# THE FERRICYANIDE METHOD OF DETERMINING THE OXYGEN CAPACITY OF BLOOD. By JOHN HALDANE, M.D., F.R.S. (One Figure in Text.)

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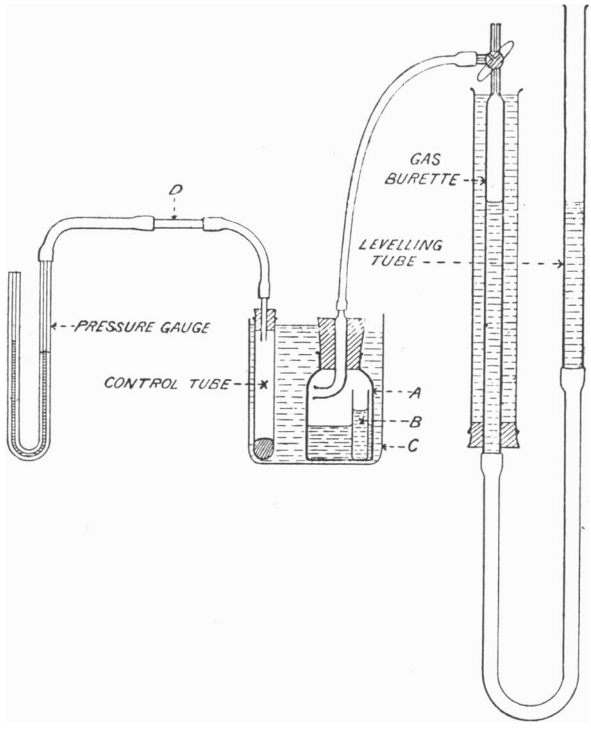
In a previous paper in this *Journal* (XXII. p. 298) I described a new and simple method of determining the percentage of oxygen capable of being taken up in combination with the hæmoglobin of blood. This method depends on the fact that the combined oxygen is liberated rapidly and completely on addition of solution of potassium ferricyanide to laked blood, and may thus be easily measured with the help of an apparatus similar to that of Dupré for determining urea in urine. Since the publication of the paper referred to I have made a number of experiments to test the method more rigorously, and have in consequence modified it with a view to avoiding possible sources of error.

The apparatus is shown in the accompanying figure, and the following is the process as now employed.

20 c.c. of the oxalated or defibrinated blood, thoroughly saturated with air by swinging it round in a large flask, are measured out from a pipette into the bottle *A*, which has a capacity of about 120 c.c. As it is important to avoid blowing expired air into the bottle the last drops of blood are expelled from the pipette by closing the top and warming the bulb with the hand. 30 c.c. are then added of a solution prepared by diluting ordinary strong ammonia solution (sp. gr. 0·88) with distilled water to $\frac{1}{500}$th. The ammonia prevents carbonic acid from coming off, while the distilled water lakes the corpuscles. The blood and ammonia solution are thoroughly mixed by shaking, and at the end of this operation the solution should appear perfectly transparent when tilted up against the sides of the bottle[1].

[1] If the solution were not transparent this would indicate that the laking was incomplete, and more ammonia solution would need to be added.

About 4 c.c. of a saturated solution of potassium ferricyanide are then poured into the small tube *B* (the length of which should slightly




exceed the width of the bottle) and placed upright in *A*. The rubber stopper, which is provided, as shown, with a bent glass tube connected with the burette by stout rubber tubing of about 1 mm. bore, is then firmly put in, and the bottle placed in the vessel of water *C*, the temperature of which should be as nearly as possible that of the room and of the blood and water in the bottle. If the stopper is not heavy enough to sink the bottle the latter should be weighted. By opening to the outside the three-way tap (or T-tube and clip) on the burette, and raising the levelling-tube, which is held by a spring clamp, the water in the burette is brought to a level close to the top. The tap is then closed to the outside, and the reading of the burette (which is graduated to ·05 c.c., and may be read to ·01 c.c.) taken after careful levelling.

The water-gauge (which has a bore of about 1 mm.) attached to the temperature and pressure control-tube is now accurately adjusted

to a definite mark. This is easily accomplished by sliding the rubber tube backwards or forwards on the piece of glass tubing *D*. The control-tube is an ordinary test-tube containing some mercury to sink it, and connected with the gauge by stout rubber tubing of about 1 mm. bore.

As soon as the reading of the burette is constant, which it will probably be within two or three minutes, the bottle is tilted so as to upset *B*, and is shaken as long as gas is evolved. During this operation *B* should be repeatedly emptied, as otherwise the oxygen dissolved in its liquid might not be completely given off. When the evolution of oxygen has ceased the bottle is replaced in the water. If, as is probable, the pressure gauge indicates an alteration in the temperature of the water, cold water from the tap, or warmed water, is added till the original temperature has been re-established, and the reading of the burette noted as soon as it is constant. The bottle is again shaken &c. until a constant result is obtained, for which about fifteen minutes from the beginning of the operations are required. The temperature of the water in the jacket of the burette, and the reading of the barometer, are now taken, and the gas evolved is reduced to its dry volume at 0° and 760 mm. To calculate the oxygen evolved from 100 c.c. of blood allowance must be made for the fact that a 20 c.c. pipette does not deliver 20 c.c. of blood, but only about 19·6 c.c. The actual amount of shortage for a given pipette can easily be determined by weighing the pipette after water, and again after blood, has been delivered from it. A further slight correction is necessary on account of the fact that the air in the bottle at the end of the operation is richer in oxygen than at the beginning, so that, as oxygen is about twice as soluble as nitrogen, slightly more gas will be in solution. With a bottle of 120 c.c. capacity, and 20 % of oxygen in the blood, the air in the bottle at the end will evidently contain about 27 % of oxygen, so that, assuming that the coefficients of absorption of oxygen and nitrogen in the 54 c.c. of liquid within the bottle are nearly the same as in water, the correction will amount at 15° to ·06 c.c. in the reading of the burette, or + ·30 % in the result.

In the method as originally described in my previous paper 20 c.c. of water and 2 c.c. of "saturated solution" of sodium carbonate were mixed with the blood previously to the addition of the ferricyanide; and in investigating the accuracy of the method I at first adhered to the original directions, but soon found that too much sodium carbonate could easily be added, the result being a shrinkage of the remains of

the corpuscles from the laked blood, in consequence of which the ferricyanide did not completely reach the oxyhæmoglobin, and the result was too low. The presence of undecomposed oxyhæmoglobin could be demonstrated by diluting the liquid in the bottle with ·6 % salt solution, and saturating part of it with carbonic oxide. The change of colour and spectrum showed the presence of undecomposed hæmoglobin. This was specially evident when the liquid was also shaken with expired air, so that the colour and spectrum of acid methæmoglobin were obtained along with those of oxy- or CO-hæmoglobin. If the carbonate of soda solution had been saturated at a low temperature, as in the previous experiments, there was no re-precipitation of the corpuscles in ox-blood provided the liquids were quickly mixed; but a solution saturated in warm weather caused a marked re-precipitation. This was accounted for by the fact that the solubility of sodium carbonate varies enormously with temperature up to about 30°. Finding that 2 c.c. of a 10 % solution of dried sodium carbonate caused no precipitation with ox-blood I made a series of test experiments, in which the results by the pump with perfectly fresh ox-blood were compared with the results by the ferricyanide method. The arrangement of pump, blood-receiver, &c. was that of Bohr[1], and the analyses were made in the apparatus which I recently described in this *Journal*[2]. As the result by the ferricyanide method represents only the oxygen in combination with hæmoglobin, while that given by the pump includes also the oxygen in simple solution, I have deducted from the result by the pump the percentage of oxygen (about 0·63 with blood saturated at 13°) which would be in solution. This amount was calculated on the assumption that the coefficient of absorption of oxygen in blood is $\frac{1}{6}$th less than in water, Paul Bert's experiments on the nitrogen and oxygen of the blood of animals in compressed air having shown that this value is probably correct[3]. I have also assumed that any excess of nitrogen over the percentage present in blood saturated with air at the same temperature was due to the accidental presence of air in the pump. This implied a

[1] *Skand. Arch. für Physiol.*, III. p. 72. In three double determinations by this method of the oxygen capacity of blood the maximum difference found by Lorrain Smith and myself was ·05 %. (This *Journal*, XVI. pp. 473, 474.)

[2] XXII. p. 465.

[3] Bert, *La Pression Barometrique*, p. 661. Bert's experiments on blood saturated with compressed air outside the body apparently indicate a much higher coefficient of absorption; but in all probability these experiments were vitiated by the presence in the saturated blood of air-bubbles caused by the shaking.

very slight additional correction. The results are given in the following table:

| | | Volumes of oxygen per 100 volumes of blood | |
|---|---|---|---|
| | | By blood-pump | By ferricyanide method |
| No. 1. | Defibrinated ox-blood | 24·38 | 24·43<br>24·35 |
| No. 2. | Oxalated ox-blood | 20·36 | 20·47<br>20·57 |
| No. 3. | Oxalated ox-blood | 22·40 | 22·20<br>22·33 |
| | Average | 22·38 | 22·39 |

The results were thus sensibly the same by the two methods.

On applying the ferricyanide method to the blood of other animals besides the ox I found that 20 c.c. of water, followed by 2 c.c. of 10 % carbonate of soda solution, often gave a turbid solution. Even with twice as much water and half as much soda human blood, for instance, was turbid. I therefore determined to give up the use of sodium carbonate, and revert to ammonia, which I had originally used in the preliminary experiments. Ammonia, unlike caustic soda or potash, can be added to blood without decomposing the hæmoglobin: nor does it cause any re-precipitation of laked corpuscles. On the other hand, even when its solutions are highly diluted they give off ammonia as gas at an appreciable tension; and it was for this reason that I at first discarded it in favour of soda. Evidently the first step was to ascertain whether it was possible to add sufficient ammonia to hold the carbonic acid, without at the same time causing errors from the presence of ammonia as gas. I found that when about the proportions recommended above were taken, and the mixing bottle closed in the usual way, there was not the slightest measurable alteration in the volume of the air in the bottle on shaking. Hence errors due to either carbonic acid or ammonia gas could be completely avoided. It remained to prove that the results with the ammonia solution were identical with those with sodium carbonate. Four successive determinations were accordingly made of the oxygen capacity of 20 c.c. of fresh ox-blood, the ammonia and soda methods being alternately employed. The results were as follows:

| | | c.c. of oxygen |
|---|---|---|
| By ammonia method | No. 1 | 4·15 |
| | No. 3 | 4·18 |
| By sodium carbonate method | No. 2 | 4·17 |
| | No. 4 | 4·18 |

The two methods thus gave identical results.

Further confirmation of the accuracy of the ferricyanide method is afforded by the fact that, as shown in a succeeding paper by Dr Lorrain Smith and myself, the results of comparative colorimetric determinations of hæmoglobin in samples of blood from different animals give parallel results to those by the ferricyanide method.

A possible source of serious error in blood-gas determinations is the presence of bacteria in the blood; and it is necessary to the attainment of an accurate result with the ferricyanide method that the blood should be collected in a cleanly manner, and kept in ice, or at a low temperature, unless the determinations can be made at once. The presence of bacteria in sufficient numbers to vitiate an analysis can easily be detected by the fact that on continuing the shaking the volume of gas begins to diminish again after the maximum has been reached. If the blood is fresh the reading should remain the same for ten minutes or more. When this is not the case the results are appreciably lower than by the pump, and until I realised the importance of keeping the blood free from bacteria I was inclined to suspect that some substance which absorbs oxygen is formed during the reaction. The following are samples of results (obtained with sodium carbonate) in which the blood, though not showing any of the ordinary signs of putrefaction, was not sufficiently fresh to give a constant reading of the burette by the ferricyanide method. In No. 3, for instance, the oxygen was observed to be disappearing at a rate of about ·05 c.c. in 15 minutes.

| | | Volumes of oxygen per 100 volumes of blood | |
|---|---|---|---|
| | | By blood-pump | By ferricyanide method |
| No. 1. | Oxalated ox-blood | 21·70 | 21·11<br>20·95<br>21·14 |
| No. 2. | Oxalated sheep's blood | 19·42 | 18·27<br>18·47 |
| No. 3. | Defibrinated sheep's blood | 20·31 | 19·70<br>19·60 |

There is little doubt that in these analyses not only the ferricyanide method, but also the pump, gave results which were somewhat too low: yet the error from the presence of bacteria was at any rate less by the pump. It seems probable that the high oxygen tension, and possibly also the alkaline reaction, prevailing in the liquid during the carrying out of the ferricyanide process hastens the disappearance of oxygen by

bacterial action. The time required for the ferricyanide process was less than that required for pumping, so that the low results by the former process could not be due to more time having elapsed since the blood was saturated with air.

In blood or hæmoglobin solutions containing bacteria the oxygen capacity could still be determined with ferricyanide by operating in the absence of oxygen, and with the blood saturated with carbonic oxide.

In two recent papers v. Zeynek[1] and Hüfner[2] have, independently of my own work on the subject, described the action of ferricyanide and other substances in liberating oxygen from oxyhæmoglobin. They found that the oxygen liberated by ferricyanide was distinctly less (about 13 % in the best experiments) than that calculated as being present in oxyhæmoglobin from determinations with the spectro-photometer. The evidence brought forward in this and my previous paper shows, however, that the whole of the oxygen is undoubtedly liberated. Most of v. Zeynek's results were evidently too low on account of the presence of bacteria in the solutions used. In Hüfner's later experiments, however, fresh solutions were used, and the results, though much better, were still apparently too low. Hüfner bases his spectro-photometric results on the hypothesis that one gramme of oxyhæmoglobin yields 1·34 c.c. of oxygen. It appears to me, however, that the evidence in support of this hypothesis is far from satisfactory. His original data[3] gave an average of 1·26 c.c. of carbonic oxide per gramme of hæmoglobin, the results of individual experiments varying by as much as 10 % from one another. To obtain the corrected figure of 1·34 c.c. (which, assuming that Zinoffsky's and Jacquet's determinations of iron are correct, gives a ratio of 1 atom of iron to 2 of oxygen) he makes two assumptions. The first is that carbonic oxide at a tension of about 500 mm. does not saturate hæmoglobin to more than about 93 %, while at a tension of about 700 mm. the saturation becomes complete. This assumption is inadmissible in view of what is now known as to the very powerful affinity of carbonic oxide for hæmoglobin[4]. Moreover, as the dissociation curve of CO-hæmoglobin is undoubtedly a rectangular hyperbola, even if we were to admit that the saturation is a good way from being complete at 500 mm. tension of carbonic oxide we should also have to admit that at 700 mm.

[1] *Arch. f.* (*Anat. u.*) *Physiol.* 1899, p. 460.
[2] *Ibid.* 1899, p. 491.
[3] *Ibid.* 1894, p. 128.
[4] Haldane and Lorrain Smith. This *Journal*, xxii. p. 253.

the incompleteness of saturation is nearly as great. His second assumption is that the coefficient of absorption of carbonic oxide in a 3 or 4 % solution of hæmoglobin, as deduced from his actual experiments, is incorrect, and that a value about 10 % lower is correct. The hæmoglobin solutions employed by Hüfner were dilute, and consequently the correction needed on account of the coefficient of absorption of carbonic oxide amounted to as much as 50 % of the result. The hypothetical correction introduced thus makes a considerable difference in the value obtained. It appears, at any rate, to be clear that the basis of Hüfner's determinations of the oxygen capacity of hæmoglobin from spectro-photometric observations is uncertain, and that this uncertainty may explain the apparent want of agreement between his results with ferricyanide and those with the spectro-photometer, though possibly there may also have been an error due to bacteria.

## Conclusion.

When the fallacies due to incomplete laking of the blood, and to the presence of bacteria, are eliminated, the ferricyanide method of determining the oxygen capacity of the hæmoglobin in blood gives exact results.