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THE FERRICYANIDE METHOD OF BLOOD-GAS ANALYSIS

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It has long been recognized (Morawitz, 1909; Plesch, 1909; Douglas, 1910) that an error occurs in the determination of the percentage oxygen capacity of the blood by Haldane's ferricyanide method, since on adding ferricyanide to liberate the oxygen from its combination with oxyhaemoglobin a slow secondary oxidation also takes place, with the result that the estimated volume of oxygen liberated from combination is too low. In van Slyke's method such an error is inappreciable, since any oxygen set free in the reaction is instantly evacuated from the laked blood, and it has therefore been customary in recent years to regard van Slyke's method as the standard method for blood-gas analysis. On the other hand, Haldane's method has the advantage that the apparatus required is simple and cheap to construct, nor does it require the considerable quantity of mercury necessary in the van Slyke apparatus. We have therefore examined the Haldane method to see if it is possible to overcome the fallacy to which it is liable.

Parsons & Parsons (1927), Litarczek (1928), Wright & Arthur (1931) and Wright, Conant & Kamerling (1931) have shown that the error arises, at least in the main, from the oxidation of lipoid material in the blood, and is the greater in conditions of lipaemia; possibly the proteins may be concerned as well. Haldane originally mixed the blood with dilute ammonium hydroxide (2 ml. ammonium hydroxide solution, sp.gr. 0·880, later increased to 4 ml., diluted to 1 l.) or with 1% (w/v) anhydrous Na_2CO_3 in order to ensure that CO_2 should be retained in combination while the reaction between oxyhaemoglobin and potassium ferricyanide was taking place, a small quantity of saponin being added to ensure laking of the corpuscles, but the observations of Wright *et al.* as well as those of Woodhouse & Pickworth (1930) point to the fact that the speed of the secondary oxidation is dependent on the alkalinity. The latter authors found that it was possible to inhibit the secondary oxidation by working close to the neutral point, but it should be noted that in determining the percentage oxygen capacity they first thoroughly aerated the

blood so that its natural CO_2 content was greatly reduced. But even if a correct estimate of the percentage oxygen capacity could be found in this way the use of the Haldane method for accurate work would still be limited, since blood-gas analysis is often needed of samples of blood containing their natural CO_2 content. It therefore seemed to us worth investigating whether it was possible to use in the Haldane method a buffer solution having a pH sufficiently low to render the secondary oxidation inappreciable and yet at the same time sufficiently high to keep the CO_2 in the blood from escaping. This could obviously not be very close to the neutral point. We first tried a borate buffer solution of pH 9·5, which showed considerable promise, but following a suggestion made to us by Dr F. J. W. Roughton we increased the pH to 10·0 and with this got very satisfactory results.

In making the buffer solutions we have followed the directions given by Walpole (1911): 'prepare a solution of "NaH_2BO_3" containing in 1 l. 12·404 g. boric acid and 100 ml. N-sodium hydrate,

for pH 9·5 add 2 ml. N/10-NaOH to 8 ml. NaH_2BO_3 solution,
for pH 10·0 add 4 ml. N/10-NaOH to 6 ml. NaH_2BO_3 solution,
for pH 12·4 add 6 ml. N/10-NaOH to 4 ml. NaH_2BO_3 solution.'

In all our experiments the blood has been rendered incoagulable by the addition of 0·2% potassium oxalate and 0·1% sodium fluoride. Human blood samples were taken by venepuncture.

We have used the last form of constant-pressure blood-gas apparatus designed by Haldane (1920, cp. also Douglas & Priestley, 1937). As has been our practice for many years we have immersed the complete apparatus in a water-bath with a glass front, the upper ends of the levelling tubes projecting above the water level, and have adopted mechanical shaking of the reaction flask. The stout wire frame to which the gas burette and gauge tubes are secured is firmly held by a clamp, and the position of the liquid menisci can be accurately read without interruption of the shaking in spite of the movement of the reaction flask and rubber connecting tube. In all our experiments the water-bath has been at room temperature.

Fig. 1 shows the mechanical device for shaking the reaction flask and also shows the form of reaction flask which we have adopted. Ferricyanide solution is placed in one of the small side tubes which slopes at an angle of 45° from the lower part of the neck of the flask; tartaric acid solution can be placed in the other side tube which slopes in the opposite direction. On tilting the reaction flask appropriately, either the ferricyanide solution or the tartaric acid (used to liberate CO_2) can be run into the flask: to do this the shaking gear to which the reaction flask is attached must be lifted sufficiently from the bath to permit the requisite tilting, and then replaced. The glass tube fitted with a tap, which penetrates the rubber stopper, fits into clips on the shaking gear and also

allows the air in the reaction flask, connecting tube and burette system, to be displaced by any gas mixture that may be desired.

As designed by Haldane, the reaction flask had a volume of about 20 ml., while we have used a larger flask holding 34 ml., and sometimes one holding 44 ml., which we felt might allow more effective shaking of the blood-buffer mixture. An error, which is the more significant the larger the reaction flask, can arise if the small amount of CO_2 in the normal air in the flask at the

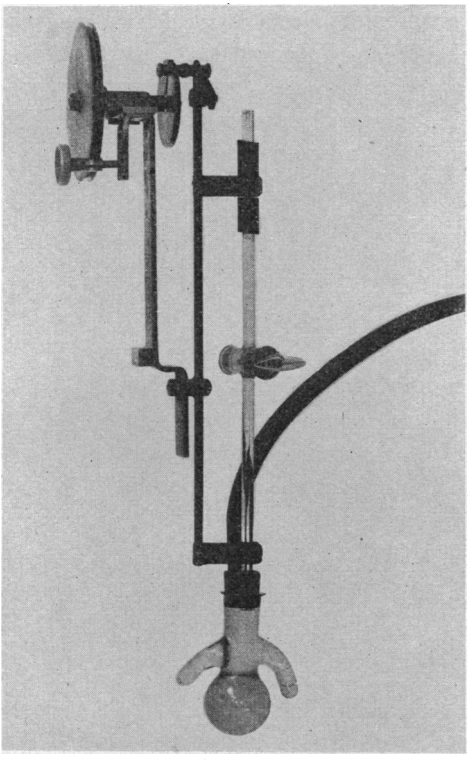

Fig. 1. Shaking gear and reaction flask used with the Haldane blood-gas apparatus.

beginning of the experiment has not been completely absorbed by the buffer solution before the first reading is made, and this may require many minutes if the flask is stationary in the water-bath owing to the slowness of diffusion. To avoid this error we always blow CO_2-free air through the apparatus, introducing it by means of the glass tube shown in Fig. 1, after the reaction flask has been immersed in the water-bath before the taps are closed.

On adding a saturated solution of potassium ferricyanide to water or to a mixture of the buffer solution and blood plasma, we have found that a small diminution of volume is registered in the gas burette, possibly because of a

change of vapour pressure. A correction can be applied for this change in volume, but it is simpler to substitute a 6% (w/v) solution of potassium ferricyanide for the saturated solution, as with this the change in volume is inappreciable; 0·25 ml. of this 6% solution is ample to expel the oxygen from 2 ml. of normal blood, even though a little of the solution is unavoidably left in the side tube on tilting the reaction flask. The dilute solution has, too, the advantage over the more concentrated, since the rate of the secondary oxidation is considerably diminished.

Our procedure has therefore been as follows: 1·5 ml. borate buffer solution of pH 10·0 (or such pH as is desired) is placed in the reaction flask, and 0·25 ml. 6% potassium ferricyanide solution, in which a small pinch of saponin (the so-called 'saponin white') has been dissolved, is placed in one of the side tubes; 1·0 ml. of the blood to be analysed is then run carefully from a pipette (Ostwald or narrow straight pipette) under the buffer solution and the reaction flask is pushed on to the rubber stopper, clipped to the shaking gear and immersed in the water-bath. Care is taken not to disturb the two layers of blood and buffer. Air which has been freed from CO_2 is then run through the apparatus and the taps are closed to air. With the shaking gear stationary, temperature equilibrium is reached in 2 or 3 min. if the bath is efficiently stirred; a steady reading of the burette is obtained up to the 10th or 15th minute, after which time a very slow diminution of volume may become apparent if the blood has been taken from a saturator at 38° C., as in determining the oxygen-dissociation curve, because of the slow diffusion of oxygen and nitrogen from the air in the flask through the buffer solution into the blood which has now cooled to the lower temperature of the water-bath and can hold a larger amount of oxygen and nitrogen in solution and combination. If the blood has been previously fully saturated with air at about the same temperature as the water-bath, the readings of the burette remain steady for many minutes. An example of this is shown in Table 1. The temperature of the water-bath was 16·5° C.; blood sample A had previously been saturated at

TABLE 1. Readings in ml. of gas burette after closing taps with the reaction flask stationary. To show the influence of diffusion of gases through the buffer solution into the blood

Min. after closing taps	Blood sample A	Blood sample B
2	0·153	—
3	0·151	0·182
4	0·151	0·181
5	0·150	0·180
6	0·150	0·180
8	0·150	0·180
10	0·150	0·180
12	0·150	0·180
14	0·150	0·180
19	0·149	0·180
22	0·148	0·180
25	—	0·180

38° C. with air containing 5·52% CO_2, 6·90% O_2 and 87·58% N_2, while sample B had previously been saturated at 16·5° C. with normal air.

When the initial steady reading has been verified the shaking is started, and any oxygen uptake is noted when the readings have been steady for 5 min.; we have found no change in volume if the blood has previously been thoroughly saturated with normal air at room temperature. The ferricyanide solution is then run into the reaction flask and shaking continued until a steady reading shows that the combined oxygen has been set free from the laked blood.

That the borate buffer of pH 10·0 can prevent any significant escape of CO_2 from the blood while one of pH 9·5 is hardly adequate is shown in Table 2.

TABLE 2. Change in gas volume in ml. on shaking blood with buffer solutions. Blood saturated at room temperature with air or air + CO_2, and measurement of volume made at the same temperature

Borate pH 9·2		Borate pH 9·5		Borate pH 10·0	
CO_2 content ml./100 ml. blood	Change on shaking	CO_2 content ml./100 ml. blood	Change on shaking	CO_2 content ml./100 ml. blood	Change on shaking
60·5	+0·009	54·0	+0·002	61·4	0·000
60·0	+0·007	63·9	+0·004	62·5	0·000
		67·6	+0·003	63·1	0·000
				67·1	−0·001
		Low	0·000	67·6	0·000
		CO_2	+0·001	68·2	0·000
		content	+0·001	71·0	0·000

Blood was saturated with oxygen at room temperature by exposure in a saturator either to normal air, which also greatly reduced the CO_2 content of the blood, or to air to which 2·4–4·3% of CO_2 had been added, in which case the total CO_2 content of the blood reached a high value. The blood was then placed under the buffer solution in the reaction flask in the usual way, and after the initial steady reading had been noted shaking was started. Whereas no further change in volume occurred with the buffer of pH 9·5 when the CO_2 content of the blood was low, when the CO_2 content was high there was a distinct, though slight, *increase* in volume with the buffer of pH 9·5 and a still greater increase with a buffer of pH 9·2 ('NaH_2BO_3' solution without addition of sodium hydroxide), although the volume remained unchanged with the buffer of pH 10·0, suggesting that at the lower pH some CO_2 passed into the gaseous phase.

Fig. 2 shows the degree to which the borate buffer of pH 10·0 inhibits the secondary oxidation in blood and plasma. The upper two graphs were obtained from a single specimen of human blood and the plasma separated from it; the lower two graphs give the mean results of experiments on the blood and plasma of each of two rabbits. The blood or plasma was put under the buffer or sodium carbonate solution and shaking started. When equilibrium had been reached the ferricyanide was run in. Since the plasma was practically free

from haemoglobin, readings of the diminution of volume due to the secondary oxidation could be started at once. In the case of blood, oxygen had first to be expelled from combination, and the graphs therefore start when a maximum reading was first reached 4–5 min. after running in the ferricyanide.

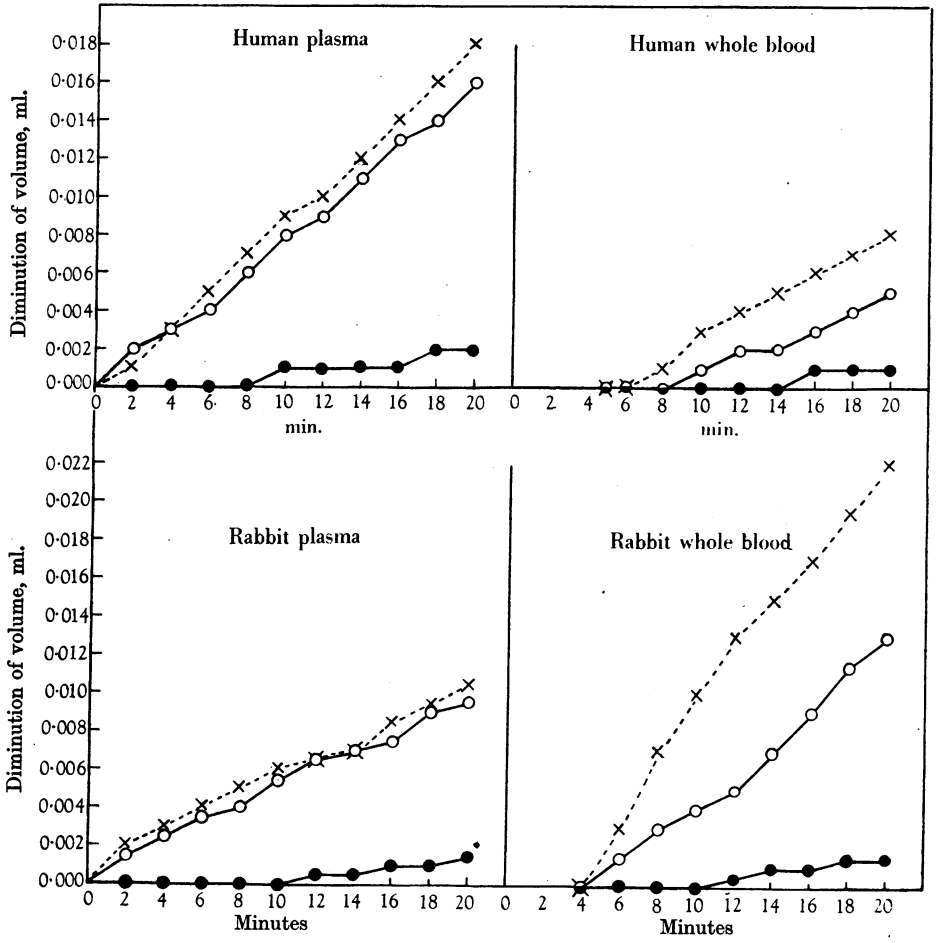

Fig. 2. Rate of secondary oxidation after adding 0·25 ml. 6% potassium ferricyanide to 1·0 ml. plasma or whole blood and 1·5 ml. buffer or sodium carbonate solution. • Borate buffer pH 10·0. o Borate buffer pH 12·4. × 1% Na_2CO_3.

Using a borate buffer of pH 10·0 the initial reading remains steady for some minutes, after which the volume begins to show a just perceptible diminution. It seems clear, therefore, that with the buffer of pH 10·0 the secondary oxidation is so slow that it should not cause any appreciable error in the measurement of the oxygen liberated from combination with haemoglobin.

This is certainly not the case when a borate buffer of pH 12·4 or 1% Na_2CO_3 is used. The rate of secondary oxidation is now so much increased that it is clear that it must influence the reading while oxygen is being liberated and make the estimate of the oxygen combined with haemoglobin too low. Ox blood and plasma behave very much like human blood, the rate of secondary oxidation being faster in plasma than in whole blood, but in the blood of several rabbits which we have tested the rate of oxidation was faster in the blood than in the plasma separated from it. The rate of the secondary oxidation varies with blood from different species, and with blood from different individuals of the same species; it is particularly troublesome in rabbit's blood, but even in this case Fig. 2 shows that the use of a borate buffer of pH 10·0 ought to render it negligible.

The percentage oxygen capacity of samples of blood which had been thoroughly aerated at room temperature with normal air, to which in some cases CO_2 was added so as to maintain a high concentration of CO_2 in the blood, was determined by both the van Slyke method and by the Haldane method, using in the latter case borate buffer solutions of pH 9·5, 10·0 and 12·4 or a 1% solution of Na_2CO_3. In calculating the results by the van Slyke method the coefficients of solubility of oxygen given by Sendroy, Dillon & van Slyke (1934) have been used. The results are given in Table 3.

TABLE 3. O_2 capacity of whole blood, ml. O_2/100 ml. blood, determined by van Slyke and Haldane methods

		Haldane			
	van Slyke	Borate pH 9·5	Borate pH 10·0	Borate pH 12·4	1% Na_2CO_3
Human	18·3	18·5	—	18·1	17·9
	18·4	18·4	—	18·0	17·6
	19·6	19·7	—	19·0	18·8
	17·6	—	17·8	17·4	17·0
	20·4	—	20·5	20·0	19·6
	18·8	—	18·8	18·4	17·6
	18·3	—	18·2	17·7	17·6
	15·4	—	15·4	14·9	14·7
	20·5	—	20·5	—	—
	15·8*	—	15·6*	—	—
Rabbit	12·8	12·8	—	12·4	11·9
	14·1	14·1	—	13·6	13·3
	12·8	—	12·9	12·4	11·5
	14·4	—	14·3	14·0	13·5
Sheep	16·6	—	16·7	16·2	15·9
	17·1†	—	17·1†	—	—
Ox	20·3	—	20·4	19·9	19·5
	21·8	—	21·8	21·4	21·5
	21·5	—	21·5	21·2	21·1
	21·4	—	21·4	—	—
	21·8‡	—	21·7‡	—	—

* 61·4 ml. CO_2/100 ml. blood.
† 67·6 ml. CO_2/100 ml. blood.
‡ 71·0 ml. CO_2/100 ml. blood.

Whereas the Haldane method with the borate buffer of pH 12·4 gives results which are lower in every case than those given by the van Slyke method, and lower still when Na_2CO_3 solution is used (which is in agreement with the results recorded by others who have used Na_2CO_3 or NH_4OH solutions, e.g. van Slyke & Stadie, 1921; Price-Jones, Dill & Wright, 1931; Bierring, Nielsen & Nielsen, 1936; Douglas, Jope, Jope, Macfarlane & O'Brien, 1943), with the borate buffer of pH 9·5 when a low CO_2 content is present in the blood, and with one of pH 10·0 even when the CO_2 content of the blood is high, the results obtained by both the Haldane method and the van Slyke method are practically identical. A borate buffer of pH 10·0 seems therefore to fulfil the required conditions.

As a further test of the validity of the Haldane method with the modifications which we have introduced we have determined the oxyhaemoglobin dissociation curve in the blood of three different individuals. The blood was equilibrated at 38° C. by the method described in detail by Douglas & Priestley (1937) with mixtures of nitrogen and different concentrations of oxygen, CO_2 being added in each instance so as to afford a partial pressure of this gas of about 40 mm. Hg. To obtain the lowest concentrations of oxygen the blood was first brought into equilibrium with nitrogen at 38° C. so that most of the oxygen was removed from it; the saturator was then refilled with nitrogen containing the required amount of CO_2 and equilibration completed. The percentage saturation of the haemoglobin with oxygen was determined independently by C. G. D. using the Haldane method with borate buffer of pH 10·0 and by F. C. C. using the van Slyke method. The results are given in Table 4.

With the gas burette which we have used in the Haldane apparatus we cannot detect a change in volume of less than 0·001 ml., corresponding to an alteration in volume of 0·1 ml./100 ml. blood when using 1·0 ml. blood for analysis. When calculating the percentage saturation of haemoglobin with oxygen this means that differences of less than 0·5 are certainly without significance. We doubt whether in our hands the van Slyke apparatus gives results of any greater accuracy than this when using 1 ml. blood if these have to be based on single determinations, as is the case in the figures shown in Table 4. We have therefore expressed the percentage oxygen saturation to the nearest 0·5. In the Haldane method the amount of oxygen taken up to complete saturation of the haemoglobin, and the amount subsequently expelled by ferricyanide, are determined in the same sample of blood, and a small error in the volume of blood delivered from the pipette has no significance. The van Slyke method involves the measurement of the actual oxygen content in two separate samples of blood of known volume, and there is the possibility of a small error arising in the delivery of blood from the pipette. In calculating the results by the van Slyke method we have in each instance taken for the

percentage oxygen capacity of the fully oxygenated blood the figure given by the Haldane method on the same sample of blood, a procedure which we feel is justified by the results given in Table 3.

TABLE 4. Oxyhaemoglobin dissociation curve of human blood determined by van Slyke and Haldane methods

		% saturation of Hb with O_2		
			Haldane	
Subject	O_2 tension mm. Hg	Van Slyke	Bohr coeff.	van Slyke coeff.
C. G. D.	1·4	1·5	1·5	2·0
	9·9	10·0	10·0	10·5
	17·7	24·0	24·0	25·0
	20·4	30·5	31·5	32·0
	30·0	52·0	53·0	53·5
	40·8	73·0	73·0	74·0
	48·3	83·5	83·0	83·5
	60·3	91·5	91·0	91·5
	79·2	95·0	95·0	96·0
	98·1	96·5	97·0	97·5
F. C. C.	1·0	1·0	1·5	2·0
	8·5	8·5	10·0	10·0
	11·6	14·5	13·5	14·0
	18·1	30·5	30·0	30·5
	28·1	53·5	54·0	54·5
	37·6	72·5	73·5	74·5
	49·0	86·5	86·0	86·5
	59·5	91·5	93·0	93·5
	79·3	97·0	95·5	96·0
	101·9	98·0	97·5	98·0
T. J. M.	0·9	1·0	0·5	1·0
	11·2	12·0	11·5	12·0
	16·6	21·0	20·0	20·5
	22·8	38·0	37·0	37·5
	30·6	56·0	56·0	56·5
	40·1	72·0	73·5	74·0
	49·0	83·5	84·0	84·5
	63·6	90·5	91·5	92·0
	80·5	96·5	95·0	95·5
	100·4	99·0	97·0	98·0

The results obtained by the Haldane method have been calculated (*a*) using the Bohr coefficients of solubility of nitrogen and oxygen in blood since these have been used by many observers in the past, and (*b*) using the more recent coefficients determined by van Slyke, Dillon & Margaria (1934) and Sendroy *et al.* (1934). In the latter case we have used for oxygen the coefficients of solubility of 0·023 at 38° C. and 0·038 at 15° C., and for nitrogen 0·013 at 38° C., but in the absence of exact data at lower temperatures we have assumed a coefficient of 0·018 at 15° C., and this figure may not be quite correct. The substitution of the van Slyke for the Bohr coefficients increases the apparent percentage saturation with oxygen by about 0·5 on the average, but, whichever figures are taken, agreement between the results obtained both

by the Haldane method and by the van Slyke method is extremely close. Out of the total of thirty observations the percentage saturation with oxygen determined by the Haldane method only differs from that determined by the van Slyke method by more than 1·0 in seven instances using the van Slyke coefficients, and in six instances using the Bohr coefficients. The close agreement between the results obtained by the two methods is clearly seen in

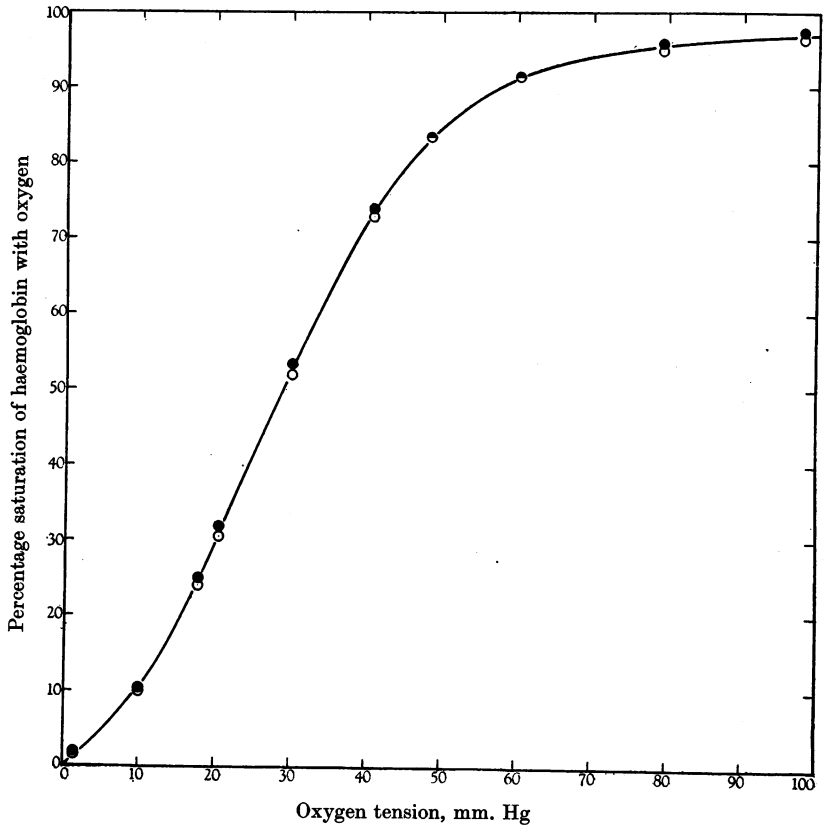

Fig. 3. Oxyhaemoglobin dissociation curve of blood of C. G. D.
• Haldane method. o van Slyke method.

Fig. 3, which shows the dissociation curve drawn for the blood of C. G. D. from the data in Table 4 which were obtained by the van Slyke method and by the Haldane method using the van Slyke coefficients of solubility. We feel therefore that, with the modifications which we have described, the Haldane method will yield results for the percentage saturation of haemoglobin in the blood which are to all intents and purposes identical with those given by the van Slyke method.

The values given by the Haldane method should be a little more accurate if 2 ml. blood were used, as Haldane originally recommended, since the volume of gas to be measured would be doubled while the minimal detectable alteration in volume would be unchanged, but our results show that adequate accuracy can be secured using only 1 ml. blood provided that the readings are made with care. The smaller volume of blood has, too, the advantage that a shorter time is required for oxygen saturation and subsequent oxygen liberation, and, when required, the CO_2 content can afterwards be determined in the same sample of blood.

Only normal blood has been used in our experiments: we have had no opportunity for testing lipaemic blood in which the secondary oxidation on adding ferricyanide is more rapid, although we made quite satisfactory determinations of the percentage oxygen capacity of samples of normal rabbit's blood in which the rate of secondary oxidation on adding ferricyanide was far quicker than in normal human blood.

SUMMARY

1. In the Haldane ferricyanide method of blood-gas analysis an error in the measurement of the oxygen content of the haemoglobin is caused by a secondary oxidation due to the action of ferricyanide on lipoid substances and perhaps proteins in the blood.

2. This error can be eliminated by the substitution of a borate buffer of pH 10·0 for the sodium carbonate or ammonia solution which has usually been employed, and the substitution of a 6% solution of potassium ferricyanide for a saturated solution.

3. A further source of error can be avoided by running CO_2-free air through the apparatus before closing the taps.

4. With this modified procedure the Haldane method has been found to give results which are to all intents and purposes identical with those given by the van Slyke method. This has been confirmed by determinations by the two methods of the percentage oxygen capacity of fully oxygenated blood and the haemoglobin dissociation curve.

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