hydrate is unsuitable because in itself it is a nitrogen compound capable of yielding a haemochromogen [Anson and Mirsky, 1925]. Haurowitz does not seem to have realised any difference between reduced haematin and haemochromogen.

Hill and Holden [1926] prepared methaemoglobin by direct combination of haematin and globin. In view of the discrepancies in the above results it was decided to attempt to titrate directly with a reducing agent solutions of methaemoglobin, of haemin in alkali, and of haemin in alkali in the presence of pyridine.

The essential requirement of such a research was that it should be simple and direct both in theory and technique.

The choice of a suitable reducing agent was the first consideration. From the limited number of available reducers ferrous tartrate in alkaline solution was selected. In practice, standard ferrous sulphate solution was added to an alkaline solution of the pigment containing sodium tartrate. By this means errors due to atmospheric oxidation of the standard reducing agent itself were reduced to a negligible amount. The only state to which a ferrous salt is readily oxidised is that of a ferric salt. The reduction potential of the simpler ferrous compounds increases greatly on passing from an acid to an alkaline reaction. Excess of ferric tartrate (the product of its oxidation) was found to be without appreciable influence. No additional standardisation is necessary if pure ferrous ammonium sulphate is used. The possibility of the reaction stopping at the formation of ferrosferric compounds is negated by the indifference of excess of ferric tartrate.

As ordinarily prepared, methaemoglobin contains undesirable amounts of ferricyanide which are not readily removable by dialysis. Nicloux's method gives a 60 % conversion of oxyhaemoglobin to methaemoglobin, but such a solution was unsuitable for these experiments. The preparation of a sufficient quantity of pure globin was too tedious owing to its high equivalent weight. Accordingly advantage was taken of the fact that at an acid reaction at which oxyhaemoglobin is still stable ferric tartrate oxidises it completely to methaemoglobin. If such a solution be immediately made alkaline the ferrous salt formed reduces some of the methaemoglobin. It was found possible to remove the ferrous salt and the bulk of the ferric salt by suitable treatment without such reduction occurring.

In the case of haematin, since both reduced haematin and pyridine haemochromogen are insoluble, they can be separated from the soluble haematin by centrifuging. The end-point can thus be determined.

In the case of methaemoglobin the oxyhaemoglobin produced by a known amount of reducer followed by oxygen was estimated by determining the oxygen capacity of the solution.

The preparation of methaemoglobin.

1 litre of fresh defibrinated ox blood was centrifuged and the corpuscles washed three times with 0.9 % sodium chloride solution. To 500 cc. of corpuscles so obtained were added 4.5 l. of water, 500 cc. 25 % sodium tartrate (Rochelle salt is not suitable) and 250 cc. 10 % acetic acid by volume. After

thorough mixing, 60 g. sodium tartrate and 40 g. iron ammonium alum dissolved in 500 cc. of cold water were slowly added with stirring. Bubbles were given off in the liquid and the methaemoglobin spectrum rapidly replaced that of oxyhaemoglobin. After standing the turbid liquid was filtered from the stroma, etc., the clear filtrate was diluted with twice its volume of water and allowed to stand overnight at 0°. The methaemoglobin was practically completely precipitated as a granular precipitate. This precipitate contained ferric tartrate and was completely insoluble in distilled water. It was soluble in very dilute alkalis yielding first a clear neutral solution of methaemoglobin, from which the iron could be dialysed. That the precipitating agent was ferric and not ferrous tartrate could be shown as follows. On suspending the washed precipitate in distilled water and adding a small amount of sodium hydrosulphite, as reduction proceeded the precipitate dissolved, finally yielding a perfectly clear solution of haemoglobin from which oxyhaemoglobin was obtained. The granular precipitate of methaemoglobin was washed with 2 l. of distilled water containing 2 cc. 10 % acetic acid and 20 cc. 25 % sodium tartrate. After expressing the superfluous liquid the precipitate was ground into a cream with water and dissolved with the minimum amount of 10 % sodium carbonate, added slowly from a burette, about 100 cc. being required. To the 620 cc. of solution so obtained were added 5 cc. of 25 % sodium tartrate and enough saturated ammonium sulphate to make a total volume of 1 l. The solution was then filtered. To the filtrate 1.5 l. saturated ammonium sulphate were added and, after standing overnight at 0°, the precipitate of methaemoglobin was filtered off. The iron remained in the filtrate. After washing with saturated ammonium sulphate the precipitate was pressed as dry as possible between filter papers and dissolved in half its weight of distilled water. This strong methaemoglobin solution was used directly for the experiments. After complete reduction of a measured sample with ferrous tartrate its oxygen capacity was determined by Barcroft's method. The tint of the oxyhaemoglobin of this sample was compared at known dilution with diluted defibrinated blood of known oxygen capacity. They were found to agree, showing that all the haematin present was in the form of methaemoglobin. Hence the precipitation of methaemoglobin by ferric tartrate under the conditions of the experiment does not alter the essential properties of the conjugated protein. When freed from the iron tartrate the oxyhaemoglobin finally obtained resembles in solubility, oxygen capacity, and colour the original oxyhaemoglobin.

The reduction of haematin.

The experiments were conducted in small centrifuge tubes about 62×18 mm. The haematin solution was made by dissolving a known weight of about 0.3 g. in 25 cc. of dilute alkali. It was used immediately. Recrystallised ferrous ammonium sulphate was dissolved in water containing a trace of sulphuric acid to give a 0.02 *N* solution. A suitable amount of an alkaline tartrate

solution was used. A layer of toluene prevented reoxidation. To a series of four tubes were added equal amounts of haematin, alkaline tartrate solution and toluene, and an amount of water to make the total volume after addition of the reducer up to 8 cc. Four different volumes of the ferrous sulphate solution were added to the different tubes. They were stirred gently and immediately centrifuged after cautious removal of the stirrers. After 5 minutes the tubes were removed from the centrifuge and observed. In Exp. 1 they were observed in sunlight without centrifuging. The details of two typical experiments are recorded and the results for a series given in Table I.

Table I.

For 2 cc. haematin titrated with standard ferrous iron solution as reducer.

Remarks	cc. reducer added to effect reduction	Observed correction in cc. of reducer for dissolved oxygen	cc. reducer used in reducing haematin	Theoretical cc. reducer for 1 equivalent	Equivalent
Pyridine haemochromogen in sodium carbonate	2.40	0.35	2.05	2.31	0.89
Same, with 1.5 cc. <i>N</i> /5 ferric iron	2.30	0.35	1.95	2.31	0.84
Reduced haematin in sodium hydroxide	2.10	0.30	1.80	1.81	0.99
Pyridine haemochromogen in sodium hydroxide	2.30	0.35	1.95	1.95	1.00
Pyridine haemochromogen in sodium hydroxide	2.20	0.35	1.85	1.95	0.95

Exp. 1. The alkaline tartrate is a mixture of 20 cc. 10 % Na_2CO_3 + 10 cc. 25 % sodium tartrate. Ferrous ammonium sulphate, 0.4015 g. in 50 cc. Haemin 0.3329 g. in 25 cc. containing 0.2 cc. purified pyridine. Each tube contained 2 cc. haematin solution + 2 cc. alkaline tartrate + 1 cc. toluene and water to make 9 cc. total volume.

Tube	Ferrous sulphate cc.	Observation
1	2.3	Nearly reduced
2	2.5	Reduced
3	2.7	"
4	4.0	"

Zero correction for dissolved oxygen using very dilute haematin.

1	0.1	Not reduced
2	0.2	"
3	0.3	"
4	0.4	Reduced

Result. 2 cc. of 0.0237 *N* haematin required 2.40–0.35 cc. of 0.0205 *N* iron.

Exp. 2. The alkaline tartrate is a mixture of 1 cc. 25 % sodium tartrate, 1 cc. 40 % sodium hydroxide and 1 cc. of water. Ferrous ammonium sulphate; 0.8073 g. in 100 cc. Haemin; 0.6054 g. to 50 cc. Each tube contained 2 cc. haematin solution + 2 cc. alkaline tartrate + 2 cc. toluene + water to make 9 cc. total volume.

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Tube	Ferrous sulphate cc.	Observation
1	1.8	Not reduced
2	2.0	"
3	2.2	Reduced
4	2.5	"
Zero correction using very dilute haematin.		
1	0.1	Not reduced
2	0.2	"
3	0.3	Just reduced
4	0.4	Reduced.

Result. 2 cc. of 0.0186 *N* haematin required 2.1–0.30 cc. 0.0206 *N* iron.

The reduction of methaemoglobin.

5 cc. of a strong solution of methaemoglobin prepared as described above were measured into a test-tube. To this was added 0.7 cc. of a solution prepared by mixing 50 cc. 25 % tartrate, 36 cc. water and 14 cc. of strong ammonia solution (Sp. Gr. 0.880). A measured quantity of water was added so that, with the volume of the reducing agent to be added subsequently, the total volume of liquid would be 8.5 cc. A stirrer was then placed in the tube and a layer of liquid paraffin 1 cm. deep carefully poured on. The ferrous ammonium sulphate solution (containing 0.2023 g. of the salt in 25 cc. of water) was run in from a burette with a long drawn-out jet reaching nearly to the bottom of the tube. The stirring was continued for 1 minute after the reducer had been added and the mixture allowed to stand 15 minutes. The solution of haemoglobin was then drawn up in a pipette from beneath the paraffin and transferred to a conical flask where it was gently shaken for 15 minutes with oxygen from a cylinder. 3 cc. of the resulting solution of oxyhaemoglobin were measured out into the bulbs of two Barcroft differential gas apparatus. The oxygen capacity was then directly determined by the addition of ferricyanide. The results of the experiments are set forth in Table II.

Table II. *Reduction of methaemoglobin as measured by oxygen capacity with standard ferrous iron solution.*

cc. reducer added	Observed oxygen capacity cc.	Calculated oxygen capacity for 1 equivalent cc.	Calculated oxygen capacity for 2 equivalents cc.	Observed equivalent
2.35	{ 838 848	948	474	{ 1.11 1.10
2.30	{ 806 788	920	460	{ 1.12 1.15
2.30	{ 781 751	920	460	{ 1.16 1.20

A similar series of experiments was now made using a Haldane evolution apparatus. Different amounts of reducer were added and the corresponding oxygen capacities determined by adding ferricyanide. The zero correction for

dissolved oxygen was determined as in the case of haematin, using a very weak solution of methaemoglobin. The results of this series are shown in Fig. 1. The ordinates represent the oxygen capacity in cc. in terms of 5 cc. of the original methaemoglobin solution, the abscissae represent cc. of a standard iron solution (0.02 *N*) added. The dotted curves represent the theoretical oxygen capacities for the reduction involving one and two equivalents of iron per molecule of methaemoglobin.

DISCUSSION OF RESULTS.

The high equivalent weights of haemin (651) and of methaemoglobin (16,700) militate against the experimental accuracy with which the addition to either of one equivalent of hydrogen can be determined. In addition, both substances are known to undergo certain changes in solution. The reaction between reducing agents and such decomposition products is as yet obscure. Such decomposition might be expected to affect the two methods of estimation employed in opposite directions decreasing the amount of oxygen obtainable

Fig. 1.

from the conjugated protein after treatment with a given amount of reducer and decreasing the amount of reducer required for a known volume of haematin solution. The mean value for the case of haemochromogen and reduced haematin is 93 % of the theoretical value for one equivalent of hydrogen. Of the two samples of haemin used the more recently prepared one gave the values most nearly approaching to the theoretical value. The determinations of the resulting oxygen capacity on reduction of a single specimen of methaemoglobin showed a progressive decrease from day to day. The mean value of four determinations carried out within 2 days after the preparation of the methaemoglobin gave a ratio of equivalents of hydrogen/molecular oxygen of 1.12. The effect of reducible impurities in the methaemoglobin is clearly shown by the curve. If the methaemoglobin were perfectly pure, a sharp break in the

curve would be expected to occur when the theoretical amount of reducer had been added. The curve, however, tails off gradually to a constant oxygen capacity corresponding to complete reduction. The methaemoglobin, however, is completely reduced long before two equivalents of reducer have been added, and for smaller amounts of reducer closely follows the slope of the theoretical curve for one equivalent.

It would seem probable that the change of methaemoglobin to haemoglobin or of haematin to either reduced haematin or haemochromogen is one and the same, namely, the addition of one equivalent of hydrogen per atom of iron. The protein would appear to play no essential part in this reduction and does not react with the reducing agent. Methaemoglobin is a compound of haematin and globin while haemoglobin is a compound of reduced haematin and globin. Küster's hypothesis [1910] is thus experimentally confirmed.

SUMMARY.

1. Methaemoglobin has been prepared by the use of ferric tartrate instead of potassium ferricyanide.
2. The protein can be completely precipitated by ferric tartrate without denaturation.
3. The reduction of methaemoglobin to haemoglobin requires one equivalent of hydrogen per atom of iron.
4. The reduction of haematin to reduced haematin or to pyridine haemochromogen requires one equivalent of hydrogen per atom of iron.

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REFERENCES.

- Anson and Mirsky (1925). *J. Physiol.* **60**, 50.
 Conant (1923). *J. Biol. Chem.* **57**, 401.
 Conant and Scott (1926). *J. Biol. Chem.* **69**, 575.
 Haurowitz (1927). *Z. physiol. Chem.* **66**, 249.
 Hill and Holden (1926). *Biochem. J.* **20**, 1326.
 Keilin (1926). *Proc. Roy. Soc. Lond. B.* **94**, 266.
 Küster (1910). *Z. physiol. Chem.* **66**, 249.
 Nicloux and Roche (1926). *Bull. Soc. Chim. Biol.* **8**, 71.
 Roche (1926). *Bull. Soc. Chim. Biol.* **8**, 362.