A CONTRIBUTION TO THE CHEMISTRY OF HÆMOGLOBIN AND ITS IMMEDIATE DERIVATIVES.

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THE investigations described in the present paper arose from the observation that when ferricyanide of potassium is added to a not too dilute solution of blood in water bubbles of gas are evolved at the same time as methæmoglobin is formed. On examination the gas proved to be nearly pure oxygen. This fact was exceedingly surprising, since Hüfner and Külz[1] have shown that although methæmoglobin yields no oxygen to a vacuum, it gives up to nitric oxide (a reducing substance) exactly the same amount of oxygen as oxyhæmoglobin does, and must therefore contain just the same amount of easily available oxygen.

Reduced blood was found to yield no gas. Blood saturated with carbonic oxide yielded just as much gas as blood saturated with air, and this gas was found to be inflammable.

The quantities of gas obtained evidently corresponded more or less closely to the quantities present in loose combination in the hæmoglobin; but in order to obtain more definite information as to the nature and exact volume of the gas liberated I made the following experiment with the blood-pump. Fresh ox-blood was saturated with air and diluted with an equal volume of water to dissolve the corpuscles, since ferricyanide does not act on undissolved corpuscles. A receiver of known capacity (41 c.c.), with two air-tight taps, and made to fit into the froth-chamber of the blood-pump, as described by Bohr[2], was then filled with the diluted blood, and fixed in position. After the froth-chamber had been evacuated two or three c.c. of the blood were allowed to pass inwards, and a corresponding quantity of saturated solution of ferricyanide was allowed to flow into the receiver through the other

[1] *Zeitschrift f. physiol. Chemie*, VII. p. 366. 1883.

[2] *Skand. Archiv für Physiol.* III. p. 72.

tap. The whole of the blood, mixed with the ferricyanide solution, was now allowed to gradually pass into the froth-chamber, care being taken to prevent fouling of the pump in consequence of the very energetic frothing caused by the action of the ferricyanide. The blood was then pumped out, and the gas collected in the ordinary way. A corresponding experiment was made with blood which had been saturated with a mixture of about equal parts of carbonic oxide and air; and in a third experiment the blood was saturated with air, but no ferricyanide was added. The same stock of blood was of course used for all the experiments, and care was taken to avoid any fallacy due to subsidence of the corpuscles. In calculating the volumes of gas given off from the hæmoglobin allowance was made (1) for the gas calculated as having been present in simple solution in the water and in the blood, and (2) for a small amount of oxygen which, to judge from the nitrogen found, had, as usual in blood-pump determinations, in some way found its way in as air. The gas present in simple solution was calculated on the assumption that the coefficients of absorption of gases are the same for blood as for water. The gas was measured and analysed in the apparatus described in a succeeding paper. The temperature of the gas-burette was 20·0°, and the barometer 765·2 mm., at the time of closing the control tube, so that all the measurements were referred to this temperature and pressure, with the corresponding tension of aqueous vapour. The results are given in the following table.

		From 20·5 c.c. of blood (at 20° C. and 759 mm.)			From 100 c.c. of blood (at 0° C. and 760 mm.)		
		Oxygen	Carbonic oxide	Nitrogen	Oxygen	Carbonic oxide	Nitrogen
No. 1	Total Gas	4·38	—	·785	19·42	—	3·54
	Liberated from hæmoglobin	4·105	—	—	**18·20**	—	—
No. 2	Total Gas	·245	4·305	·535	1·09	19·09	2·37
	Liberated from hæmoglobin	—	4·075	—	—	**18·07**	—
No. 3	Total Gas	4·375	—	·78	19·40	—	3·52
	Liberated from hæmoglobin	4·10	—	—	**18·18**	—	—

It will be seen from the above table that the volume of oxygen obtained from the oxyhæmoglobin is the same with ferricyanide as with

the vacuum alone: also that the volume of carbonic oxide obtained from the carbonic-oxide-hæmoglobin is the same as the volume of oxygen obtained from the oxyhæmoglobin. The carbonic oxide was determined by combustion with a platinum spiral. The contraction on combustion (2·155 c.c.) was exactly half the volume of carbonic acid formed (4·305 c.c.), so that there was no doubt as to the combustible gas being pure carbonic oxide. It is also evident that no other gas, such as nitrogen, is given off in the reaction between the ferricyanide and the blood, since the residue ("nitrogen") is exactly the same in nos. 1 and 3, which are comparable in this respect. The percentage of nitrogen is unusually high, both in no. 1 and no. 3, but this is simply due to the fact that the usual error due to mixture of air with the gas pumped off tells more seriously than it otherwise would, owing to the volume of undiluted blood employed being so small.

In a second experiment the oxygen obtained from the hæmoglobin of 20·5 c.c. of ox-blood by the pump alone was 4·20 c.c., and with ferricyanide 4·24 c.c.

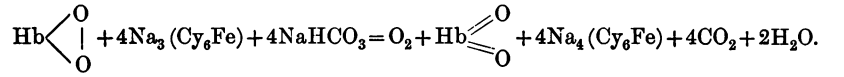
It is evident from these experiments that the quantity of oxygen or carbonic oxide liberated in the ferricyanide reaction corresponds exactly to the quantities previously present in loose combination with the hæmoglobin. It would thus seem that the result of the action of the ferricyanide is that the affinity of the hæmoglobin molecule for the oxygen or carbonic oxide in loose combination with it is simply abolished, and that these gases are consequently liberated. It is of course conceivable that the gas liberated might in whole or in part have some other source than the gas previously combined in the form of oxyhæmoglobin or carbonic-oxide-hæmoglobin, but against such an interpretation stand the facts: (1) that although methæmoglobin is formed in exactly the same manner as before, no gas is liberated when ferricyanide acts on reduced hæmoglobin; (2) that carbonic oxide is liberated from its combination with hæmoglobin in precisely the same way as oxygen. It is exceedingly difficult to interpret these facts on any other hypothesis than that given.

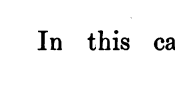
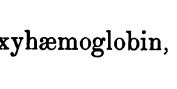
What, then, is the source of the oxygen which Hüfner's experiments have shown to be present in methæmoglobin? If ferricyanide be added to diluted blood it will be found that the solution now gives with ferric chloride the blue colour indicating the presence of ferrocyanide. Evidently, therefore, the ferricyanide is reduced to ferrocyanide. The oxygen rendered available in this reaction doubtless passes into the hæmoglobin molecule. The whole process thus conceived may be

provisionally represented by the following equation when the ferricyanide is acting on reduced hæmoglobin.

$$Hb + 4Na_3(Cy_6Fe) + 4NaHCO_3 = HbO_2 + 4Na_4(Cy_6Fe) + 4CO_2 + 2H_2O.$$

In this case the symbol HbO_2 represents methæmoglobin. When the ferricyanide is acting on oxyhæmoglobin the following equation represents the supposed process.



In this case  represents oxyhæmoglobin, and  methæmoglobin. The reason for employing these different symbols is that as shown below there are some grounds for believing that in oxyhæmoglobin the oxygen atoms are united together, whereas in methæmoglobin this is probably not the case.

It is usually stated that in methæmoglobin the oxygen is more firmly united than in oxyhæmoglobin. This statement is, I think, somewhat misleading, and has led to the misinterpretation of a number of well-known facts. It is of course undoubtedly the case that methæmoglobin will not yield up its oxygen to a vacuum, while oxyhæmoglobin will; but in other ways methæmoglobin gives up its oxygen far more readily than oxyhæmoglobin, and in fact methæmoglobin must be regarded as an oxidising agent of some activity, whereas oxyhæmoglobin has no greater oxidising power than free oxygen. Oxyhæmoglobin, indeed, very readily acts as a reducing agent, as in the case of its reaction with ferricyanide discussed above.

The readiness with which methæmoglobin parts with its oxygen to a reducing agent is strikingly shown by the effect of adding (in the cold) a drop of ammonium sulphide to a few c.c. of a dilute solution of methæmoglobin. The effect is that (unless an excess of ferricyanide is still present) the spectrum of oxyhæmoglobin at once appears, and remains for long before the oxyhæmoglobin is finally reduced to hæmoglobin. What happens is that the methæmoglobin, which very readily gives up its oxygen, is reduced to hæmoglobin almost instantly, but this hæmoglobin at once combines with the free oxygen present and forms oxyhæmoglobin. The ammonium sulphide acts very slowly on this oxygen, whether it be free or combined as oxyhæmoglobin: consequently the spectrum of oxyhæmoglobin remains for long unaltered. The explanation commonly given is that oxyhæmoglobin is a stage in

the reduction of methæmoglobin to hæmoglobin. That the oxyhæmoglobin is formed simply in consequence of the presence of free oxygen in the solution is, however, easily proved by the following experiment. Solution of methæmoglobin, diluted so far that its spectrum can be easily observed, is placed in a glass receiver provided with taps at both ends, one of the taps being a three-way one. The free oxygen is then entirely boiled out with the help of a filter-pump, the receiver being placed in warm water. After the oxygen has been removed the tap is closed and the solution cooled if it is at all warm. Two or three drops of a dilute solution of ammonium sulphide are then allowed to pass in through the three-way tap, after all air has first been displaced by the ammonium sulphide solution, which is first warmed so as to get rid of any free oxygen. It will now be found that the methæmoglobin solution gives absolutely no oxyhæmoglobin spectrum, but only reduced hæmoglobin when the ammonium sulphide acts on the methæmoglobin. The same arrangement can be employed for showing that when ferricyanide acts on reduced hæmoglobin there is no intermediate stage of oxyhæmoglobin.

From the facts given above it would seem that the chemical differences between oxyhæmoglobin and methæmoglobin may be explained on the theory that in oxyhæmoglobin the oxygen atoms are united firmly with one another, but very loosely with the hæmoglobin molecule, while in methæmoglobin the oxygen atoms are either not united together at all, or very loosely united, while they are also somewhat loosely united with the hæmoglobin molecule. Oxyhæmoglobin may thus perhaps be regarded as a compound of a molecule of oxygen with hæmoglobin, while methæmoglobin is a compound of two atoms of oxygen with hæmoglobin. This view seems to explain the fact that though methæmoglobin will not part with its oxygen to the pump it will part with it far more readily than oxyhæmoglobin to reducing agents.

With sodium nitrite oxyhæmoglobin yields no free gas. With potassium permanganate there is also little or no evolution of gas. With a neutral hydroxylamine chloride solution there is an abundant evolution of gas, which, however, is not simply oxygen.

As the action of ferricyanide in liberating oxygen and carbonic oxide from their combination with hæmoglobin is so very definite and sharp I made experiments with a view to ascertaining whether the reaction may be utilised for determining the amount of oxygen or carbonic oxide capable of being absorbed by a blood or hæmoglobin

solution. The alternative means of making this determination is to pump out, measure, and analyse, the gas; and this is a somewhat troublesome plan, requiring a good deal of time, apparatus, and skill; hence a more ready method would be of considerable use.

I at first tried the plan of confining the blood solution over mercury in a closed vessel, measuring the volume of mercury driven out by the evolution of the gas. It was difficult however to devise an apparatus which was quite satisfactory for this purpose and which could be freely shaken to facilitate the reaction: moreover the ferricyanide acts on mercury, and thus causes much inconvenience. The most convenient arrangement was finally found to be an apparatus arranged similarly to the well-known urea apparatus of Dupré[1]. The Dupré apparatus itself answers fairly well, but as the present method appeared to be one admitting of much greater accuracy than the determination of nitrogen in urine by the hypobromite method it seemed worth while to endeavour to introduce one or two modifications with a view to making the measurements more definite and accurate. In the first place a test-tube containing some mercury to sink it, with a few drops of water added to keep the contained air moist, and connected with a manometer made of narrow bore (1 mm.) glass tubing, was kept in the water-bath of the apparatus to indicate any differences of pressure or temperature arising during the analysis. By pouring in cold or warm water until the water in this gauge returned to its initial position errors of measurement due to variations in temperature of the bath or barometric pressure were entirely eliminated. A simple calculation will show that even a very slight variation of temperature in the bath or barometric pressure would make a considerable difference in the results. Thus a variation of 0·5° C. would cause an error of more than 2% in the readings of the gas-burette. In the second place an accurately graduated burette, allowing of readings to ·02 c.c., was substituted for the wide burette which is supplied for urea determinations. The burette was connected below in the ordinary way with a levelling tube, water being the confining liquid. For further convenience and certainty in measuring the temperature of the air in the burette the latter was enclosed in a water-jacket, though this was less important since slight errors as regards the temperature of the burette would make little difference in the results. The burette was connected with the mixing bottle by pressure tubing of narrow bore.

[1] *Journ. Chem. Soc.* I. p. 534. 1877.

It is evident that along with the oxygen a certain amount of carbonic acid would be given off by the blood solution to the air in the mixing bottle unless precautions were taken to prevent this. The addition to the blood of 5 c.c. of a saturated solution of sodium carbonate was found to prevent the giving off of carbonic acid and not to interfere with the reaction. When this precaution was not taken the results were 10% or more too high. With the sodium carbonate the air in the mixing bottle was found to contain only ·06% of carbonic acid, and there was no sensible alteration of the volume of the air on shaking without the addition of ferricyanide.

The procedure adopted in the case of blood was as follows. 50 c.c. of the blood (which was first well saturated with air) were measured off with a pipette into the mixing bottle, which had a capacity of 220 c.c.[1] 50 c.c. of water were added and the whole mixed. In adding the blood and water care was taken not to blow expired air into the bottle, as the carbonic acid might have caused gross error. The addition of the water produced rapid solution of the corpuscles, and after this had occurred 5 c.c. of saturated sodium carbonate solution were added. About 5 c.c. of saturated solution of potassium ferricyanide were then placed inside the bottle in the little tube ordinarily used for holding the urine in a urea determination. The stopper was then inserted, the bottle placed in the water-bath, and the level of the burette read off as soon as the temperature was constant. At the same time the water-gauge of the control tube standing in the water-bath was adjusted to a definite mark. The ferricyanide was now mixed with the blood, and the bottle shaken until gas ceased to come off. Most of the oxygen came off very rapidly under these circumstances, but some time was necessary in order to get off the last two or three tenths of a c.c. The whole process required about ten minutes. Meanwhile the temperature of the bath had probably risen slightly. The temperature was therefore adjusted by pouring in cold water until the water in the gauge came back to its former level. The burette was then levelled and read off, its temperature being at the same time noted, as well as the height of the barometer. The results were corrected to dry air at 0° and 760 mm., and calculated for 100 c.c. of blood.

It is evident that the result thus obtained is the oxygen combined

[1] 20 c.c. of blood and a mixing bottle of 100 c.c. capacity answered quite as well, or better, with a burette graduated to ·05 c.c. The determinations of sample nos. 4 and 5 in the table given below were made with 20 c.c. of blood, and much smaller volumes might if necessary be employed.

in the hæmoglobin, and that the oxygen in simple solution in the blood remains in solution and is therefore not measured. The volume of gas in the bottle and burette is about 120 c.c. At the end of the experiment this will contain about 29% of oxygen, and the blood solution will now be saturated with an atmosphere containing 29% of oxygen and 71% of nitrogen. As oxygen is about twice as soluble in liquids as nitrogen a slight correction is in very accurate experiments necessary for the extra volume of gas dissolved from this atmosphere. This correction amounts to only about ·11 c.c. for oxygen and ·06 c.c. for carbonic oxide in the case of a bottle of 220 c.c. capacity, containing 100 c.c. of mixed blood and water.

The following table gives the results of some experiments in which several determinations of the same sample were made. In the case of carbonic oxide determinations the blood was first saturated with coal-gas, and then shaken with air to avoid any fallacies due to the differences in the coefficients of absorption of coal-gas and air. The carbonic oxide comes off a good deal more slowly than oxygen does.

		Volumes of gas per 100 c.c. of blood
Sample No. 1 (saturated with air)	(*a*)	21·57
	(*b*)	21·48
	(*c*)	21·34
Sample No. 2 (saturated with air)	(*a*)	19·70
	(*b*)	19·72
Sample No. 3 (saturated with CO)		19·52
Same blood (saturated with air)		19·38
Sample No. 4 (saturated with air)	(*a*)	16·91
	(*b*)	16·91
	(*c*)	17·05
	(*d*)	16·86
Sample No. 4 (saturated with CO)	(*e*)	17·00
	(*f*)	17·22
Sample No. 5 (saturated with air)	(*a*)	21·07
	(*b*)	21·07

These results show that the method is a very exact one. With a first-rate pump, and accurate methods of gas-analysis, more even results can be obtained, but for many purposes the present method will probably be preferred. For rapidly demonstrating the volume of oxygen absorbed by blood this method will be found particularly convenient.

Summary of Chief Conclusions.

1. When ferricyanide is added to solutions of oxyhæmoglobin or carbonic-oxide-hæmoglobin, the gas combined with the hæmoglobin is set free and froths off, while methæmoglobin is formed.

2. By taking advantage of this reaction the volume of gas capable of being absorbed by the hæmoglobin of blood may be rapidly and accurately determined without the use of the blood-pump.

3. Although methæmoglobin yields no oxygen to a vacuum it parts with its oxygen to reducing substances far more readily than oxyhæmoglobin does.