The Precise Determination of Haemoglobin in Blood by Abridged Photoelectric Spectrophotometry

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Many workers (e.g. Karr & Clark, 1941; Macfarlane, 1945; Macfarlane, King, Wootton & Gilchrist, 1948) have drawn attention to the inaccuracy of most methods for the determination of haemoglobin in routine laboratory practice. McCarthy & Van Slyke (1939) have shown that there is a diurnal variation in the haemoglobin content of the blood of normal men amounting to a range of 7% on the Haldane scale. If it is desired to follow closely the progress of an anaemia it is therefore essential that the already large uncertainty should not be increased still further by the use of a method only accurate to 10% or worse.

Precise photoelectric methods of haemoglobin determination have been described by Szigeti (1940) and Bell, Chambers & Waddell (1945) among others. These workers use an incandescent light source, with approximately monochromatic filters. Their results, therefore, though highly reproducible, require the calibration of the individual instrument and filters against a blood solution whose concentration has been determined by other means. Absolute methods in general use depend on chemical determination of the iron content (e.g. Delory, 1943), or on manometric determination of the gas capacity (e.g. Van Slyke & Neill, 1924), and both these techniques are too time-consuming for frequent use. Spectrophotometry could also yield absolute values, provided an agreed spectrophotometric standard existed. The British Standards Institution (1942) has published the absorption curve of carboxyhaemoglobin used in calculating the tristimulus colour values by which the British Standard is defined; this curve is intended to be used as a whole, and therefore constitutes another tedious method. Because of the sharpness of the absorption bands of most blood pigments, spectrophotometric constants determined at single wavelengths are markedly influenced by small errors in wavelength setting, or differences in slit width. For this reason Butterfield (1912) suggested the use of the mercury arc with a spectrophotometer. Heilmeyer (1933) quotes Butterfield as having found an absorption ratio at 546 m μ . for oxyhaemoglobin corresponding to a specific extinction ($c = 1$ g./ml.) of 800.

Nicholas & Pollak (1950) have described filters which give sharp isolation of the mercury lines and

therefore bring true spectrophotometric measurements within the realm of routine absorptiometry. It is the purpose of this paper to try to establish the spectrophotometric or abridged spectrophotometric* determination of haemoglobin as a method as fundamental as the iron content or gas capacity, and far more rapid.

A variety of haemoglobin derivatives have been used in colorimetric estimations. King *et al.* (1948) found oxyhaemoglobin itself to be one of the two most reliable of the six they examined. It has obvious advantages in convenience and in avoiding the introduction of errors by additional manipulation, and has therefore been chosen for the present method.

While this paper was in the press Donaldson, Sisson, King, Wootton & Macfarlane (1951) published spectrophotometric curves of haemoglobin derivatives, and deduced by interpolation between the 540 and 550 m μ . extinctions a value for oxyhaemoglobin corresponding to $E_{546}^{1\text{ g./ml.}} = 810$.

METHODS

For the determination of the specific extinction of HbO₂ at 546 m μ ., and for the investigation of the turbidity correction and the stability of HbO₂ the photoelectric absorptiometer described by Nicholas (1951) was used. The green line of the mercury arc (546 m μ .) and a band in the orange-red (designated '607 m μ .', but consisting largely of background spectrum, and therefore not strictly monochromatic) were isolated by the filter combinations of Nicholas & Pollak (1951). For experiments in routine determinations a model H 602 Spekker absorptiometer with mercury vapour lamp (made by Adam Hilger Ltd.) was also used, with the precautions detailed later. Oxalated human venous blood was diluted with 0.007N-NH₄OH, which Szigeti has shown to favour the formation of methaemoglobin less than the more usually recommended higher concentrations. Carboxyhaemoglobin where required was prepared by shaking the diluted blood with excess of pure CO prepared from A.R. formic acid and H₂SO₄. The solution cell used for the determination of the specific extinction was specially selected and measured by the makers (The Tintometer Ltd.). A blank on

* This term is used here to denote the measurements of true densities at exact wavelengths by a shortened procedure. Its use as by some American authors to denote any absorptiometric procedure is considered incorrect.

water was measured in the same cell and subtracted. Comparison with the British Standard was made by the National Physical Laboratory on their comparator.

Choice and use of absorptiometer. An instrument is required equipped with a mercury vapour lamp and capable of having its density scale checked over the entire range used. The H 602 Spekker is the most commonly available instrument fulfilling these requirements, but the newer H 676 instrument is preferable. If facilities for the filter construction are at hand the Nicholas (1951) absorptiometer is especially suitable. Precautions necessary in using the Spekker for precision work are discussed at length by Pollak & Nicholas (1951). In particular it should be noted that the H 602 Spekker has the monochromatic filters in approximately the plane of the calibrated variable aperture, and the H 676 model has them in an optically equivalent position. Density gradients or other imperfections in the filter will therefore affect the calibration of the instrument (see example given by Nicholas, 1951). This means that if the instrument is not modified the same individual filter which was used during calibration must always be used for readings, and must always be inserted the same way up. Heat affects the transmission of filters as well as damaging them, so it is advisable to modify the H 602 Spekker as recommended by Hadley (1945) to bring them into a cool, constant and accessible position on the right-hand side of the drum. It is even better, if possible, to modify the instrument more drastically so that the filters are immediately adjacent to the right-hand photocell (the position of, and variations in, the left-hand filter are unimportant). If this is done the calibration not only becomes independent of variations in individual filters but also becomes the same for all wavelengths. It is essential, of course, that any modification made in the photocell housing should not allow the entry of stray light, which in this position would be especially detrimental to accuracy. The elimination of the left-hand water cell is recommended to avoid the zero-drift which results from air-bubble formation there; Nicholas & Pollak (1951) have found that this cell serves no useful purpose. The possibility of lost motion exists in the drum of the H 602 Spekker (but not the H 676), and the balance point should, therefore, always be approached in the direction of closing the drum aperture, where movement is positive. For convenience it is preferable to adopt a modification of Vaughan's (1941) 'water-to-water' setting and set the instrument 'air-to-air'. To ensure reproducibility the setting on the H 602 model should be at a drum reading not greater than 0.800. A lens should be used for both setting and reading; the latter should be by interpolation to 0.001.

Calibration of density scale. The Spekker scale, although bearing an approximately linear relationship with density, cannot be considered as a density scale. It should therefore be recalibrated by the following simple and rapid method. The procedure is described more fully by Pollak & Nicholas (1951), who also describe the process of cutting a new scale. In the following abridged description it is assumed that all that is required is the construction of a correction graph, or preferably, table. Using the 546 m μ . line the Spekker is set air-to-air 0.800, and a filter of density d (approximately 0.07), conveniently made by binding together two pieces of plain glass, is placed in a suitable holder in the cell carrier and a reading obtained for it. Leaving the drum at the reading so reached the galvanometer is restored to zero by the zero adjustment and a second reading of the filter taken. By this means drum readings corresponding to d , $2d$, $3d$, ..., are found. Finally the drum is reset to 0.800 and a plane-parallel

glass neutral filter of known density D (in the region of 0.5) calibrated, for example, at the National Physical Laboratory is read. By linear interpolation D is expressed in terms of d , and thus the true value of d is found. A calibration graph can then be drawn, which will probably be nearly linear.

Filters. Owing to the sharpness of the β -absorption band of oxyhaemoglobin stray light either in the form of background radiation adjacent to the line or in the form of near infrared will cause large errors. Filters used therefore require to be chosen with care (Nicholas & Pollak, 1951). The combination Wratten 16 + Wratten 74 + Ilford 804 is recommended to isolate the 546 m μ . line for the actual determination. If it is desired to apply the turbidity correction (see p. 3) the band in the orange-red can be isolated by Wratten 26 + Ilford 804, but this requires a more sensitive galvanometer than the Cambridge Spot usually used with the Spekker; a much wider band not requiring a sensitive galvanometer could be isolated by Wratten 25 + Ilford 801. It is convenient to cement the individual gelatin films in the above combinations into compound filters. Light should traverse them in the order in which the components are listed. Manufacture of Wratten 16 and Wratten 26 has recently been discontinued in England, although stocks should last some time yet (Kodak Ltd., personal communication). Two thicknesses of Ilford 111 can be substituted for Wratten 16, and two thicknesses of Wratten 25 for Wratten 26 (Nicholas & Pollak, 1951).

Preparation and examination of blood. Oxalated, heparinized, or fresh blood may be used. A dilution of 1 in 250 in 0.007 N-NH₄OH gives a suitable density. If large numbers of examinations are being made a dilution of 1 + 200 will be found more convenient, the blood being measured in a 0.05 ml. capillary pipette and the diluent in a 10 ml. automatic pipette. The extinction of the solution is measured at 546 m μ . in a 10 mm. cell. The extinctions given in Table 2 will be found convenient in calculating results. For measurements of the greatest precision the dilute blood should be filtered (e.g. through Whatman no. 541 paper), but this is normally unnecessary. It should be remembered that tolerances on the nominal size of both pipettes and cells are relatively large (see p. 4); checking is therefore recommended.

RESULTS

Reproducibility

Ten replicate 1 in 250 dilutions of blood were prepared. The oxalated blood was mixed before sampling by gently inverting the bottle once a second for 60 sec. The same Ostwald-Folin pipette calibrated to contain 0.2 ml. and the same 50 ml. volumetric flask were used throughout. Densities were read on the author's absorptiometer on the day of dilution, and on the Spekker set air-to-air 0.5 on the following day. The absorptiometer gave a mean value of 0.3853, with a range of 0.385–0.387, and a coefficient of variation of 0.175 %. The Spekker gave a mean of 0.3855, range 0.385–0.387, coefficient of variation 0.184 %. It should be noted that this coefficient of variation takes account of all random errors of sampling from a bulk specimen of blood, pipetting, diluting and reading, but not of errors in the calibration of glassware.

Determination of specific extinction of oxyhaemoglobin

Blood was diluted 1 in 100 and filtered through Whatman no. 541 paper. Portions were saturated with carbon monoxide and submitted to the National Physical Laboratory (N.P.L.) for standardization. Further portions of the original ungasged solution were diluted 1+1 with 0.007 N-ammonia and their densities measured at 546 m μ . in a depth of 10.000 $\pm$ 0.005 mm. As a precaution against errors in diluting the same 10 ml. pipette was used for measuring both blood solution and diluent, and the dilution was performed in quadruplicate. Table 1 gives the

Table 1. *Determination of $E_{546 \text{ m}\mu}$ of oxyhaemoglobin corresponding to 100 % on British Standard Scale*

(Each mean represents three readings on each of four dilutions. The maximum deviation from the mean was 0.001, which occurred five times in all thirty-six readings.)

Exp. no.	% B.S.I. (N.P.L. result)	Mean extinction (1 in 200 dilution)	Extinction of 100 % B.S.I.	Mean
206	92.7	0.552	0.595	0.5963
207	106.9	0.635	0.595	
327	87.6	0.525	0.599	

results. The mean extinction of a 1 in 100 dilution of blood of 100 % haemoglobin on the British Standards Institution (B.S.I.) scale is therefore 1.192₆, with a possible error (see later) of ± 0.59 %. Taking 100 % B.S.I. as being 148 g. haemoglobin/l. (King *et al.* 1947) the specific extinction $E_{546 \text{ m}\mu}^{1 \text{ g./ml.}}$ becomes 806. This value assumes a factor for the iron content of haemoglobin of 0.334 %, based on the results of Hüfner (1894). If this factor is eliminated as possibly unreliable, and the haemoglobin molecule is assumed to contain four atoms of iron the molecular extinction $\epsilon_{546 \text{ m}\mu}$ is 53,910.

Table 2. *Extinctions of oxyhaemoglobin solutions at 546 m μ . for routine determinations*

Concentration of original blood	Dilution	
	1 in 250	1 + 200
100 % B.S.I.	0.477	0.593
100 g./l.	0.322	0.401
1 mm	0.216	0.269

Stability of oxalated blood

In view of the possibility of a choice arising between doing a haemoglobin estimation immediately by a less accurate method and sending it elsewhere to be done accurately after one or more days' delay the effect of delay was investigated. Ten oxalated bloods which had been taken at intervals over a period of 12 days were diluted afresh and read 13 days after taking the first sample. The same glassware was used throughout. A comparison

between the immediate and delayed readings is given in Table 3, which shows that in no case does the difference exceed 0.002 (approximately 0.5 % of reading).

Table 3. *Stability of oxalated blood*

Exp. no.	Age of specimen (days)	Original $E_{546 \text{ m}\mu}$	Delayed $E_{546 \text{ m}\mu}$	Difference
102a	13	0.385	0.383	-0.002
101a	12	0.402	0.402	0.000
102b	11	0.402	0.400	-0.002
101b	10	0.408	0.408	0.000
102c	9	0.387	0.386	-0.001
102d	6	0.363	0.365	+0.002
101c	5	0.363	0.365	+0.002
102e	4	0.389	0.389	0.000
101d	3	0.386	0.386	0.000
101e	2	0.386	0.385	-0.001

Effect of turbidity

It has sometimes been adduced against photo-electric haemoglobinometry that slight turbidities due to lipaemia may cause falsely high results. Such turbidities will be visible to the eye, and can be allowed for by making an extra reading at about 607 m μ . If the ratio $E_{546 \text{ m}\mu}/E_{607 \text{ m}\mu}$ is R , and the added turbidity is assumed to have equal densities at the two wavelengths, it can be calculated that the true extinction at 546 m μ is

$$(\text{measured } E_{546 \text{ m}\mu} - \text{measured } E_{607 \text{ m}\mu}) \times R/(R-1).$$

To test this equation in use replicate dilutions of the same oxalated blood were made in which were incorporated varying amounts of milk, producing in some cases a turbidity in excess of anything likely to be found in practice. Corrected and uncorrected readings are given in Table 4. The value of $R/(R-1)$

Table 4. *Use of turbidity correction*

Exp. no.	$E_{546 \text{ m}\mu}$	$E_{607 \text{ m}\mu}$	$E_{546 \text{ m}\mu}$ corrected	Difference from expectation
91a (control)	0.389	0.025	0.388	-0.001
92a	0.449	0.084	0.388	-0.001
92b	0.515	0.155	0.383	-0.006
92c	0.571	0.209	0.386	-0.003
92d	0.416	0.048	0.392	+0.003
92e	0.407	0.041	0.390	+0.001
	Mean			-0.001
	Standard deviation			0.0031
	Coefficient of variation			0.81 %

used was 1.063 (mean of twenty-four estimations, s.e.m. 0.001). It should be emphasized that this correction should only be applied to a turbidity in fresh blood due to lipaemia, and cannot be used in the presence of methaemoglobin. The waveband designated '607 m μ .', although narrow is not strictly monochromatic. R cannot, therefore, be considered an absolute constant, and should be checked with the filters and instrument actually used.

DISCUSSION

The method described occupies less time than the Haldane or Sahli methods, and its reproducibility is shown to be high. The overall error depends on a number of individual errors, and is calculated as the root of the sum of their squares: (1) The systematic error in the calibration of the absorptiometer. This depends on a neutral filter whose density is given by the National Physical Laboratory to $\pm 0.5\%$. (2) The random error of reading the author's absorptiometer. For a density of 0.600 this is $\pm 0.2\%$ (twice coefficient of variation, based on figures of Nicholas, 1951). (3) The error in sampling, pipetting, diluting and reading the H 602 model Spekker has been shown above to be $\pm 0.368\%$. (4) The error in calibration of glassware may be large; British Standards (1938*a, b*) require only $\pm 3\%$ accuracy for the 0.2 ml. Ostwald-Folin pipette or $\pm 1\%$ for the same size capillary pipette. Pipettes should therefore be calibrated for content by weighing water, when accuracy to $\pm 0.1\%$ is attainable. Absorptiometer cells usually used are not guaranteed by the makers to better than $\pm 1\%$, but special cells can be obtained from them accurate to $\pm 0.1\%$ or better. (5) The error in the comparison with the British Standard scale by the National Physical Laboratory is estimated by them to be $\pm 0.5\%$. Triplication is here considered to have reduced this to $\pm 0.29\%$. (6) The error in the determination of the extinction coefficient of oxyhaemoglobin corresponding to the British Standard depends on (1), (2), (4) and (5) above, but (2) becomes negligible because of replication, and only the cell need be considered under (4). The error therefore becomes $\pm 0.59\%$.

In comparing an unknown sample with the British Standard on an unspecified instrument (1), (3), (4) and (6) above are operative, and the error is $\pm 0.87\%$. In comparing two samples, both by this method, on unspecified instruments (6) need not be considered, and the error becomes $\pm 0.64\%$. When maximum accuracy is desired in a comparison, and the same instrument and the same glassware can be

used, an error of $\pm 0.37\%$ can be achieved, and can be reduced further by replication.

The possibility of interference from turbidity, carboxyhaemoglobin and methaemoglobin should be considered. It has been shown above that a turbidity due to lipaemia can be approximately corrected for. Carboxyhaemoglobin does not interfere appreciably in physiological quantities, being read as oxyhaemoglobin. The ratio $E_{546\text{ m}\mu}$ oxyhaemoglobin/ $E_{546\text{ m}\mu}$ carboxyhaemoglobin is 1.041 (mean of sixteen determinations, s.e.m. 0.0014), so at 5% carbon monoxide saturation the error from this cause will be only 0.2% of the reading. Methaemoglobin has an appreciably lower extinction coefficient than oxyhaemoglobin, so bloods containing more than minimal quantities of this pigment should not be examined by the method described.

It is suggested that the method here described provides immediately a means whereby any worker may relate his results to the British Standard with an accuracy sufficient for all clinical purposes, and one with another with an accuracy sufficient for most research purposes. It is further suggested that ultimately the British Standard should be redefined in terms of the extinction of oxyhaemoglobin at 546 m μ , with the tristimulus specification of the Haldane colour tube becoming a substandard until such time as the use of the Haldane method could be abandoned altogether.

SUMMARY

1. A precise photoelectric method is described for the determination of haemoglobin in blood, suitable for routine, research or standardization purposes, and depending on the measurement of its extinction as oxyhaemoglobin at the 546 m μ . line of the mercury arc. The latter can be isolated by a special filter combination.

2. On the British Standard haemoglobin scale 100% corresponds to an extinction in a 1 in 100 dilution of 1.193. By calculation from this value the specific extinction $E_{546\text{ m}\mu}^{1\text{g./ml.}}$ is 806, and the molecular extinction $\epsilon_{546\text{ m}\mu}$ is 53,910.

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The Activation of a Proteolytic Enzyme in Blood by Animal Tissue

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This paper presents an investigation of the activation of the proteolytic enzyme in blood (plasmin, fibrinolysin) by an interaction between its pro-enzyme (plasminogen, profibrinolysin) and the insoluble tissue activator (fibrinokinase) recently discovered (Astrup & Permin, 1947, 1948; Permin, 1947, 1949, 1950b). This reaction appears to be of a stoicheiometrical and not of a catalytic nature (Astrup, 1949).

METHODS AND MATERIALS

The fibrin plate method (Permin, 1947, 1949, 1950a; Astrup, 1948), as modified by Astrup, Crookston & MacIntyre (1950) was used for measuring the activity of the enzyme. Activities were recorded as the product of two perpendicular diameters of the lysed zones, usually the mean value of three single determinations being taken. Although the proportionality between enzyme concentration and this product, as recorded by Permin (1950a), could not be confirmed (Astrup *et al.* 1950), it has been found convenient to use it as an estimate of the enzymic activity, when carrying out comparative investigations. The recorded results, therefore, do not represent a precise quantitative estimation of the enzyme concentration, more especially at higher activities. The applicability of the fibrin plate method, as a general method of estimating fibrinolytic enzymes, is treated in detail in papers now under preparation.

Ox fibrinogen, bovine plasminogen and fibrinokinase from pig heart were prepared as previously described (Permin, 1950a, b; Astrup *et al.* 1950). Ox thrombin was obtained from Løvens kemiske Fabrik, Copenhagen.

RESULTS

The activation time. The time needed for activation was determined in the following way

Tubes containing 10 ml. each of bovine plasminogen and a few drops of toluene were slowly rotated at room temperature for different lengths of time. One series (A) contained

50 mg. of fibrinokinase, the second (B) 250 mg. in each tube. The insoluble fibrinokinase was removed by centrifugation and the samples stored in the ice box until all samples could be tested. Drops were then placed on the fibrin plates and the results recorded after 20 hr. at 32° (Table 1). A sample of plasminogen and samples containing fibrinokinase plus saline or buffer were rotated for 24 hr. at room temperature as controls. They did not show any activity.

The experiment shows that maximal activation is obtained after about 4 hr. The activity obtained at the lower concentration of fibrinokinase is only a fraction of that obtained with the higher concentration, and is not increased significantly by increasing

Table 1. *Activation of bovine plasminogen by means of fibrinokinase*

Time of rotation	Diameter products (mm. ²)	
	A, 50 mg.	B, 250 mg.
Zero	0	0
15 min.	(0)	36, 49, 64
30 min.	(0)	90, 100
60 min.	25, 20, 25	100, 121, 144
2 hr.	49, 42, 42	169, 156, 169
4 hr.	56, 42, 36	196, 182, 182
24 hr.	56, 72, 64	169, 169, 225

the reaction time. Moreover, the large amount (B) yields a much higher activity after only 30 min. than the maximal activity obtained with the small amount (A). It may be concluded that a small amount of fibrinokinase is unable to produce as much activation in a plasminogen sample as a larger amount, even if the reaction time is increased. This means that the reaction is not of a catalytic nature, but is one in which a component is used up during the process.

It was found in subsequent experiments that the activity gained by increasing the activation time