SPECTROPHOTOMETRIC STUDIES

XVI. DETERMINATION OF THE OXYGEN SATURATION OF BLOOD BY A SIMPLIFIED TECHNIQUE, APPLICABLE TO STANDARD EQUIPMENT*

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With a Note by Julian B. Marsh‡

(Received for publication, December 17, 1956)

The metabolism, as measured by oxygen consumption, of organs *in situ* is being increasingly investigated by the vascular catheterization technique (see Drabkin (3) for pertinent literature). The oxygen consumption is derived from the arteriovenous difference in the oxygen saturation or percentage of oxyhemoglobin (4) of blood entering and leaving the tissue. Such work has pointed to the need of a practical and withal reliable method for the routine analysis of the oxygen saturation of small blood samples (5, 6). Probably the most accurate determination of the percentage of oxyhemoglobin available at present is the direct spectrophotometric method of Drabkin and Schmidt (7), which avoids certain inaccuracies inherent in the standard, indirect gasometric technique employed for many years (8, 9). The extension of optical instrumentation to the analysis of oxygen saturation was made possible by the use of the Drabkin and Austin special cuvette of calibrated 0.007 cm. depth (10), which had been introduced much earlier for measurements of this type upon whole blood or concentrated hemoglobin solutions unexposed to air. An important advantage of the 0.007 cm. cuvette is that it permits the examination of undiluted blood and thereby circumvents the volumetric measurement of the sample, which remains one of the least accurate steps of analytical

* This work was done under contract between the Office of Naval Research and the Bureau of Medicine and Surgery, Department of the Navy, and the University of Pennsylvania. Reproduction in whole or in part is permitted for any purpose by the United States Government. Preliminary reports have been made (1, 2).

The data in this paper are largely taken from a portion of a thesis on "The optical properties of hemolyzed and whole blood," presented by Edwin Gordy to the Faculty of the Graduate School of Medicine of the University of Pennsylvania in partial fulfilment for the degree of Doctor of Medical Sciences.

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procedures upon this viscid fluid. However, the very thin layer cuvette of Drabkin and Austin, designed originally for measurements largely in the green spectral region (Fig. 1, Region 2), is of delicate construction (see Drabkin and Austin (10), and Drabkin (11)) and is not ideally suited for routine practice.

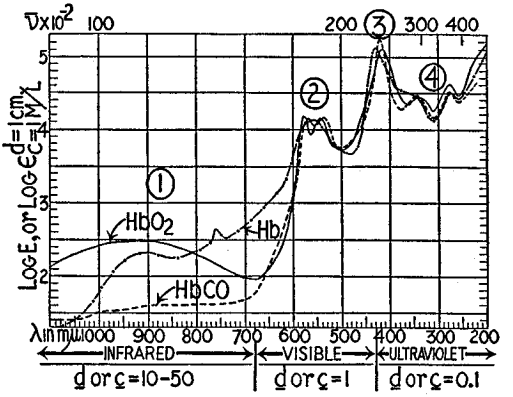

FIG. 1. Absorption spectrum curves, plotted as log E (log of the *molecular extinction coefficient* (11)) against wave length in millimicrons, for oxyhemoglobin, HbO_2, deoxygenated hemoglobin, Hb, and carbonyl hemoglobin, HbCO. The curves illustrate how the quantity of selective absorption in different spectral regions determines the choice of depth of layer, d, or concentration, c, to secure optimal spectrophotometric measurement of transmission, T (11). A pigment such as HbO_2 has about a 500-fold difference in density over the range of the infrared, visible, and ultraviolet regions, and three different cuvette depths with the same concentration, or three different ranges of concentration with the same cuvette depth are required for accurate measurement (11). Isosbestic points (regions of equal absorption), as at 805 and 505 mμ, are evident in spectral Regions 1, 2, and 3. Each of these regions may be suitable for the determination of two or all three of these pigments when they are present together in the sample. The preferential choice of one of the regions depends upon the nature of the problem (see Drabkin (11)). Owing to the similarity of the absorption curves in Region 4, the ultraviolet region, this portion of the spectrum is obviously unsuitable for the determination of mixtures of hemoglobin derivatives.

The principles of the thin layer spectrophotometric technique in the determination of oxygen saturation (7, 10) have been the basis of a number of proposed modified methods (12–15) in which one compromise or another has been employed to attain simplification. It may be seen from the selective absorption characteristics of oxyhemoglobin, HbO_2, and deoxygenated hemoglobin, Hb (Fig. 1), that two factors, dilution and spectral region, determine the optimal depth to be used in spectrophotometry. In an appropriate spectral wave band in the red (700 to 600 mμ), a cuvette of much greater depth than 0.007 cm. can be employed. In this communi-

cation a simplified direct spectrophotometric technique for the measurement of the percentage of HbO_2 in undiluted blood will be described. The procedure retains desirable features in the original Drabkin and Schmidt method (7), but is applicable to standard photoelectric photometric equipment such as the Beckman DU spectrophotometer and the recently available Bausch and Lomb Spectronic 20 instrument, with only minor modifications in the commonly employed 1 cm. depth cuvettes. The reliability of this technique in comparison with the classical indirect gasometric procedure for oxygen saturation of Van Slyke and Neill (16) has been verified in extensive field trials, summarized in the "Note" appended to this paper by J. B. Marsh.

Equipment and Analytical Procedure

Modification of Beckman 1 Cm. Cuvette—The 1 cm. cuvette is converted to one with a nominal depth of 0.05 cm. by means of a snugly fitting, polished, parallel glass prism (Fig. 2).[1] Our cuvette-prism combination has a calibrated optical depth of 0.067 cm., 10 times the depth of the Drabkin and Austin cuvette. An optical depth up to about 0.1 cm. can be employed effectively in the technique. The modified cuvette is sealed with a stopper cut from sponge rubber (Fig. 2).

Preparation of Blood-Collecting Vessel—A 6 ml. capacity tonometer, as shown in Fig. 2 (7), is used in the anaerobic collection and hemolysis of the blood samples. Such vessels are prepared in advance as follows: 1 ml. of anticlotting hemolyzing solution is introduced into each and allowed to dry slowly on the inner surface in a thin uniform layer by gently drawing a stream of air through the tonometer. Care must be taken to avoid trapping of air bubbles during drying. The anticlotting hemolyzing solution contains 50 mg. of saponin (Merck, purified), 12 mg. of ammonium oxalate, 8 mg. of potassium oxalate, and 2 mg. of sodium carbonate per ml. The prepared tonometric vessels are connected (Fig. 2, *a*) to a small leveling bulb which serves as a mercury reservoir, and to a male needle adapter (Fig. 2, *b* and *c*). Through the reservoir, the tonometers, including their exit capillaries, are filled with thoroughly clean mercury, thereby completely expelling the air. The blood-collecting assemblies are now ready to receive the samples.

Collection of Blood Samples—The male needle adapter (Fig. 2, *c*) is attached to a needle and, with the lower stopcock of the collecting vessel

[1] Such a prism (3 cm. high, 0.98 cm. wide, and 0.90 to 0.95 cm. thick) can be made to specification at low cost by any good opticians' establishment, which does its own lens grinding. Only the two optical faces of the prism are polished. The snug fit of the prism in the transverse direction of the cuvette (internal width of about 0.99 cm.) is essential to insure alignment of the prism and reproducibility of depth.

open and the upper stopcock closed, the needle is introduced into the blood source (vein, artery, or catheter). During ensuing operations, the mercury-leveling bulb is kept at appropriate, slight negative pressure. By means of

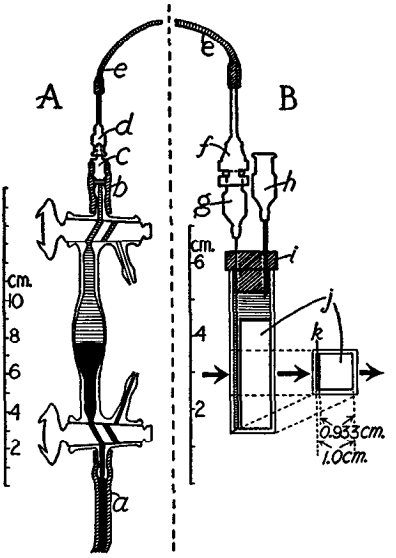

FIG. 2. Sketch, drawn to scale, showing transfer of anaerobically collected and hemolyzed blood sample from glass tonometric collecting vessel of 6 ml. capacity, *A*, into modified Beckman cuvette, *B*. The cuvette is represented in vertical and transverse sections, the latter with entry and exit ports (needles) removed, ready for spectrophotometric measurement. Arrows indicate the direction of luminous flux. *a*, 18 inch length of plastic tubing (Tygon, outside diameter 7 mm., inside diameter 4 mm.) to mercury reservoir in small leveling bulb, held at slight positive pressure; *b*, 1 inch length of Tygon tubing (same as in *a*), connecting collecting vessel with male metal needle adapter, *c*; *d*, female half of B-D type LLX 3 inch metal needle extension (see the text); *e*, 6 inch length of thin, flexible polyethylene tubing (outside diameter 2 mm., inside diameter 1.5 mm.); *f*, male half of B-D type LLX (see the text); *g*, 25 gauge, 2 inch needle (entry port); *h*, short 20 gauge needle (exit port); *i*, tightly fitting stopper cut from sponge rubber; *j*, snugly fitting polished glass slab, which reduces the optical depth of the Beckman cuvette from 1 cm. to 0.067 cm.; *k*, thin (0.067 cm.) layer of sample.

the upper stopcock, the small amount of dead space air is eliminated through the side arm of the tonometer, which is then filled with the blood sample. The blood is allowed to displace all but 1 ml. of mercury, retained to aid mixing. Both stopcocks are now closed, and the tonometer is disconnected from the blood source. The sample is thoroughly mixed and hemolyzed by gently rocking the tonometer for about 1 minute. We regard this method of preparing the blood sample as the most reliable

from the standpoint of ultimate accuracy. However, it will be evident that acceptable results can be obtained in a further simplification of technique in which the samples are collected and somewhat diluted in a syringe (see the "Note" by J. B. Marsh).

Transfer of Sample to Modified Cuvette—Fig. 2 is practically self-explanatory as to the method of filling the cuvette. The connection between the collecting vessel, *A*, and the cuvette, *B*, is accomplished by means of the connector, *d*, *e*, and *f*, and a 2 inch, 25 gauge needle, *g*. The connector is made by cutting a 3 inch B-D type LLX metal needle extension into equal length halves (female *d*, and male *f*), filing the cut ends smooth, and joining them with a 6 inch length of thin flexible polyethylene tubing, *e* (outside diameter 2 mm., inside diameter 1.5 mm.). The male end of the connector is attached to the needle, *g*, which is inserted through the sponge rubber stopper, *i*. The point of this needle should touch the bottom of the cuvette. A short 20 gauge needle, *h*, inserted through the stopper (Fig. 2), is a vent for the escape of air and excess blood, as the cuvette is slowly and completely filled from the bottom up with the sample. During the transfer of the sample the tonometer is held vertical at slight positive pressure. About 1 ml. of blood is needed for filling the modified cuvette; since the tonometer contains 5 ml., the sample is sufficiently large to allow for a liberal overflow through *h*. This provision insures samples uncontaminated by air. After the cuvette has been properly filled, both needles are removed and the sample is ready for measurement.

Spectrophotometry—Measurements are carried out in the usual manner with the Beckman DU spectrophotometer. The method depends upon optical density (*D*) readings at two wave lengths, 660 and 805 mμ. The narrowest possible slit is used, namely 0.015 to 0.02 mm., which corresponds to a spectral band width (span or wave band) of 2.5 to 3 mμ. Fig. 1, showing the absorption spectrum curves of HbO_2, Hb, and HbCO in the infrared, visible, and ultraviolet regions, supplies the information upon which the choice of spectral region and cuvette depth is based (11). It may be seen that wave length 660 mμ is a region of large spectroscopic difference between HbO_2 and Hb, whereas at 805 mμ the absorption of the two pigments is isosbestic (11), *i.e.* the same. The measurement in the latter region is an effective and convenient substitute for an independent determination of total pigment, but is utilized with the assumption that one is dealing only with mixtures of the two pigment species, HbO_2 and Hb. This assumption of a two-component system is inherent in the proposed method of handling the data (below) and appears to hold for most blood samples, which normally contain only traces of methemoglobin, MHb (4, 7) and, with the possible exception of samples from heavy smokers (9), only negligible amounts of carbonyl hemoglobin, HbCO.

The spectrophotometric constants, ϵ values, supplied in Tables I to III, are *fractional molar extinction coefficients* (11).[2]

Determination of Percentage of HbO_2—A knowledge of the optical density, D, of the sample at wave lengths 660 and 805 mμ and the spectrophotometric constants of HbO_2 and Hb in these spectral regions, obtained from Table I, are all that is required to calculate the per cent of HbO_2 present. The validity of the use of these constants and the working equation (Equation 8, below) based upon them may be inferred from the good agreement between results obtained by this method and by the gasometric procedure. However, the four groups of investigators who participated in the trial of the optical method used the Beckman DU spectrophotometer (see the "Note" by J. B. Marsh). It should be cautioned that the ϵ values are not necessarily transferable to photometric equipment or conditions of measurement which do not allow the narrow spectral isolation used by us. For those employing filter photometers or wide spectral bands for measurement, Equation 5 (below) may be used by substituting in it the values for ϵ determined in the particular instrument. The development of the working equation parallels that of Drabkin (11) for mixtures of carbonyl and oxyhemoglobin and is applicable to any two-component mixture at any two appropriate wave lengths, with at least one of which advantage may be taken of a large difference in the spectral absorption between the two components. The following symbols are used: A and B, the two components; $\epsilon_{\lambda 1A}$, $\epsilon_{\lambda 2A}$, $\epsilon_{\lambda 1B}$, and $\epsilon_{\lambda 2B}$, the extinction at definite concentration and depth of layer for the respective components, established at the two wave lengths, $\lambda 1$ and $\gamma 2$; x, the fraction of component A, and $1 - x$, the fraction of component B; r, the ratio of optical densities, $D_{\lambda 1}$ and $D_{\lambda 2}$, obtained by measurement on the mixture. The relationship between r, x, and extinction coefficients may be at once formulated as

$$r = \frac{x\epsilon_{\lambda 1A} + (1 - x)\epsilon_{\lambda 1B}}{x\epsilon_{\lambda 2A} + (1 - x)\epsilon_{\lambda 2B}} \tag{1}$$

Solving for x, dividing by $\epsilon_{\lambda 2B}$ and collecting terms, the yield is

$$x = \frac{r - \frac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}}{r\left(1 - \frac{\epsilon_{\lambda 2A}}{\epsilon_{\lambda 2B}}\right) + \left(\frac{\epsilon_{\lambda 1A} - \epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}\right)} \tag{2}$$

Equation 2 applies to the general case, but in this particular instance $\epsilon_{\lambda 2A}$

[2] ϵ, at a concentration of 1 mmole per liter, referable to a 1 iron atom, equivalent weight of 16,700 for hemoglobin, and a cuvette depth of 1 cm. The concentration in all cases was determined spectrophotometrically upon aliquots converted into cyanmethemoglobin with the constant $\epsilon = 11.5$ at wave length 540 mμ (17).

and $\epsilon_{\lambda 2B}$ are identical (isosbestic absorption at 805 mμ (Table I)) and the first term in the denominator drops out, yielding

$$x = \frac{r - \frac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}}{\frac{\epsilon_{\lambda 1A} - \epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}} \tag{3}$$

For practical convenience in later handling in this particular instance, the signs are reversed, obtaining

$$x = \frac{\frac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}} - r}{\frac{\epsilon_{\lambda 1B} - \epsilon_{\lambda 1A}}{\epsilon_{\lambda 2B}}} \tag{4}$$

Equation 4 is now rewritten specifically to obtain Equation 5, applicable to the photometric determination of the *fraction of* HbO_2 by any optical equipment, when one of the two wave lengths used for measurement is an isosbestic region for the two components:

$$\textit{Fraction of } HbO_2 = \frac{\frac{\epsilon_{660}\text{Hb}}{\epsilon_{805}\text{Hb}} - \frac{D_{660}}{D_{805}}}{\frac{\epsilon_{660}\text{Hb} - \epsilon_{660}\text{HbO}_2}{\epsilon_{805}\text{Hb}}} \tag{5}$$

The established ϵ values at wave lengths 660 and 805 mμ (Table I) are substituted in Equation 5 to give

$$\textit{Fraction of } HbO_2 = \frac{\frac{0.820}{0.196} - \frac{D_{660}}{D_{805}}}{\frac{0.820 - 0.100}{0.196}} \tag{6}$$

which becomes

$$\textit{Fraction of } HbO_2 = \frac{4.18 - \frac{D_{660}}{D_{805}}}{3.67} \tag{7}$$

Therefore,

$$\%\ HbO_2 = \left(\frac{4.18 - \frac{D_{660}}{D_{805}}}{3.67}\right) 100 \tag{8}$$

It is obvious that the *per cent of Hb* in the mixture is obtained from 100 less *per cent of* HbO_2, as given by the working Equation 8. It should be

evident that the exact optical depth of the cuvette employed need not be known for the determination of per cent of HbO_2 by the present method, provided that a reliably calibrated spectrophotometer affording narrow spectral isolation, such as the Beckman DU instrument, is employed, that the cuvette depth is between 0.05 and 0.10 cm., permitting the instrument to be used over its most accurate density range, when measuring undiluted samples of blood, and that the spectrophotometric constants established by us (Table I) are accepted as applicable. On the other hand, for the establishment of the ϵ values and for the determination of total pigment as cyanmethemoglobin, an accurate calibration of cuvette depth is necessary.

EXPERIMENTAL

The ϵ values in Table I were derived from measurements with the Beckman DU spectophotometer and our modified 0.067 cm. depth cuvette on nine samples of fresh human blood. The fully oxygenated specimens (HbO_2) were obtained by equilibrating 10 ml. volumes of the hemolyzed blood in an atmosphere of oxygen in a 400 ml. capacity tonometer. The deoxygenated samples (Hb) were obtained by adding sufficient active dithionite, $Na_2S_2O_4$ (Eimer and Amend, low iron content), in solid form.

In published simplifications (12–14) of the original Drabkin and Schmidt technique (7), the possibility has been neglected that under certain conditions blood samples may contain more than minimal quantities of carbonyl hemoglobin, HbCO, or methemoglobin, MHb. In such cases the above method, designed for a two-component mixture of HbO_2 and Hb, would yield erroneous information. With a view of extending the technique to the determination of the per cent of HbO_2 in the presence of HbCO or MHb, the extinction coefficients for HbCO, MHb_{acid} (pH 6.1), and $MHb_{alkaline}$ (pH 8.8) were obtained for the red and near infrared spectral regions, wave lengths 620 to 900 mμ (Table II). The data in Table II disclose that wave length 805 mμ can no longer serve for the total pigment estimation in hemolyzed blood samples containing several per cent of HbCO or MHb. At 805 mμ the extinction of radiant flux by HbCO is only one-fifth that of HbO_2 or Hb, whereas, dependent on the pH of the sample, MHb in this spectral region will have more than 2-fold greater absorption than either the HbO_2 or Hb species. However, it may also be seen (Tables I and II) that the spectral absorptions of HbO_2 and HbCO are practically identical at wave length 660 mμ. Moreover, at this wave length and at an intermediate pH of 8.0, the extinction coefficients of Hb and MHb are virtually the same (Table I and Table II, footnotes). This information is utilized in the following further modification, designed by one of us (D. L.

D.), to afford effective correction for the presence of HbCO or MHb in blood suspected of containing abnormal amounts of either of these derivatives. It is assumed that ϵ values at the wave length of 805 mμ less than 0.196 will reflect the presence of HbCO, whereas values greater than 0.196

TABLE I

Fractional Molar Extinction Coefficients, ϵ, in Red and Near Infrared Spectral Regions for HbO_2 and Hb Obtained from Nine Samples of Whole Hemolyzed Blood*

HbO_2, sample oxygenated; Hb, sample treated with $Na_2S_2O_4$; measurements in modified cuvette of 0.067 cm. depth with Beckman DU spectrophotometer; spectral span = 2.5 to 3 mμ.

Wave length	HbO_2		Hb		Wave length	HbO_2		Hb	
	ϵ	S. D.†	ϵ	S. D.†		ϵ	S. D.†	ϵ	S. D.†
mμ					mμ				
600	1.06	0.03 ±0.01	3.40	0.03 ±0.01	755	0.129	0.011 ±0.003		
605	0.674	0.013 ±0.003	2.61	0.05 ±0.01	760			0.378	0.006 ±0.002
610	0.464	0.011 ±0.003	1.96	0.04 ±0.01	800	0.189	0.010 ±0.003	0.200	0.007 ±0.002
625	0.228	0.011 ±0.003	1.22	0.04 ±0.01	805	0.196‡		0.196‡	
650	0.118	0.006 ±0.002	0.872	0.019 ±0.005	810	0.202	0.010 ±0.003	0.191	0.005 ±0.001
660	0.100	0.018 ±0.004	0.820	0.006 ±0.002	850	0.233	0.005 ±0.001	0.167	0.005 ±0.001
695	0.089	0.014 ±0.003	0.475	0.016 ±0.004	900	0.256	0.006 ±0.002	0.170	0.003 ±0.001
735	0.104	0.004 ±0.001	0.307	0.009 ±0.002					

* See the text, footnote 2.

† Standard deviation = $\sqrt{\Sigma d^2/(n-1)}$; the values after plus-minus are the standard errors of the standard deviation = S.D./$\sqrt{2n}$.

‡ Obtained by interpolation.

will indicate the presence of MHb (Tables I and II). Since such ϵ values are obtained from D_{805}/cd, where c is the concentration (in millimoles per liter) and d the cuvette depth in cm., the modified procedure demands that the concentration of total hemoglobin pigments be determined independently on an aliquot of the sample converted into cyanmethemoglobin and that a cuvette of exactly calibrated depth be used. Another aliquot, or the remainder of the sample, unexposed to air, is hemolyzed and at the same time adjusted to pH 8.0 by including appropriate buffer solution with

the saponin in the preparation of the tonometers. In the handling of the data three quantities are needed, $D_{660,\text{pH }8.0}$, $D_{805,\text{pH }8.0}$, and theoretical $D_{805,\text{pH }8.0}$ (for a mixture of HbO_2 and Hb). The first two are obtained by measurement, and the last is derived from $D_{805,\text{pH }8.0} = \epsilon_{805,\text{Hb}}cd = 0.196cd$. The ratio, $D_{660,\text{pH }8.0}$ (measured) to $D_{805,\text{pH }8.0}$ (derived), is substituted in

TABLE II

Fractional Molar Extinction Coefficients, ϵ, in Red and Near Infrared Spectral Regions for HbCO, Acid MHb, and Alkaline MHb†*

Measurements in unmodified Beckman cuvette 1 cm. in depth on solutions prepared from hemolyzed washed red cells at a concentration of 0.680 mmole per liter, with a spectral span of 2.5 to 3 mμ; HbCO samples obtained by equilibration with alkali- and water-washed illuminating gas; MHb samples prepared by addition of $K_3Fe(CN)_6$ to appropriate buffered solutions at 0.1 ionic strength (see Austin and Drabkin (18)).

Wave length	HbCO	MHb_{acid}‡	$MHb_{alkaline}$§	Wave length	HbCO	MHb_{acid}‡	$MHb_{alkaline}$§
	ϵ	ϵ	ϵ		ϵ	ϵ	ϵ
mμ				mμ			
620	0.424	3.38	3.24	760	0.041	0.198	0.360
630		3.88	2.14	800	0.041	0.326	0.488
640	0.207	3.85	1.53	805	0.040	0.345	0.506
650		2.45	1.24	810	0.040	0.364	0.523
660	0.105	1.05	0.517	820	0.040	0.403	0.544
675		0.320	0.320	840	0.040	0.475	0.517
680	0.068	0.224	0.301	845		0.499	0.502
720	0.047	0.131	0.336	880	0.040	0.601	0.351
740		0.155	0.345	900	0.040	0.660	0.298

* See the text, footnote 2.

† Data obtained by one of us (D. L. D.).

‡ pH 6.1, measured by glass electrode.

§ pH 8.8, measured by glass electrode. ϵ for MHb at intermediate pH between 6.1 and 8.8 may be calculated (18); ϵ for MHb at pH 8.0 = 0.826 at λ660 mμ and 0.412 at λ805 mμ.

Equation 8, which is now used to calculate the per cent of HbO_2. This relatively simple procedure for obtaining the per cent of HbO_2 in a mixture of HbO_2, Hb, and MHb is possible because the absorption spectra of HbO_2 and Hb are invariant with pH and only that of MHb is pH-dependent (18), and because the adjustment of the pH to 8.0 renders identical the extinction coefficients of Hb and MHb. Owing to the near identity of the ϵ values (at 660 mμ and at pH 8.0) of Hb and MHb on the one hand, and of HbO_2 and HbCO on the other, the evaluation of Hb in a mixture of HbO_2, Hb,

and MHb or of HbO_2 in a mixture of HbO_2, Hb, and HbCO requires the use of appropriate correction factors, supplied in the following formulations:

$$\% \, MHb = \left(\frac{\epsilon_{805,\, pH\ 8.0} - 0.196}{0.216} \right) 100 \tag{9}$$

$$\% \, HbCO = \left(\frac{\epsilon_{805,\, pH\ 8.0} - 0.196}{0.156} \right) 100 \tag{10}$$

$\epsilon_{805,pH\ 8.0}$ is obtained from the measurement of $D_{805,pH\ 8.0}$, since $\epsilon_{805,pH\ 8.0} = D_{805,pH\ 8.0}/cd$. In the above equations the numerators represent, respectively, the partial change in extinction between HbO_2 and $MHb_{pH\ 8.0}$ and between HbO_2 and HbCO, whereas the denominators are, respectively, the total change or difference in ϵ between the components ($\epsilon_{MHb_{pH\ 8.0}} - \epsilon_{HbO_2} = 0.412 - 0.196$ and $\epsilon_{HbO_2} - \epsilon_{HbCO} = 0.196 - 0.04$). The method is not applicable to the simultaneous presence of both HbCO and MHb, but this situation should be encountered only rarely. The absorption curves in Fig. 1 suggest that wave length 1050 mμ should be an ideal region for the determination of HbO_2 in mixtures of HbO_2, Hb, and HbCO, since the two latter species are isosbestic at this wave length. However, the Beckman DU spectrophotometer and other usually available equipment are inaccurate beyond 900 to 1000 mμ (11).

Table III furnishes extinction coefficient values for HbO_2 and Hb, obtained with the Bausch and Lomb Spectronic 20 spectrophotometer, used in combination with the Arthur H. Thomas Roto-Cell assembly. For the present purpose the latter adjunct is essential. It is a water-cooled carrier which permits both the use of parallel side cuvettes of exact optical depth and the rapid interchange in the light path of blank and sample.[3] While the ϵ values yielded by this equipment are not identical with those obtained with the Beckman DU spectrophotometer, nevertheless, as plot-

[3] The wave length scale of the Spectronic 20 instrument should be calibrated by the user and, if necessary, reset. An ϵ value of 11.5 at 540 mμ for cyanmethemoglobin was found to be applicable to the Spectronic 20 instrument, provided with the blue-sensitive phototube, R. M. A. type 5581, and the Thomas double chambered cuvette of Corex brand glass. Solutions of cyanmethemoglobin of known concentration were then used for the calibration of the depth of modified cuvettes. For modifying the depth of each chamber of the cuvette, a pair of very snugly fitting glass prism spacers, 0.93 cm. in optical depth and 2.8 cm. high, was ground and polished to our specifications by the Arthur H. Thomas Company. The calibrated optical depth of the modified partitioned cuvette was 0.0697 cm. With these particular spacers, 0.3 ml.of hemolyzed blood will not only fill the optical area but will allow for 0.2 ml. of sample above the spacers. It is possible to use spacers of the same height as the cuvette, and under these conditions about 0.12 ml. of blood will suffice.

ting of the data in Tables I and III will show, the relatively inexpensive Spectronic 20 spectrophotometer does a good over-all job in furnishing the absorption patterns of HbO_2 and Hb. Hence, the equipment can serve for a relatively accurate determination of per cent of HbO_2 in hemolyzed blood samples unexposed to air. It may be calculated that the ratio of $\epsilon_{Hb}/\epsilon_{HbO_2}$ is very slightly greater here (Table III) at 650 than at 660 mμ, and the isosbestic point is at 810 rather than at 805 mμ. The ϵ val-

TABLE III

Fractional Molar Extinction Coefficients, ϵ, in Red and Near Infrared Spectral Regions for HbO_2 and Hb Obtained on Whole Hemolyzed Blood†*

HbO_2, sample oxygenated; Hb, sample treated with $Na_2S_2O_4$; measurements with the Bausch and Lomb Spectronic 20 diffraction-grating spectrophotometer, provided with red and infrared sensitive phototube, type 1P40, a Corning red glass for filtering out interference from the second order spectrum produced by the grating, Arthur H. Thomas Roto-Cell assembly, which accommodates the partitioned cuvette, here modified to a calibrated depth of 0.0697 cm. by means of glass prism spacers and a voltage stabilizer.

Wave length	HbO_2	Hb	Wave length	HbO_2	Hb
	ϵ	ϵ		ϵ	ϵ
mμ			mμ		
600	0.848	2.74	750	0.183	0.459
610	0.530	1.96	760	0.198	0.454
620	0.348	1.50	800	0.248	0.285
630	0.263	1.24	805	0.254	0.269
650	0.178	0.997	810	0.261	0.263
660	0.165	0.913	850	0.304	0.254
700	0.143	0.537	900	0.337	0.267
740	0.176	0.417			

* See the text, footnote 2.

† Data obtained by one of us (D. L. D.).

ues at wave lengths 650 and 810 mμ are used as in Equation 5, and the following working equation applicable to the Spectronic 20 instrument is derived:

$$\% \; HbO_2 = \left(\frac{3.79 - \frac{D_{650}}{D_{810}}}{3.11} \right) 100 \qquad (11)$$

Because of the comparatively small volumetric size of the optical compartments of the Thomas cuvette, this equipment is particularly suitable for small blood samples.

Note on Results with Present Optical Method in Comparison with Those by Gasometric Technique

By Julian B. Marsh

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The technique of Gordy and Drabkin was adapted for use with ordinary syringes. The method of obtaining and hemolyzing the blood was essen-

Table IV

Comparison of Results by Gordy and Drabkin Spectrophotometric Procedure with Those by Gasometric Technique

Investigators	No. of individual sample comparisons	Mean difference in per cent saturation between spectrophotometric and gasometric analysis	Standard error of the mean difference
		per cent	
Marsh, J. B., Khouri, E., and Jetton, M.*	39	+0.62	±0.579
Williams, M. H.*	86	+1.97	±0.368
Cooper, D. Y., Billman, D. E., and Cooper, H. R.†	29	+1.00	±0.593
Wyeth, J., Ecker, P., and Polis, B. D.‡	14	+0.41§	±0.252

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§ Calculated from the data of Wyeth, Ecker, and Polis (19), assuming an oxygen capacity of 20 volumes per cent for all samples.

tially that of Hickam and Frayser (14), except that the saponin was introduced into the syringe through a 3-way metal stopcock. After hemolysis, a 3 inch, 22 gauge needle, bent at right angles, was attached, and the sample was delivered at the bottom of a Beckman cuvette 1 cm. deep but modified to a depth of 0.10 cm. by means of a lucite (Plexiglas) block, 9 × 9 mm. in cross section and 3.5 cm. high. It was convenient to place a small screw in the top of the lucite block so that it could be removed easily. Such plastic prisms were inexpensive substitutes for the polished glass prisms used by Gordy and Drabkin, and were discarded when their optical faces became marred after a period of service.

Measurements of the optical density at 660 and 805 mμ were made with

the Beckman DU spectrophotometer, and the per cent saturation (per cent of HbO_2 of the total hemoglobin) was calculated by means of the working Equation 8, based on the constants established by Gordy and Drabkin (Table I). The optical density readings remained unchanged for at least 15 minutes, and presumably it was not necessary to prevent exposure of the solution above the light path to the air, as in the cuvette described by Nahas (13). The chief source of difficulty in the present method was in the unreliability of commercially available saponin preparations. We found some samples of Merck and Eastman Kodak saponin to be satisfactory. With hemolytically potent saponin preparations, it was not necessary to use a 30 per cent solution, the concentration employed by Hickam and Frayser (14). In many of the determinations (Table IV), the blood was hemolyzed with 0.2 volume of 10 per cent saponin (Eastman Kodak, special) buffered at pH 7.4 with phosphate buffer. Wyeth, Ecker, and Polis (19) used a detergent, Triton X 100 (0.05 volume of a 33 per cent solution in 0.1 M borax), as the hemolytic agent.

The data in Table IV are a summary of results in four independent field trials of the Gordy and Drabkin method, carried out during the past 4 years. In confirmation of earlier findings (9), the per cent of oxygen saturation was slightly higher by the spectrophotometric than by the classical Van Slyke and Neill (16) gasometric method. However, the agreement of the independent techniques was most satisfactory.

DISCUSSION

The present simplified spectrophotometric method for the determination of the per cent of HbO_2 in hemolyzed blood unexposed to air retains the essential desirable features in the original Drabkin and Schmidt technique (7). The method is easily adaptable to standard equipment, such as the Beckman DU spectrophotometer and the new inexpensive Bausch and Lomb Spectronic 20 instrument. The manipulative steps involved are simple and rapid, and only small blood samples are required. Disadvantages in other modifications of the Drabkin and Schmidt thin layer technique (13, 14) have been overcome.

Under optimal conditions, a single component of a mixture of several species, the absorption constants of each of which have been established, can be determined spectrophotometrically with an accuracy of 1 per cent (7, 11, 18). The agreement between per cent of oxygen saturation (*i.e.* per cent of HbO_2 of total hemoglobin) determined independently by the present, direct spectrophotometric technique and that determined by the indirect gasometric method was easily within 2 per cent.

SUMMARY

A simple, rapid, and accurate method for the spectrophotometric analysis of the per cent of HbO_2 in hemolyzed blood unexposed to air has been described.

The method is applicable to standard equipment, such as the Beckman DU and the Bausch and Lomb Spectronic 20 spectrophotometers.

Suitable modifications in procedure permit the per cent of HbO_2 to be determined also in blood samples which may contain more than negligible amounts of carbonyl or methemoglobin.

BIBLIOGRAPHY

1. Gordy, E., and Drabkin, D. L., *Am. J. Med. Sc.*, **221,** 231 (1951).
2. Drabkin, D. L., *Proc. 1st International Instrument Congress and Exposition, Instrument Soc. Am.*, No. 54-13-2 (1954).
3. Drabkin, D. L., *J. Biol. Chem.*, **182,** 317 (1950).
4. Drabkin, D. L., in Roughton, F. J. W., and Kendrew, J. C., Haemoglobin, London, 35 (1949).
5. Drabkin, D. L., in Comroe, J. H., Jr., Methods in medical research, Chicago, **2,** 159 (1950).
6. Comroe, J. H., Jr., and Wood, E. H., in Comroe, J. H., Jr., Methods in medical research, Chicago, **2,** 144 (1950).
7. Drabkin, D. L., and Schmidt, C. F., *J. Biol. Chem.*, **157,** 69 (1945).
8. Roughton, F. J. W., Darling, R. C., and Root, W. S., *Am. J. Physiol.*, **142,** 708 (1944).
9. Lambertsen, C. J., Bunce, P. L., Drabkin, D. L., and Schmidt, C. F., *J. Appl. Physiol.*, **4,** 873 (1952).
10. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112,** 105 (1935–36).
11. Drabkin, D. L., in Glasser, O., Medical physics, Chicago, **2,** 1039 (1950).
12. Lowry, O. H., Smith, C. A., and Cohen, D. L., *J. Biol. Chem.*, **146,** 519 (1942).
13. Nahas, G. G., *Science*, **113,** 723 (1951).
14. Hickam, J. B., and Frayser, R., *J. Biol. Chem.*, **180,** 457 (1949).
15. Wood, E. H., Geraci, J. E., and Groom, D. L., *Federation Proc.*, **7,** 137 (1948); Wood, E. H., in Glasser, O., Medical physics, Chicago, **2,** 664 (1950).
16. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61,** 523 (1924).
17. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112,** 51 (1935–36).
18. Austin, J. H., and Drabkin, D. L., *J. Biol. Chem.*, **112,** 67 (1935–36).
19. Wyeth, J., Ecker, P., and Polis, B. D., United States Naval Air Development Center, Aviation Medical Acceleration Laboratory, Report No. NADC-MA-5408, Johnsville (1954).