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The Routine Estimation of Haemoglobin as Oxyhaemoglobin

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It has become evident that visual (subjective) colorimetric estimations of haemoglobin are, perhaps even for clinical purposes, not sufficiently accurate because of the wide scatter in determinations by the same observer and the still greater variations between the results of different observers using samples of the same blood. Photoelectric colorimeters, on the other hand, have no subjective errors and do not cause visual fatigue. Moreover, they are now cheaper (Bell, 1944) than colorimeters of the Duboseq type. A number of procedures suitable for photoelectric colorimetry have already been closely investigated, viz. the alkaline haematin method (Clegg & King, 1942; Peterson & Strangeways, 1944), the cyanmethaemoglobin method (Stadie, 1920; Austin & Drabkin, 1935; King, Gilchrist & Delory, 1944) and the pyridine haemochromogen method (Rimington, 1942). Although these three methods seem to be the most favoured they are moderately complicated, and the latter two use dangerous or objectionable chemicals. The use of oxyhaemoglobin, the most easily prepared derivative, has been suggested by Szigeti (1940), Karr & Clark (1941), Bell & Guthmann (1943) and Reeve (1944). This paper describes our experiences with the oxyhaemoglobin method using the balanced modification of the photoelectric colorimeter described by Bell & Guthmann (1943).

METHODS

Measurement of optical densities of haemoglobin derivatives with various light filters. The densities of oxyhaemoglobin and of the three pigments mentioned above were measured in the green, blue and violet regions of the spectrum. The oxyhaemoglobin solution was made by diluting 0.2 ml. of oxalated venous blood to 25 ml. with approximately 0.007 N-NH₃ (0.04 ml. ammonia solution, sp.gr. 0.88, to 100 ml. with water (Szigeti, 1940)); the other three pigments by

the methods quoted in the introduction. The final concentration of blood was the same in all cases and the results have been referred to oxyhaemoglobin as 100 (Table 1).

Measurement of optical densities with green light filters. The green region of the spectrum was investigated by means of a number of filters (Ilford Micro 3 and Spectrum 604; and the Wratten nos. 40, 44, 53, 58A, 60P, 61N, 63, 65, 65A and 67; cf. Ilford Ltd. (1944) and Eastman Kodak Co. (1940)) in the hope of finding a region where all the pigments likely to be found in circulating blood had approximately the same density. The results for three of these filters are given in Table 2.

The first four pigments were prepared from each of eight blood samples. Sulphaemoglobin (four samples) was prepared by bubbling H₂S through the oxyhaemoglobin solution for 1½ min., centrifuging and measuring the density before any precipitate of colloidal sulphur appeared. A marked change in colour was seen; there was a dense absorption band in the red (c. 620 mμ) with faint bands of residual haemoglobin; the band in the red persisted after treatment with Na₂S₂O₄. The haemoglobin was largely (probably over 80%) converted to sulphaemoglobin (cf. Drabkin & Austin, 1935).

Comparison of oxyhaemoglobin method with colorimetric iron analyses. The accuracy of the oxyhaemoglobin method was investigated by the Wong (1928) thiocyanate method, but it was found that duplicate estimations did not agree closely. A slight alteration of Ponder's (1942) modification was suggested to us by Dr A. B. Anderson and the final procedure was as follows:

To 0.5 ml. of blood in a 50 ml. flask were added gradually 2 ml. conc. H₂SO₄, swirled to mix and allowed to stand overnight. Potassium persulphate (2 ml. saturated K₂S₂O₈ solution) was added, mixed and allowed to stand 1 hr. Distilled water was added to approximately 25 ml. and mixed. Sodium tungstate (2 ml. 10%) was added, mixed and allowed to stand 1 hr. The volume was made to 50 ml., the fluid mixed and filtered. To 20 ml. of the filtrate were added 1 ml. saturated K₂S₂O₈ solution and 4 ml. 3 N-KCNS. The density of the Fe(CNS)₃ colour was read exactly 3 min. later, with a Micro 2 light filter (Ilford).

Comparison of oxyhaemoglobin method with TiCl_3 iron analyses. The titanous chloride method described by Delory (1943) and applied to blood by King *et al.* (1944) was then employed as follows. 5 ml. duplicates of oxalated blood (Wintrobe) were treated with 1 ml. conc. HNO_3 and evaporated slowly to dryness and subsequently to complete charring; then ashed at about 450° in an electric muffle furnace overnight. The stock solution of TiCl_3 was diluted with 0.1 N-HCl saturated with CO_2 from a Kipp's apparatus. The method was checked against pure iron wire. Pipettes were calibrated to 'contain'.

RESULTS

Within limits, the denser the colour used for any estimation the greater the accuracy. Table 1 shows that the density of oxyhaemoglobin in green light is greater than that of alkaline haematin and is not much less than that of pyridine haemochromogen.

Table 1. *Relative optical densities of pigments used for the estimation of haemoglobin*

Ilford light filters ...	Optical density (% of haemoglobin) with Ilford light filters			Optical density (% of haemoglobin with Micro 3 (green) filter) with Ilford light filters		
	Micro 1 (violet) (%)	Micro 2 (blue) (%)	Micro 3 (green) (%)	Micro 1 (violet) (%)	Micro 2 (blue) (%)	Micro 3 (green) (%)
Oxyhaemoglobin	100	100	100	129	87	100
Cyanmethaemoglobin	119	123	96	154	107	96
Pyridine haemochromogen	125	152	128	161	132	128
Alkaline haematin	109	110	68	141	95	68

An advantage of the alkaline haematin and the cyanmethaemoglobin methods is that all the haemoglobin is converted to one pigment and accordingly the total pigment is estimated. Clegg & King (1942) consider it desirable that 'the method employed should estimate the concentration of all forms of haem-pigment circulating in the blood'. With the oxyhaemoglobin method any reduced haemoglobin is converted to oxyhaemoglobin but methaemoglobin and other abnormal pigments (reviewed by Medical Research Council, 1943) are unaffected. If a wave-band could be found at which all the pigments likely to be found in blood had the same density, then the measurement would be independent of the type of haemoglobin. With violet

and blue filters the differences in density are great, but with properly chosen green filters there is very little difference in the density of these pigments (Table 2; cf. also Drabkin & Austin (1935) and Austin & Drabkin (1935)). Even if 10% of the haemoglobin in a blood sample were in the form of one of these pigments, there would be less than 1% alteration in the density reading (and in the haemoglobin estimation), no matter which of the three green filters were used.

The accuracy of the oxyhaemoglobin method was investigated by comparison with the colorimetric iron method in blood samples from 23 blood-bank donors. The ratio of the density of the oxyhaemoglobin measured with a green (Micro 3) filter to the density of the ferric thiocyanate measured with a blue (Micro 2) filter was calculated in each case. The coefficient of variation of this ratio was 1.67%.

There is thus no doubt that the colorimetric iron method is consistent with the oxyhaemoglobin method. To determine whether it gives results in agreement with the titanous method the iron content of 12 blood samples was estimated by both methods. The mean ratio of the colorimetric Fe value to that by TiCl_3 was 1.045 with a standard deviation of 0.009. Slightly higher values given by the colorimetric method were due to iron washed out of the filter paper. Many commercial filter papers contain sufficient iron to interfere with the accuracy of the method.

The method of titration of ferric iron with a titanous salt seems to be the accepted standard method for the estimation of haemoglobin (Subcommittee Report to the Medical Research Council, 1943). There are substantial objections to using oxygen capacity (Ammundsen, 1941).

After preliminary experiments had shown that our technique was satisfactory, 31 consecutive blood samples from the blood bank were estimated simultaneously by the TiCl_3 method and the oxyhaemoglobin method. The samples contained from 41.0 to 61.4 mg. Fe/100 ml. (i.e. from 12.3 to 18.4 g. Hb/100 ml.), and the corresponding range of optical density, with a 0.5 cm. glass cell, was 0.249–0.358 with the green Micro 3 filter. The Fe estimations and the density measurements were in duplicate.

Table 2. *Optical densities of haem-pigments found in circulating blood, relative to oxyhaemoglobin, in the green region of the spectrum*

Light filters ...	Micro 3*	Wratten 40*	Wratten 60 P*
	(%)	(%)	(%)
Oxyhaemoglobin	100	100	100
Reduced haemoglobin	96	91	93
CO-haemoglobin	110	107	108
Methaemoglobin	94	102	100
Sulphaemoglobin	94	101	99

* These filters have their maximum transmission at about 520 m μ .

The ratio—density of oxyhaemoglobin $\times 10^5$ divided by Fe in mg./100 ml.—was calculated in each case with the results shown in Table 3.

Table 3. *Ratio of optical density of oxyhaemoglobin solutions to iron analyses by the titanous chloride method*

Green light filters	Mean ratio*	Corrected standard deviation	Coefficient of variation (%)
Micro 3	598	7.09	1.18
Wratten 40	530	5.18	0.98
Wratten 60P	554	8.26	1.49

* Density of the oxyhaemoglobin sample (0.2 ml. blood in 25 ml. 0.007N-NH₃) multiplied by 10^5 and divided by mg. Fe/100 ml. of the same blood. Thirty-one blood samples were used with a mean value of 15.0 g. haemoglobin/100 ml.

These results show even less scatter than do the results of the cyanmethaemoglobin method which King *et al.* (1944) consider is the most accurate colorimetric method. Any of the three filters gives satisfactory results, but the data of Tables 2 and 3 taken together suggest that Wratten 40 is the best.

Macfarlane & O'Brien (1944) suggested that there might be a sex difference in haemoglobin. Our 31 cases were made up of 14 men and 17 women (11 of the latter were over 45 years). The Fisher *t* test indicated that the differences are just statistically significant. They are in line with those of Macfarlane & O'Brien (1944), and suggest that there is more non-haemoglobin iron in men than in women (Table 4). This sex difference obviously

Table 4. *Ratios of optical density of oxyhaemoglobin to iron analyses of blood of men and women*

Green light filters	Mean ratio*		Difference of means	Difference of means $\times 100 \div$ mean	<i>t</i> (Fisher's test)
	Men	Women			
Micro 3	594.8	600.6	5.80	0.97	2.44
Wratten 40	527.9	532.4	4.49	0.85	2.047
Wratten 60P	551.5	556.6	5.15	0.93	2.35

* Same ratio as in Table 3 calculated separately for 14 men and 17 women. Fisher *t* test shows a significant difference between the ratios for the two sexes. Where $n=29$, $t=2.046$ when $P=0.05$, and $t=2.462$ when $P=0.02$.

causes a scatter of the results when men and women are taken together, as shown in Table 3. If this factor is taken into account the coefficients of variation in Table 3 are reduced still further.

DISCUSSION

We have presented ample proof that the photoelectric estimation of haemoglobin as oxyhaemoglobin under the conditions specified is a very accurate method. Its simplicity and accuracy

should appeal to those who have to make numerous routine estimations. Nothing is gained by using more complicated procedures.

The reasons for the choice of weak ammonia as the diluting fluid are that the alkalinity (pH about 10) prevents significant turbidity due to lipids and proteins and allows the haemoglobin to be more readily converted to oxyhaemoglobin, that the maximum colour is reached at once, and the density remains unchanged for a considerable time. The greater stability of oxyhaemoglobin in weak ammonia compared to stronger alkali was first pointed out by Szigeti (1940) and was confirmed by Hill & Pincock (1941). We have found that the density of a solution of oxyhaemoglobin in 0.007N-ammonia measured with any of the three green filters remains substantially unchanged even after 4 days at room temperature.

It must be remembered, in using the oxyhaemoglobin procedure, that the form of the absorption spectrum, especially of methaemoglobin, depends on pH. Furthermore, the use of any diluting fluid other than that recommended by us would mean the abandonment of one of the main advantages of our method, namely, that, under the conditions we have described, the densities of normal and abnormal pigments are very nearly the same.

SUMMARY

1. The use of oxyhaemoglobin in a routine method for the photoelectric estimation of haemoglobin is recommended. The oxyhaemoglobin formed when blood is mixed with 0.007N-ammonia is very stable. With an appropriate green light filter the presence of abnormal pigments in the blood makes no appreciable error in the method. The optical density of the oxyhaemoglobin solution is of the same order as that of other derivatives (cyanmethaemoglobin, pyridine haemochromogen, alkaline haematin) advised by other workers for the estimation of haemoglobin.

2. The photoelectric estimation of haemoglobin as oxyhaemoglobin, when standardized by the TiCl₃ method of iron estimation, is in our experience the most simple and accurate method for routine purposes.

3. There is a small but significant difference between the ratio of haemoglobin to total iron in the blood of men and women.

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The Mode of Occurrence of Carotene and Vitamin A in Human Blood Plasma

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This work was undertaken primarily in the hope that a knowledge of the way in which carotene and vitamin A are carried in blood would aid in solving the problem of the apparently erratic occurrence of vitamin A in abnormal urines.

The existing statements are conflicting, and the evidence in support of them is scanty. Palmer (1915), impressed by the impossibility of extracting more than traces of carotene from cow serum by ethanol-free ether, and by the different behaviour of cow and hen sera, postulated a carotene-albumin compound. Pett & Le Page (1940) believed that their experiments on fractional precipitation showed the carotene to be bound to serum globulin, and Bennhold's (1938) measurements of cataphoretic migration support this view. On the other hand, Van den Bergh & Müller (1920) showed that simple aqueous colloidal solutions of carotene resembled serum in that the carotene was not readily extracted by organic solvents such as ether and, though there were some differences, they considered carotene as existing in serum in simple colloidal solution. Their observations were confirmed by Drummond & McWalter (1935). The existence in nature of carotene- or carotenoid-proteins has been demonstrated, e.g. lycopene-protein in tomato (Kuhn & Bielg (1940), astacene-protein and rhodopsin, vitamin A-protein (Wald, 1938; Lovern & Morton, 1939), ovoidin, astaxanthin-protein (Kuhn & Sorensen,

1938). However, the fact that these substances exist, though it shows the possibility, does not indicate whether or not carotene and vitamin A are actually carried in blood plasma as protein (or other) compounds or in simple colloidal solution.

METHODS

The plan was to examine the power of ether and other organic solvents to extract carotene and vitamin A from citrated human plasma (prepared from a mixture of 9 vol. of blood and 1 vol. of 3.8% trisodium citrate) under various conditions—adjustment of pH over a wide range, freezing in presence of the solvent, addition of protein precipitants, addition of aqueous colloidal solution of carotene, etc. Under all suitable conditions, comparison was to be made with simple aqueous colloidal solutions of carotene prepared as described by Van den Bergh & Müller (1920). These experiments are described later in the paper. The methods of extracting and determining the carotene and vitamin A were common to all.

Extraction

The standard method was to make three successive extractions of the material (plasma, serum, precipitated protein, etc.) with peroxide-free ethyl ether or other solvent. For each extraction, 2 vol. of solvent were added to 1 vol. of the material, and the mixture was shaken vigorously by hand for 10 min., after which the ether layer was allowed to separate spontaneously or by centrifuging and was removed. From the combined extracts, the ether was completely distilled in an atmosphere of CO₂ and with avoidance