LXXIII. THE OXYGEN CONTENT OF METHAEMOGLOBIN.

By HERBERT ELDON ROAF

AND WILLIAM ARTHUR MERRETT SMART.

Eliza Ann Alston Scholar, London Hospital Medical College.

From the Department of Physiology, London Hospital Medical College.

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SOME years ago one of us made measurements of the osmotic pressure of haemoglobin with a view to correlating changes in osmotic pressure of haemoglobin with haemolysis [Roaf, 1910, 1, and 1912]. In certain cases, notably in the action of alkali, there was a distinct relation between the effect of alkali on haemolysis and on the osmotic pressure of haemoglobin. In the case of acid, however, the results did not show so great an increase in osmotic pressure and it was suggested that some secondary change might have occurred.

The results were explained on the basis that haemoglobin acted as an amphoteric electrolyte with the formation of ionising salts, thus producing a corresponding rise of osmotic pressure and the establishment of an electrical potential.

Recently Mrs Wilson [1923] has published the results of experiments with carbon dioxide and acetic acid which confirm the result that acid causes a rise of osmotic pressure and she obtained higher pressures per 1 % of haemoglobin than were obtained by Roaf. Unfortunately, her paper does not indicate that spectroscopic examination, accompanied by reduction, was made when the pressure was high. In view of the fact that haematin may be recombined with protein [Menzies, 1894, 2], it is possible that the highest pressures obtained were due to some splitting of the haemoglobin and the subsequent fall due to recombination. It was for fear of this condition that dialysis experiments were not carried out with haemoglobin, but Mrs Wilson overlooks the fact that such experiments were carried out with serum proteins [Roaf, 1910, 2]¹.

¹ Mrs Wilson whilst confirming my results and agreeing with my conclusions states that they were based on slight evidence. The evidence was that haemoglobin although showing no evidence of decomposition into haematin gave higher osmotic pressures than could be accounted for by single molecules of haemoglobin. This evidence is good enough for the guarded statement: "It is most likely that the correct explanation is that haemoglobin forms ionising salts with alkalies and acids, and that the observed pressures are due to the sum of the pressure caused by haemoglobin and the ions into which the salt can split, for instance sodium and 'haemoglobyl' ions."

The view that proteins form ionising salts is based on a much wider basis than one set of experiments. The starting point for this belief is the work of Emil Fischer in showing that

In the previous experiments methaemoglobin was tentatively considered to be the stable form of oxyhaemoglobin on the acid side of the isoelectric point although that view did not harmonise with the formation of alkaline methaemoglobin.

Buckmaster [1914] has published results in which he found that methaemoglobin formed by the action of hydroxylamine yields with hydrazine hydrate only half the nitrogen that is furnished by oxyhaemoglobin. The bearing of this point on the osmotic pressure measurements is that acid oxyhaemoglobin may have a high osmotic pressure but it readily passes into methaemoglobin with a lower osmotic pressure. If one could measure the osmotic pressure of oxyhaemoglobin in acid before methaemoglobin was formed, sufficient rise of pressure might be recorded to account for haemolysis by acid.

We thought this subject of sufficient interest to merit investigation by a different method. We measured the amount of oxygen given off when methaemoglobin was formed by the action of acetic acid on oxyhaemoglobin and compared this with the amount given off when another portion of the same oxyhaemoglobin solution was acted on by potassium ferricyanide.

EXPERIMENTAL METHODS.

The method finally adopted was to use the principle of van Slyke's blood gas apparatus. In order to carry out a series of observations we used Haldane sampling bulbs for the containers. A tube over 76 cm. long was used to produce the vacuum (Fig. 1). On the upper end of this tube was a bulb (*B*) to receive the solution on evacuation. The lower end of the tube (*A*) was furnished with pressure tubing and mercury bulb in the usual manner. The Haldane sampling tube (*C*) was united with the bulb (*B*) by pressure tubing. After testing the apparatus to see that the taps did not leak the whole was filled with mercury. The haemoglobin solution was run in from a pipette through the upper opening of the sampling tube. The pipette was filled above the mark and the excess allowed to escape through the side outlet of the Haldane tube, so that all the connections up to the tap were filled with the solution. The tap was then turned through 180° so that the haemoglobin solution ran into the sampling tube above the mercury.

The connections were washed out with water through the side tube and the reagent added in the same way that the haemoglobin solution was added.

proteins are composed of amino acids. Other evidence was briefly quoted in the paper to which Mrs Wilson refers. She evidently overlooks the large amount of work published in this Journal from the Laboratory of the late Professor Benjamin Moore, F.R.S., which shows that the inorganic constituents are an integral part of the protein when they increase the osmotic pressure of the protein, otherwise the inorganic constituents would escape through the membrane. This work has been corroborated by other observers.

One point in connection with that work is frequently overlooked, viz. the importance of changes in the osmotic pressure of colloids on cell mechanics. If other cell proteins are as easily affected as haemoglobin considerable effects may be produced in cells by changes in osmotic pressure.

When ferricyanide was used the apparatus was immediately evacuated. After evacuation was completed the solution was collected in the bulb (*B*), the lower tap of the sampling tube was turned and the solution run out through the lower side tube for spectroscopic examination. The lower tap was then reversed and the mercury run into the Haldane tube.

Fig. 1.

When acid was used, the reagent was added to the haemoglobin, both taps were closed, the tube disconnected, and left (usually for twenty-four hours at room temperature) until the acid had converted the oxyhaemoglobin into methaemoglobin. It was then re-connected and evacuated as described above.

The gas was analysed by running it into a tube containing alkaline water. This tube was connected to the Haldane tube by pressure tubing and the connections filled with water by running the alkaline solution through the upper side outlet of the Haldane tube. The upper tap was then reversed and by raising the mercury the gas was forced into the bulb (*D*).

By disconnecting the analysing tube from the Haldane tube and attaching a rubber tube with reservoir to the end which had been connected to the Haldane tube the gas can be forced along the measuring tube (*E*).

The absorption of gas was carried out in the far bulb (*F*). This bulb is furnished with two side tubes each with a right-angled tap so that either of two supply tubes, making four in all, could be connected with the bulb. The first reservoir contained the alkaline water for manipulation of the gas along the measuring tube. The second contained saturated sodium hydroxide for absorption of any carbon dioxide which escaped absorption by the alkaline water. The third contained concentrated pyrogallol which when run into the strong alkali in the bulb absorbed the oxygen. The fourth opening was attached to a waste tube for the purpose of washing out the apparatus.

The amount of oxygen given off was measured by the difference in volume after the action of strong alkali followed by alkaline pyrogallol, the usual assumption made in gas absorption analysis.

The haemoglobin solution was prepared as follows. The blood was centrifugalised and the corpuscles twice washed with saline. The corpuscles were haemolysed by an equal volume of distilled water. At first as the result of osmotic pressure experiments we did not dare to use a preservative but later we found that toluene did not interfere with the results but actually improved them.

Caprylic alcohol was added to the alkaline water in the measuring tube to decrease the difficulty due to frothing.

RESULTS.

The results show unmistakably that addition of acid sufficient to convert oxyhaemoglobin into methaemoglobin liberates approximately half the amount of oxygen that would be given off by the same quantity of haemoglobin when acted on by ferricyanide. A few of the results are given in tabular form.

DISCUSSION.

Before deducing that methaemoglobin contains only half the replaceable oxygen that oxyhaemoglobin contains we must examine all other possible explanations. As oxygen is given off the product formed by acid must contain less oxygen than oxyhaemoglobin and there is no source of oxygen other than the oxyhaemoglobin.

*Tabular Statement of Results in those experiments in which
toluene was used as a preservative.*

Date	Amount of haemoglobin solution cc.	Treatment of solutions	Amount of oxygen given off cc.		Percentage of oxygen capacity	Spectrum examination
Exp. 1	10	5 cc. saturated ferricyanide	1.68	av. of 2 readings	100	Methaemoglobin
19. ii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	1.54	„ 2 „	45.8	Methaemoglobin + a doubtful trace of oxyhaemoglobin
	20	1.6 cc. 3 <i>N</i> acetic acid after equilibration with air	0.22		6.5	Methaemoglobin + a trace of acid haematin
Exp. 2	10	5 cc. saturated ferricyanide	1.30	av. of 2 readings	100	Methaemoglobin
26. ii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	1.275	„ 2 „	49	„
	20	1.6 cc. 3 <i>N</i> acetic acid after equilibration with 90.1 % oxygen	0.57	accounted for by solubility of oxygen	22	„
Exp. 3	10	5 cc. saturated ferricyanide	1.37	av. of 5 readings	100	Methaemoglobin
5. iii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	1.355	„ 2 „	49.5	„
	20	1.6 cc. 3 <i>N</i> acetic acid after equilibration with 56 % oxygen	0.42	accounted for by solubility of oxygen	15.3	„
Exp. 4	10	5 cc. saturated ferricyanide	1.53	av. of 3 readings	100	Methaemoglobin
12. iii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	0.44		14.4	Methaemoglobin + acid haematin
A further set was started next day containing less acid with the following result:						
	20	1.4 cc. 3 <i>N</i> acetic acid	1.39	av. of 2 readings	45.4	Methaemoglobin
Exp. 5	10	5 cc. saturated ferricyanide	1.15	av. of 2 readings	100	Methaemoglobin
28. v. 23	20	1.4 cc. 3 <i>N</i> acetic acid	1.22		53	„

The average of these five experiments is 48.5 % of the oxygen capacity is given off when oxyhaemoglobin is converted into methaemoglobin.

We must first consider whether methaemoglobin is formed by the action of acid. There may be several forms of methaemoglobin, but at present it is recognised by its colour and absorption band. We have always examined the solution from the experiments in the following manner. We examined it spectroscopically for a band in the red near the *C* line. The solution was rendered alkaline and the change to alkaline methaemoglobin observed. The solution was then reduced and examined for reduced haemoglobin and reduced haematin. Any experiment the solution from which showed oxyhaemoglobin on first examination or reduced haematin on reduction was discarded.

We observed that the spectrum of methaemoglobin formed by the addition of acid lacked the two lines between *D* and *E* which are usually seen with methaemoglobin formed by ferricyanide. We think, however, that this does not indicate any real difference [compare Menzies, 1894, 1] because the addition of alkali to methaemoglobin formed by acid causes the two bands to appear. In our opinion the methaemoglobin formed by ferricyanide is a mixture of methaemoglobin and alkaline methaemoglobin: the two bands disappear with much less acid than is necessary to form methaemoglobin from oxyhaemoglobin.

The next point is whether the oxygen given off might have come from the incomplete formation of methaemoglobin. Any oxyhaemoglobin left unchanged might give off oxygen on evacuation. This alternative is shown not to occur because in those experiments in which oxyhaemoglobin was recognised spectroscopically the volume of oxygen given off was less than when no oxyhaemoglobin was found.

The alternative difficulty, namely, that acid haematin may be formed with the liberation of oxygen is negated by the result that if reduced haematin is seen after reduction the yield of oxygen is lower (see Exp. 4). In fact we made a few experiments to see if acid haematin contained less oxygen than oxyhaemoglobin. We found that if acid haematin is formed by using larger quantities of acid or by acid plus pepsin no oxygen is given off, thus indicating that acid haematin contains as much oxygen as oxyhaemoglobin.

In some experiments in which we treated oxyhaemoglobin with acid over mercury in a closed tube we did not find any gas above the solution. We wondered if this result were due to the gas remaining in a supersaturated condition or if there were two forms of methaemoglobin, one containing more oxygen than the other [Roaf and Smart, 1922]. Experiments were carried out to test this possibility. We prepared methaemoglobin by the action of acid during exposure to various pressures of oxygen. These solutions were run into our evacuating apparatus, and in all cases the amount of oxygen obtained was well below that liberated when oxyhaemoglobin was converted into methaemoglobin in a closed vessel and then evacuated. With high concentrations of oxygen more oxygen was observed (see Exps. 2 and 3), but the amount obtained corresponded to the amount that one would expect to find dissolved at the oxygen pressure used. In one case in which air was used above the solution the oxygen content of the air was increased by the amount that would correspond to the giving off of half the oxygen of the original oxyhaemoglobin. Therefore there is no evidence that there is a form of methaemoglobin containing more oxygen at higher oxygen pressures.

Having discussed the alternative explanations of our results we wish to draw attention to some of the difficulties of the experiments. As suggested above, too much or too little acid will give results too low because acid haematin fails to give off oxygen and oxyhaemoglobin was associated with a lower yield. Therefore only those experiments in which the right amount of acid was used gave results worth recording. Incomplete saturation of the oxyhaemoglobin will give as a result less than half of the oxygen content observed by the ferricyanide method. Bacterial action will cause a loss of oxygen, and before the use of toluene as a preservative lower results were obtained. Chemical action, apart from bacterial action, might cause some loss of oxygen, so that is another possible cause of low results. In the evacuation of the methaemoglobin solution the solution to which acid had been added was viscous and it frothed badly. This frothing was one of our difficulties, and it was less easy to get off all the oxygen than from the methaemoglobin formed by ferricyanide. We attempted to use horse corpuscles obtained through the courtesy of Dr O'Brien of Messrs Burroughs, Welcome and Company (to whom our thanks are due), but we abandoned them for bullocks' corpuscles, because the horse haemoglobin became practically solid after the action of acid. We are not certain whether there is a difference due to the species or whether it is due to the absence of calcium in the case of the horse corpuscles, which were obtained from oxalated blood and washed with oxalated saline.

In view of these various reasons why the amount of oxygen obtained might be less than the theoretical amount we consider that our results show a very good approximation to 50 % of the dissociable oxygen of oxyhaemoglobin.

Hüfner and Külz [1883] are frequently quoted as having proved that methaemoglobin contains the same amount of oxygen as oxyhaemoglobin. Their experimental method was to treat solutions of oxyhaemoglobin and methaemoglobin with urea and nitric oxide. Their results show that the same amount of nitrogen is given off in each case, but that may be due to only part of the oxygen of oxyhaemoglobin being concerned in the reaction. Küster [1910], in discussing this problem, points out that hydrocyanic acid will combine directly with methaemoglobin but not with haemoglobin. Oxyhaemoglobin reacts slowly with hydrocyanic acid. He concludes that methaemoglobin is intermediate between oxyhaemoglobin and haemoglobin.

Methaemoglobin is formed by such a variety of agents, some oxidising and some reducing [for instance palladium-hydrogen, Hoppe-Seyler, 1877, 1878], that one is led to the belief that it is the stable form in acid solution. Alkaline methaemoglobin also seems to be a stable form on the alkaline side, but it is easily converted into haemoglobin by reducing agents without passing through the form of oxyhaemoglobin (Haldane).

If the deduction made in this paper is correct the equations given by Haldane [1898] for the action of ferricyanide on haemoglobin and on oxyhaemoglobin should be rewritten with two molecules of ferricyanide instead of four. If there were a reasonable method for the estimation of ferrocyanide in the mixture this might be put to the test of experimental verification.

$\text{HbO}_2 + 2\text{K}_3\text{Fe}(\text{CN})_6 + 2\text{NaHCO}_3 = \text{HbO} + 2\text{K}_3\text{NaFe}(\text{CN})_6 + 2\text{CO}_2 + \text{H}_2\text{O} + \text{O}_2$,
and

$\text{Hb} + 2\text{K}_3\text{Fe}(\text{CN})_6 + 2\text{NaHCO}_3 = \text{HbO} + 2\text{K}_3\text{NaFe}(\text{CN})_6 + 2\text{CO}_2 + \text{H}_2\text{O}$.

CONCLUSIONS.

Oxyhaemoglobin on conversion into methaemoglobin loses half of its dissociable oxygen. This is shown in our experiments by the actual liberation of oxygen as the result of the action of acid. In Buckmaster's experiments the evidence is that hydrazine hydrate liberates only half as much nitrogen when it reduces methaemoglobin as when it reduces oxyhaemoglobin.

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