Simultaneous Determination of Hemoglobin Derivatives, Oxygen Content, Oxygen Capacity, and Oxygen Saturation in 10 μl of Whole Blood

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We describe a new method for simultaneous determination of four hemoglobin derivatives (deoxyhemoglobin, Hb; oxyhemoglobin, HbO_2; methemoglobin, Hb^+; and carbon monoxide hemoglobin, HbCO) and total oxygen content in 10 μl of whole blood. Percentage HbO_2, HbCO, Hb^+, total hemoglobin (Hb_t), and oxygen capacity can also be obtained from the experimental data by simple calculations. Total analysis time is 1 min. Blood is diluted 100-fold with a buffer contained in a quasi-anaerobic cuvette, where simultaneous measurements of oxygen pressure (by a p_{O_2} electrode) and absorbance (at 497, 565, and 620 nm) are made. The decrease of oxygen pressure, as recorded by the oxygen electrode, is proportional to the amount of deoxyhemoglobin. The concentrations of HbO_2, HbCO, and Hb^+ can be obtained from absorbance measurements at the specified wavelengths. The new method eliminates the use of short-path optical cells and, due to the low sample volume requirement, makes possible the automated measurement of hemoglobin derivatives and oxygen saturation in arterialized capillary blood.

The determination of hemoglobin and its derivatives is of critical importance in the evaluation of certain physiological functions. A key factor in this regard is the knowledge of oxygen saturation in arterial or arterialized blood, which gives the best information for the evaluation of the efficiency of the oxygen transfer across the alveolar wall and of blood oxygen affinity. It is well known that gasometric methods, such as those described by Van Slyke and Neill (1), although accurate, are not easily adaptable to routine clinical work. Simpler but less accurate techniques have thus been proposed, and simultaneous spectrophotometric determination of two (or more) hemoglobin derivatives has gained much favor in recent years. All these techniques in which spectrophotometric analysis is used have primarily been derived from the classical studies of Drabkin (2) on the chemistry and optical properties of hemoglobin and its derivatives. Comprehensive and up-to-date reviews on this subject have recently been published (3, 4).

There is a common and critical step in the measurement of oxygen saturation by spectrophotometric analysis. In order not to change the proportion between deoxy- and oxyhemoglobin, it is necessary that the whole blood sample be hemolyzed and handled anaerobically. Accordingly, short-path optical cells and unnecessarily large sample volumes are usually required. These considerations have posed certain practical limitations to the design of semi-automatic spectrophotometric methods for measuring hemoglobin and its derivatives. The approach followed in this paper involves a single 100-fold dilution of the blood sample and requires only 10 μl of whole blood.

Materials and Methods

1. The Measuring System

The schematic diagram shown in Figure 1 represents the experimental setup which has been used in this study. Figure 1A shows the top view of the measuring system, which is thermostated at 32 ± 0.01 °C by heating elements and a separate electronic temperature control circuit (not shown in the diagram). L is a tungsten 10-W lamp; S, S_1, and S_2 are three reflecting mirrors. B is a beam splitter. F_1, F_2, and F_3 are three 5-nm (at half bandwidth) interference filters centered at 497, 565, and 620 nm. F_1 and F_2 can automatically be interchanged by motor M_1. P_1 and P_2 are two 1P28 type photomultipliers. The broken line indicates the optical path.

Figure 1B shows a side view of the block. T is a tonometer in which a buffer is kept in equilibrium with air by a stirrer magnetically coupled to motor M_2. C is a 10-mm (optical pathlength) stainless-steel cuvette, which is provided with glass optical windows. E is an
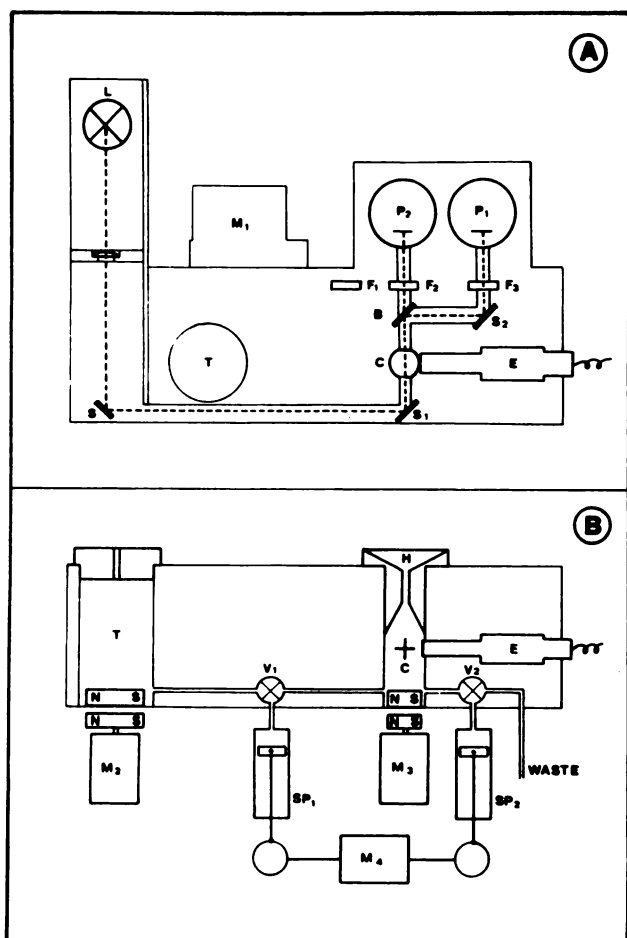
Fig. 1. Schematic diagram of the apparatus used in this study
A, top view; B, side view. For further details see text

Fig. 2. The mixing of blood at 50% oxygen saturation with a borate buffer at pH 9.1 in equilibrium with air
The insets in A and B show the oxygen dissociation curve of normal human blood (A) and the oxygen dissociation curve of human hemoglobin dissolved in the borate buffer (B). For further details see text

oxygen electrode of the Clark type. Syringes SP_1 and SP_2 and valves V_1 and V_2 provide for automatic filling and emptying of the cuvette. Stopper H decreases oxygen diffusion between C and air, providing a quasi-anaerobic environment for the buffer in C. The electrical output of photomultipliers P_1 and P_2 and of the oxygen electrode E are fed into suitable electronic amplifiers and read on separate digital voltmeters (not shown in the figure).

2. Principles of Operation

By operating syringes SP_1 and SP_2, cuvette C is filled with 10 mmol/liter borate buffer, pH about 9.1, which is derived from T. Per liter, this buffer contains 1 gram of "Sterox SE" (Baker Chemical Co.), a hemolyzing agent. After electronically zeroing the optical channels and calibration of the oxygen electrode against the p_{O_2} of the buffer solution [the p_{O_2} at 32 °C is given by the known relationship $p_{O_2} = 20.93 \times (1/100) \times$ (barometric pressure $-$ 35.6)], a small volume of blood (typically 10 μl) is introduced into C with a microsyringe or, more conveniently, by a "micropettor" (Scientific Manufacturing Industries, Emeryville, Calif.). The zero setting of the p_{O_2} electrode is occasionally checked by introducing a small amount of a 1 g/liter solution of freshly prepared dithionite into the cuvette.
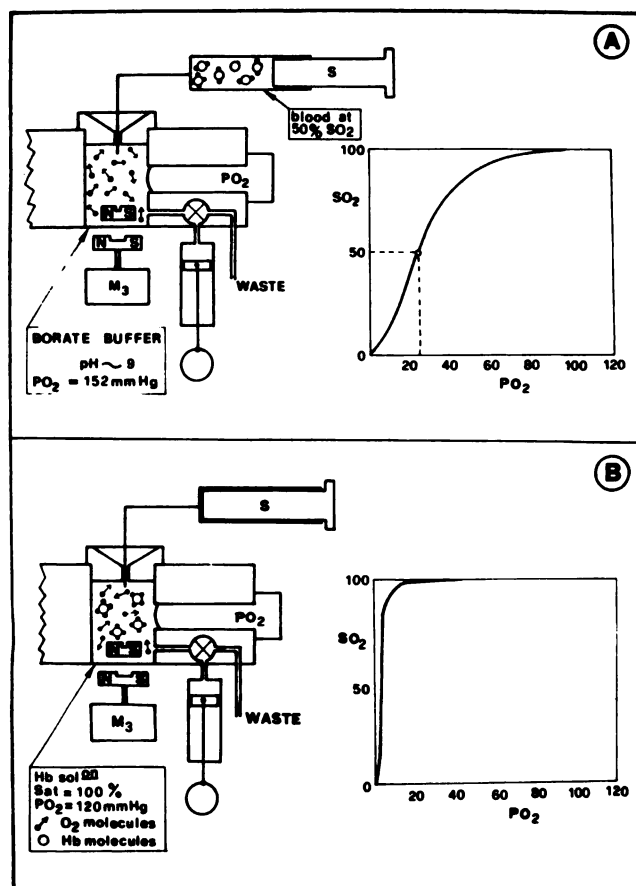
Figure 2 illustrates a practical example of the principles involved in the measurement of hemoglobin and its derivatives. Let us assume, as an example, that the blood sample to be analyzed contains 0.01 equiv. of hemoglobin, 50% saturated with oxygen, per liter. (In this paper hemoglobin concentration is expressed as equivalents of oxygen per liter; 1 equiv./liter equals 16 110 g of the protein per liter.) Figure 2A shows the oxygen dissociation curve for normal blood (inset) and the key part of the instrument. As the cuvette typically has a volume of 1 ml, the final hemoglobin concentration in C will be 1×10^{-4} equiv./liter. It will be shown later (see Results, part 5) that hemoglobin at pH about 9.1, 32 °C, can be assumed to be practically saturated with oxygen at a p_{O_2} of about 4 kPa (30 mmHg).[1] By definition (Figure 2B), of the total hemoglobin present in C, 0.5×10^{-4} equiv./liter is deoxyhemoglobin. This derivative will thus combine with the physically dissolved oxygen in the buffer and be quantitatively transformed into oxyhemoglobin. If air p_{O_2} is assumed equal to 152 mmHg, the concentration of physically dissolved oxygen will be $152 \times 1.49 \times 10^{-6} = 0.226 \times 10^{-3}$ mol/liter [1.49

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[1] 1 mmHg = 133 Pa (the SI unit, which is not yet in common use in clinical laboratories).

$\times 10^{-6}$ mol/liter per mmHg is the coefficient of oxygen solubility in the borate buffer (5)]. The total oxygen left in solution after hemoglobin has become completely oxygenated will be about $0.226 - 0.05 = 0.185 \times 10^{-3}$ mol/liter, which corresponds to a p_{O_2} of about 124 mmHg. Even if the blood sample were completely deoxygenated, the oxygen that would have been left in the cuvette (e.g., $0.226 - 0.1 = 0.126 \times 10^{-3}$ mol/liter, which corresponds to about 84 mmHg) would still be more than enough to ensure the complete transformation of all deoxyhemoglobin into oxyhemoglobin. In the previous calculations, the physically dissolved oxygen present in the original blood sample has been disregarded for simplicity. A more precise treatment will be given under *Results*, part 3.

At the end of the hemolysis step, which is completed in about 1 min, deoxyhemoglobin has disappeared from the solution. It should be remembered that this compound not only is normally present in a much higher concentration in venous blood than are Hb^+ and $HbCO$, but it also has a visible spectrum overlapping that of HbO_2 and $HbCO$. The disappearance of Hb obviously makes it easier to calculate the concentration of other hemoglobin derivatives by spectrophotometric analysis at several wavelengths. HbO_2, Hb^+, and $HbCO$ can now be determined by optical observation at three wavelengths by use of their known absorption coefficients.

3. The Determination of the Absorption Coefficients of HbO_2, $HbCO$, and Hb^+

An accurate knowledge of the absorption coefficients of HbO_2, $HbCO$, and Hb^+, at least at three wavelengths, is required in order to calculate concentrations from optical data. The wavelengths chosen for this study are 497, 565, and 620 nm. The procedure used for determination of the various absorption coefficients is as follows. Human hemoglobin has been prepared according to the method of Adair and Adair (6), and its carbon monoxide hemoglobin and methemoglobin content has been determined as described by Peters and Van Slyke (1) and Kilmartin and Rossi-Bernardi (7). Total hemoglobin has been determined according to the Drabkin method by transformation of hemoglobin derivatives into cyanmethemoglobin. Absorbance readings were determined at least 20 h after mixing blood or hemoglobin solutions with Drabkin's reagent to ensure the complete transformation of HbCO into cyanmethemoglobin. Methemoglobin was prepared from oxyhemoglobin by oxidation with 1.2 equivalents of potassium ferricyanide at pH 6.9. Excess ferricyanide was removed from hemoglobin by passing the solution through a column containing Sephadex G-25-F equilibrated with 0.1 mol/liter KCl.

Absorption measurements were obtained in a Beckman Acta III Spectrophotometer. Wavelength calibration of this instrument was checked according to the procedures indicated by the manufacturer and found to be in agreement with specifications.

The absorbance at 497, 565, and 620 nm of dilute solution of HbO_2, $HbCO$, and Hb^+ was first determined

and then corrected for the contribution of contaminating hemoglobin species. No corrections were required for the determination of ϵ_{Hb^+}, since all hemoglobin derivatives have been converted into methemoglobin by the procedure described.

Results

1. The Absorption Coefficients of HbO_2, $HbCO$, and Hb^+

Table 1 reports the values of the absorption coefficients of HbO_2, $HbCO$, and Hb^+ at 497, 565, and 620 nm. All values refer to hemoglobin (or blood) dissolved in borate buffer (10 mmol/liter, pH about 9.1, and containing 1 g of Sterox SE per liter) at 32 ± 1 °C.

2. The Kinetics of Erythrocyte Hemolysis

Figure 3 shows the change in absorbance with time when 10 μl whole blood is mixed with the borate solution. The top line of Figure 3 refers to 565 nm, the most critical wavelength of observation. The kinetics of hemolysis is complex and includes a rapid phase, which is probably of the same order as the mixing time (5 to 10 s), followed by a slow secondary process, which is completed in about 2 to 3 min. The secondary process involves a rate of change in the absorbance of about 0.0005 A/min. Readings obtained after 1 min are thus well within 0.5% of the final value. To estimate the presence of turbidity, we centrifuged the diluted hemoglobin solution obtained by mixing blood with the borate buffer at 16 000 $\times$ g for 15 min in a Sorvall RC-2B refrigerated centrifuge, or filtered it through a Millipore type 100 HAWPO-1300 filter. No further change in absorbance was measured after such procedures, indicating that the combination of alkaline pH and Sterox SE effectively prevented the formation of significant turbidity in the sample. The lower line of Figure 3 refers to an experiment in which sodium benzoate (10 g/liter) has been added to the borate solution to minimize bacterial growth. The denaturing effect of this substance on human hemoglobin at pH 9.1 (which has not been described before) is indicated by the large variation of absorbance with time.

3. The Determination of Deoxyhemoglobin and Fractional Oxygen Saturation

The total oxygen concentration (in mol/liter) of the borate buffer contained in cuvette C (Figures 1A, 1B) in equilibrium with air is given by

$$[O_2t] = \alpha p_{O_2} \qquad (1)$$

where α is the solubility coefficient for oxygen for the buffer. The total oxygen (in moles) contained in a volume V_0 (in liters) of buffer will be given by

$$O_2t = \alpha p_{O_2} \times V_0 \qquad (2)$$

Similarly, the total oxygen content of a volume (V_B) of blood is expressed by

$$O_2't = \alpha' p_{O_2}' V_B + ([O_2 \text{ comb}] \times V_B) \qquad (3)$$

Table 1. Absorption Coefficients of HbO₂, HbCO, and Hb⁺ in 0.01 mol/liter Borate Buffer, pH 9.1, Containing 0.1% Sterox SE (1 g/liter)[a]

	497 nm	565 nm	620 nm
HbO₂			
WB[b]	5.27	9.43	
WB	5.15	9.02	
WB	5.23	9.11	0.22
WB	5.29	9.21	
WB	5.29 f[c]	9.33 f	
Hb[d]	5.12	9.20	
Hb	5.21 f	9.26 f	
Hb	5.34	9.29	
Mean	5.24 ± 0.075 (±1.4%)	9.25 ± 0.129 (±1.4%)	
HbCO			
WB	5.05	13.81	
WB	5.15	13.71	0.34
WB	5.27	13.81	
WB	5.30 f	14.08 f	
Hb	5.35	13.58	
Hb	5.19	13.80	
Hb	5.15 f	13.81 f	
Mean	5.21 ± 0.10 (±2%)	13.84 ± 0.10 (±1%)	
Hb⁺			
Hb	7.59	7.84	2.67
Hb	7.65	7.93	2.69
Hb	7.64	7.86	2.65
Hb	7.66	7.85	2.69
Mean	7.63	7.87	2.68

[a] All values are expressed in liters × equiv.⁻¹ × cm⁻¹ × 10³.

[b] WB, whole blood.

[c] f, the measurements have been obtained on solutions filtered through Millipore filters.

[d] Hb, hemoglobin solution.
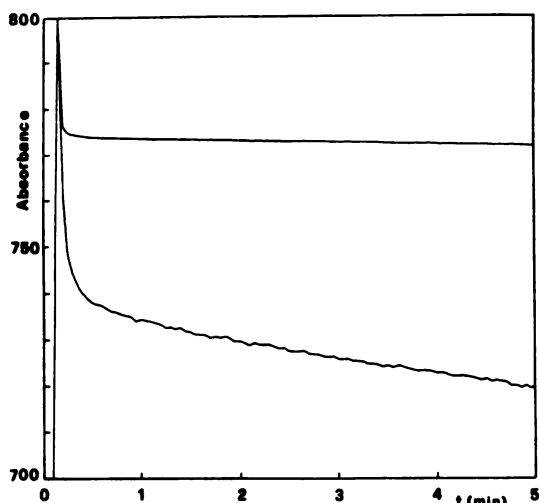
Fig. 3. Absorbance changes when whole blood (10 μl) is mixed with 1.0 ml of borate buffer containing Sterox SE (top curve)

The bottom curve shows the effect of 1 g of added sodium benzoate per liter of borate buffer

where [O₂ comb] is the concentration of oxygen combined with hemoglobin in blood, expressed in mol/liter, α' is the solubility coefficient of oxygen in blood [= 1.40 × 10⁻⁶ at 37 °C (8)] and p_{O_2}' is the oxygen pressure in

equilibrium with blood. After adding a volume V_B of blood to the buffer contained in C, but before any physical mixing or chemical reaction has occurred, the total oxygen content in C will be expressed by the sum of equations 2 and 3, i.e.,

$$\text{total oxygen} = \alpha' p_{O_2}' V_B + V_B[\text{O}_2 \text{ comb}] + p_{O_2}(V_0 - V_B) \quad (4)$$

where $(V_0 - V_B)$ is the volume of buffer displaced from C. After physical mixing and reaction of oxygen with hemoglobin,

$$\text{total oxygen} = \alpha'' p_{O_2}'' V_0 + [\text{O}_2 \text{ comb}]^m V_0 \quad (5)$$

where α'' ($\cong \alpha$) is the oxygen solubility coefficient in the borate–blood mixture, p_{O_2}'' is the oxygen pressure in equilibrium with this solution, and [O₂ comb]^m is the final concentration of oxygen combined with hemoglobin, after deoxyhemoglobin has been transformed into oxyhemoglobin.

Let us now define S as the fractional oxygen saturation of the undiluted blood sample, S' as the fractional oxygen saturation of hemoglobin after dilution in the borate buffer, and [OxyCap] and [OxyCap]^m as the oxygen combining capacities of the undiluted blood and of hemoglobin diluted in the borate buffer (in equiv./
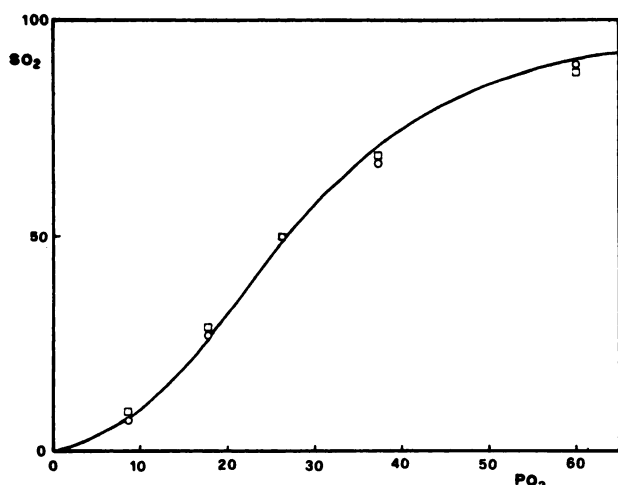
Fig. 4. Oxygen dissociation curve of normal human blood at 37 °C, p_{CO_2} 40 mmHg, pH 7.39, as determined by the Van Slyke gasometric method (*squares*) and by the microtechnique described in this paper (*circles*)

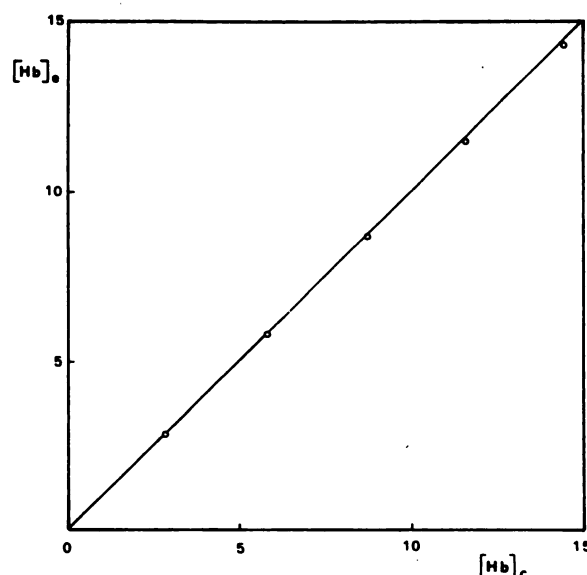The curve is the standard curve reported in the literature (*21*)

Fig. 5. Determination of deoxyhemoglobin by use of the p_{O_2} electrode technique and by application of equation 6

Experimental values of deoxyhemoglobin ($[Hb]_e$) are represented on the *ordinate* vs. the theoretical values ($[Hb]_c$) on the *abscissa*

liter), respectively. Since $S' = 1$ (see *Results*, part 5), from equations 4 and 5,

$[OxyCap](1 - S)$ = deoxyhemoglobin in the undiluted blood sample

$$= \frac{\alpha V_0(p_{O_2} - p_{O_2}'')}{V_B} - (\alpha p_{O_2} - \alpha' p_{O_2}') \qquad (6)$$

Data on the determination of oxygen saturation as obtained by the technique described in this paper and the application of equation 6 and by Van Slyke analysis on samples of whole human blood of various hemoglobin concentrations are reported in Table 2. From the deoxyhemoglobin concentration (equation 6) and the oxygen capacity, the fractional oxygen saturation, S, of the sample can be calculated by the following equation:

$$S = 1 - \frac{[\text{deoxyhemoglobin}]}{[\text{OxyCap}]} \qquad (7)$$

Figure 4 shows the oxygen dissociation curve of a human blood sample equilibrated with various oxygen pressures at a constant $p_{CO_2} = 40$ mmHg. The experimental points were obtained by Van Slyke analysis on 2 ml of whole blood, or by the technique described in this paper on 10 μl of whole blood.

4. The Linearity of the Oxygen Electrode

The method for the determination of deoxyhemoglobin according to equation 6 requires, for accurate results, a linear response between the oxygen electrode output and the p_{O_2} of the borate solution. To check this point, various amounts of a 6×10^{-3} equiv./liter solution of human deoxyhemoglobin were introduced anaerobically into cuvette C; results of a typical test are shown in Figure 5.

Figure 6 shows the change in p_{O_2} with time upon addition of a 10-μl sample of deoxygenated blood to the

Table 2. Comparison of Oxygen Capacity and Saturation Estimations, and Corresponding Standard Deviations, Obtained by the Van Slyke Manometric Method and by the Approach Described in This Paper[a]

n	Hb_f	[OxyCap]'	[OxyCap][g]	S%	S% [g]
6	4.22 ± 0.05	3.96 ± 0.03	3.81 ± 0.05	16.4 ± 1.50	15.4 ± 0.97
4	4.22 ± 0.10	3.99 ± 0.10	3.86 ± 0.12	41.0 ± 0.90	40.8 ± 0.74
6	4.22 ± 0.04	3.99 ± 0.03	3.75 ± 0.03	64.5 ± 0.40	63.5 ± 0.50
6	6.33 ± 0.01	5.71 ± 0.17	5.90 ± 0.14	17.7 ± 1.95	15.4 ± 1.00
6	8.38 ± 0.01	7.64 ± 0.12	7.92 ± 0.10	71.4 ± 0.67	71.0 ± 0.80
6	10.43 ± 0.01	9.95 ± 0.15	9.71 ± 0.11	51.2 ± 0.50	50.8 ± 0.76
6	10.68 ± 0.01	10.21 ± 0.15	9.91 ± 0.12	15.8 ± 0.61	14.6 ± 0.60
7	12.29 ± 0.01	10.48 ± 0.22	10.52 ± 0.22	73.3 ± 1.00	75.2 ± 0.90

[a] n is the number of samples; Hb_f is the concentration of hemoglobin (liters × equiv.$^{-1}$ × 10^3) as estimated from the sum of HbCO + HbO_2 + Hb$^+$; [OxyCap]' is the oxygen combining capacity (liters × equiv.$^{-1}$ × 10^3) estimated from the optical estimation of HbO_2; [OxyCap] [g] represents the oxygen combining capacity estimated from the total CO combining capacity minus the concentration of HbCO present in the original samples; S% is the percentage oxygen saturation calculated from equation 7; S% [g] is the oxygen saturation estimated from Van Slyke determinations of total oxygen content and of oxygen capacity.
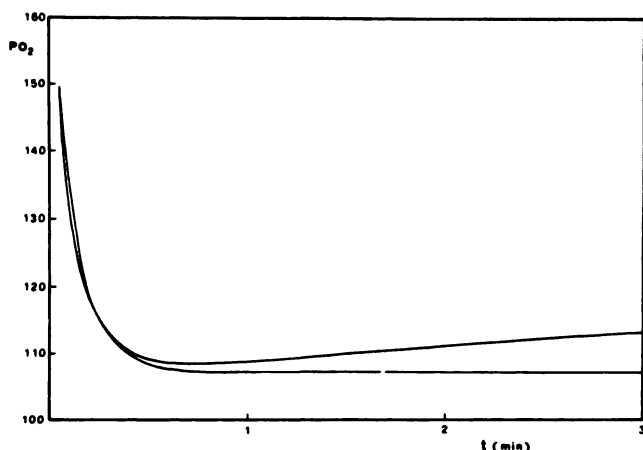
Fig. 6. Decrease with time in p_{O_2} in the borate buffer upon addition of 10 μl of deoxy blood

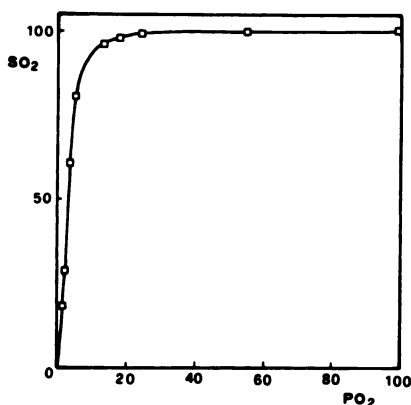The *top curve* shows the effect of a small air bubble introduced into the cuvette

Fig. 7. Oxygen dissociation curve of a human hemoglobin solution dissolved in borate buffer, pH 9.1, 32 °C

borate solution contained in cuvette C (Figure 1). After the initial large p_{O_2} drop, due to the transformation of deoxy- into oxyhemoglobin, the p_{O_2} of the solution remains almost constant (Figure 6, bottom curve). This indicates that the stainless-steel cuvette and its stopper provide a virtually anaerobic environment for the blood-plus-buffer solution. If, however, a small (about 5 μl) air bubble is introduced into the cuvette, then a large p_{O_2} drift (Figure 6, top curve) results. It is thus essential, for accurate results, that no air bubbles be trapped in the cuvette.

5. The Oxygen Dissociation Curve of Whole Blood Diluted in 10 mmol/liter Borate Buffer at 32 °C

The accurate determination of deoxyhemoglobin by equation 6 requires that *any* deoxyhemoglobin contained in a blood sample be quantitatively transformed into oxyhemoglobin upon dilution in a borate buffer. To test this point, we determined the oxygen dissociation curve of whole blood diluted 100-fold in an 10 mmol/liter borate buffer (plus Sterox, 1 g/liter) as follows. Ten microliters of whole blood was diluted in 1 ml of borate

buffer contained in cuvette C (Figure 1). Stopper H was raised, to provide an air space, and a stream of water-saturated nitrogen was passed over the hemoglobin solution. Absorbance measurements were recorded at 565 nm simultaneously with p_{O_2} electrode measurements of the oxygen pressure in solution. Deoxygenation of the solution was completed in about 20 min. Percentage saturation values were obtained from the total change in absorbance upon deoxygenation and from the absorbance values at each oxygen pressure. The oxygen dissociation curve for whole blood diluted in borate buffer is reported in Figure 7. The data clearly show that blood diluted in a borate buffer can be assumed to be saturated with oxygen at a p_{O_2} greater than 30 mmHg.

6. The Determination of "Total" Hemoglobin, Carbon Monoxide Hemoglobin, and Methemoglobin

The diluted hemoglobin solution that results from adding a normal blood sample to the borate buffer will typically contain (albeit in different concentrations) only three pigments: HbO_2, HbCO, and Hb^+. In the following treatment, it is assumed that no other pathological derivatives of hemoglobin (such as sulfhemoglobin) are present in solution. The reader is reminded that the concentration of HbO_2 in the blood-borate mixture will not correspond to the concentration of oxyhemoglobin of the undiluted blood sample, because it has been in part derived, upon dilution with the borate buffer, from the oxygenation of deoxyhemoglobin.

Total hemoglobin is estimated as follows. Table 1 shows that HbO_2 and HbCO have an absorption isosbestic point at 497 nm and very low absorption coefficients at 620 nm. Thus, if we assume $\epsilon_{620}{}^{HbO_2} \sim \epsilon_{620}{}^{HbCO}$, the following equations must hold:

$$A_{497} = \epsilon_{497}{}^{HbO_2, HbCO} ([HbO_2]$$
$$+ [HbCO]) + \epsilon_{497}{}^{Hb^+}[Hb^+] \quad (8)$$

$$A_{620} = \epsilon_{620}{}^{HbO_2, HbCO} ([HbO_2]$$
$$+ [HbCO]) + \epsilon_{620}{}^{Hb^+}[Hb^+] \quad (9)$$

In equations 8 and 9 there are only two unknowns, i.e., the sum of $[HbO_2]$ + $[HbCO]$ and $[Hb^+]$. Such unknowns can be algebraically obtained as described by Van Kampen and Zijlstra (3). The total hemoglobin concentration is obtained from the sum of $[HbO_2]$ + $[HbCO]$ + $[Hb^+]$.

Table 3 summarizes determinations of total hemoglobin concentration (on the same blood sample) by three methods of analysis: (a) determination of CO combining capacity according to Van Slyke and Neill (1); (b) the Drabkin method, and (c) the dual-wavelength approach described here. Note that all values reported appear to be within the experimental error of the various procedures used.

The concentration of HbO_2 and HbCO can be obtained by repeating the optical observations at 497 and 565 nm. The absorbance values at such wavelengths can
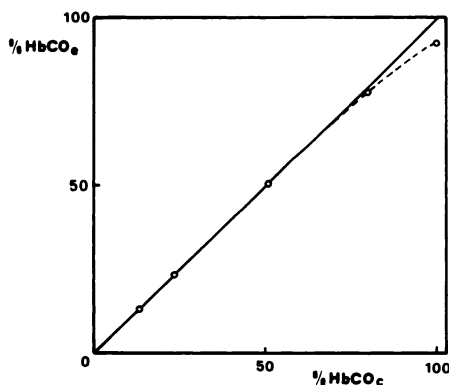
Table 3. Determination of "Total" Hemoglobin by Drabkin's Cyanmethemoglobin method, CO Combining Capacity, and Spectrophotometry at 497 and 620 nm

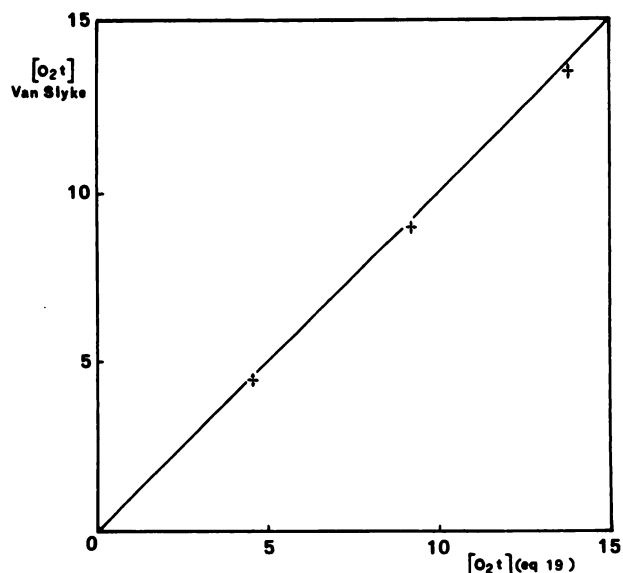| Method	Total hemoglobin (liters × equiv.$^{-1}$ × 10^3)	
Drabkin, at 540 nm	7.64 ± 0.06	(4)[a]
Van Slyke	7.59	
	7.55	
Spectrophotometry (Acta III), at 497 and 620 nm	7.66 ± 0.06	(4)
Spectrophotometry (this method), at 497 and 620 nm	7.59 ± 0.03	(10)

[a] Numbers in parentheses are number of experiments.

Fig. 9. Results of determination of total oxygen content of human blood by the Van Slyke gasometric technique on 2-ml samples vs. total oxygen determinations by the p_{O_2} electrode technique and by application of equation 11

The arrows represent standard errors for the two methods

be corrected for the contribution of [Hb$^+$] and the concentrations of HbCO and HbO$_2$ derived in the same way as indicated for the unknowns of equations 8 and 9. The value of HbO$_2$ represents, in this case, the oxygen capacity of the hemoglobin sample under analysis. This is the value that has been used to calculate oxygen saturation in equation 7.

To estimate the accuracy of the procedure for the determination of HbCO, part of a human blood sample was deoxygenated by equilibration with nitrogen and another part was first saturated with pure carbon monoxide. The excess dissolved CO was removed by equilibration of carbonmonoxy blood with nitrogen for 3 min in an IL 237 Tonometer (Instrumentation Laboratory, Inc., Lexington, Mass. 02173). Total HbCO content was estimated in both samples by Van Slyke analysis. Mixtures with various percentages of HbCO were prepared by adding different amounts of the two solutions directly into cuvette C (Figure 1). Figure 8 shows calculated [HbCO] concentrations vs. those obtained by dual-wavelength photometry at 497 and 565 nm. A significant decrease in the experimentally determined value of [HbCO] is clearly apparent above 60% [HbCO].

Fig. 8. Percentage carbonmonoxyhemoglobin as determined by optical observation at 497 and 565 nm vs. theoretical values
Total hemoglobin concentration: 10.5 × 10^{-3} equiv./liter

7. The Determination of Total Blood Oxygen Content

The total oxygen of a volume (V_0) of borate buffer is given by equation 2. After addition of a volume (V_B) of blood,

$$O_2't = \alpha p_{O_2}''V_0 + [HbO_2]V_0 - \alpha p_{O_2}V_B \qquad (10)$$

where $\alpha p_{O_2}''V_0$ is the physically dissolved oxygen; [HbO$_2$]V_0 is the combined oxygen (where [HbO$_2$] is the concentration of HbO$_2$ in the blood plus borate mixture); and $\alpha p_{O_2}V_B$ is the amount of oxygen which has been displaced from cuvette C (Figure 1) by the addition of a volume, V_B, of blood. The total oxygen concentration present in blood is obtained from the difference between equations 10 and 2 and is given by

$$[O_2{}^B] = f \times \frac{\alpha p_{O_2}''V_0 + [HbO_2]V_0 - \alpha p_{O_2}(V_0 - V_B)}{V_0}$$

$$(11)$$

where f is the dilution factor (= V_0/V_B). If the accuracy required in the determination of total oxygen is not greater than ±1 to 2%, then equation 11 can be approximated by the simpler expression

$$[O_2{}^B] = f([HbO_2] - \alpha(p_{O_2} - p_{O_2}'')) \qquad (12)$$

Because [HbO$_2$] is obtainable from the optical data and p_{O_2} and p_{O_2}'' are experimental quantities, the total oxygen content of a blood sample can be determined from equation 11 or, more simply, from equation 12. Figure 9 shows the experimental determinations of total oxygen content of a human blood sample equilibrated with pure oxygen.
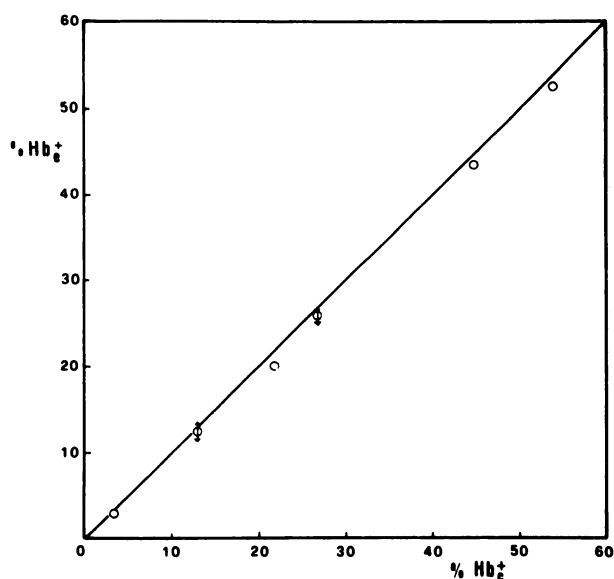
Fig. 10. Theoretical values for percentage methemoglobin (% Hb_c^+) obtained by mixing solutions of human oxyhemoglobin and methemoglobin vs. experimental values (% Hb_e^+) obtained by absorbance measurements at 497 and 620 nm and by application of equations 8 and 9

The arrows represent standard errors

8. The Determination of Methemoglobin

The value for methemoglobin can be obtained from equations 8 and 9 by assuming that, after the blood is diluted in the borate buffer, only HbO_2, HbCO, and Hb^+ are present in solution. Table 1 shows, however, that values of ϵ_{620}^{HbCO} and $\epsilon_{620}^{HbO_2}$ are similar, but not equal. If the two coefficients are assumed to be identical, then a small error in the determination of Hb^+ is introduced at HbCO concentrations higher than 30 to 40%. Figure 10 shows theoretical values of methemoglobin concentration (Hb_c^+) vs. results (Hb_e^+) obtained by the procedure previously indicated and application of equations 8 and 9. All the results reported in Figure 11 refer to an experiment in which a purified human oxyhemoglobin solution was mixed with various amounts of human methemoglobin, prepared as described in *Methods*.

Discussion

The Determination of Oxygen Capacity and Oxygen Saturation

The use of the p_{O_2} electrode for the measurement of blood total oxygen content was originally described by Clark (9). Since then, several papers have dealt with refinements of this technique. Laver et al. (10) and Tucker (11) used ferricyanide to release oxygen from hemoglobin, and Awad and Winzler (12) and Klingenmaier et al. (13) introduced the use of carbon monoxide to displace oxygen from hemoglobin without lysing the cells. Maio and Neville (14) estimated both oxygen content and oxygen capacity by the polarographic dropping-mercury electrode. All of these techniques, however, are based on the irreversible transformation

of one or various derivatives of hemoglobin. The ferricyanide method (10), for instance, involves the transformation of Hb, HbO_2, and possibly some HbCO into Hb^+, whereas the carbon monoxide method (13) causes the transformation of Hb and HbO_2 into HbCO. Thus, the former method will prevent the determination of methemoglobin on the same sample, whereas the latter method will not be suitable for the determination of the HbCO concentration.

The method reported here introduces a key variation in procedure, since the coupling of p_{O_2} electrode measurements with optical observations at several wavelengths and the complete transformation of deoxy- into oxyhemoglobin allows the simultaneous estimation of oxygen saturation and of the concentration of several hemoglobin derivatives in microsamples of whole blood. Other advantages of the approach described are: (a) absorbance measurements are made on *diluted* hemoglobin solutions in an optical cell of 1 cm pathlength, and (b) the disappearance of deoxyhemoglobin from solution allows the use of more favorable wavelengths of observation for the calculation of total hemoglobin, Hb^+, and HbCO.

In deriving equation 6, it has been assumed that the oxygen solubility coefficient of 10 mmol/liter borate with or without added blood is practically the same as for pure water. This is indicated by the findings of Laver et al. (10), who showed that the oxygen solubility coefficient for water and a 2 g/liter potassium ferricyanide solution containing 1 g of saponin per liter and for a mixture of the latter with a small volume of whole blood did not differ significantly. We confirmed this finding by Van Slyke analysis (on 7-ml samples) of total oxygen content of a 10 mmol/liter borate buffer containing Sterox (1 g/liter) in equilibrium with air. A value of $1.46 \pm 0.04 \times 10^{-6}$ mol/liter per mmHg was obtained for the oxygen solubility coefficient at 32 °C. This value should be compared with the value of 1.492×10^{-6} mol/liter calculated from the reported value of the Bunsen oxygen solubility coefficient (5), which has been used in all calculations reported in this paper.

Figure 7 shows the oxygen dissociation curve of whole blood diluted in borate buffer at pH 9.1, 32 °C. Note that hemoglobin is practically saturated with oxygen at p_{O_2}'s exceeding 30 mmHg. This large increase in oxygen affinity is a result of: the Bohr effect, according to which the oxygen affinity of hemoglobin increases with pH; the disappearance of the Margaria and Green effect (15), because p_{CO_2} in the borate buffer is close to zero; the effect of a decrease in temperature from 37 to 32 °C; and the disappearance of the effect of 2,3-diphosphoglycerate on the oxygen affinity of hemoglobin at high pH's (16). The assumption made in deriving equation 6—namely, that a normal blood sample will reach 100% oxygen saturation when diluted in a borate buffer—is thus confirmed. It is interesting to ask whether the same may also be true for blood with an abnormal oxygen affinity.

Several causes of decreased oxygen affinity should be discussed in this regard. (a) Decreased oxygen affinity

due to a variation in p_{CO_2}, pH, or organic phosphates concentration, or some combination of these. All such factors of alteration will be removed upon mixing blood with the borate buffer. (b) Decreased affinity due to an abnormal effect of protein concentration, as typically found for sickle cell hemoglobin. In this case, the dilution of SS-blood or hemoglobin solutions will restore oxygen affinity of hemoglobin S to normal (17, 18). (c) Decreased oxygen affinity due to the presence of an abnormal hemoglobin with a low oxygen affinity. From Table 15 of Baldwin's comprehensive review (19), it appears that hemoglobin Kansas is the abnormal hemoglobin with the lowest oxygen affinity so far described. Gibson et al. (20) have shown for this pathological hemoglobin: n (Hill's coefficient) = 1.3, P_{50} (at pH 7.0, 20 °C) = 20 mmHg, and $-\Delta \log P_{50}/\Delta$ pH (the Bohr coefficient) = 0.3 (60% of the normal value). If it is assumed that the effect of temperature on the P_{50} of hemoglobin Kansas is the same as for normal hemoglobin, then from the reported effect of temperature on the affinity of whole blood (21):

$$P_{50}^{32°}(\text{Hb Kansas}) = 1.25 \times P_{50}^{20°} = 25 \text{ mmHg}$$

Because $-\Delta \log P_{50}/\Delta$ pH for hemoglobin Kansas is 0.3, it follows that $P_{50}^{32°}$ (pH 9.1) for this hemoglobin will be in the order of 7 mmHg. Applying the known Hill's relationship at 50%, where y is the oxygen saturation, then

$$(P_y/P_{50})^n = y/(100 - y) \qquad (13)$$

The partial pressure of oxygen in equilibrium with a solution of hemoglobin Kansas at 32 °C, pH 9.1, y = 96%, can be estimated from equation 13 to be about 72 mmHg. The addition of a 10 μl of completely deoxygenated blood sample that has a hemoglobin concentration of 1×10^{-2} equiv./liter will reduce the oxygen pressure in cuvette C to about 83 mmHg. Because hemoglobins with altered oxygen affinity comprise only a fraction of the total hemoglobin content of a pathological blood sample, the figures reported demonstrate that the procedure indicated for estimating oxygen saturation should be correct also for abnormal blood samples.

Equation 6 shows that in order to obtain an exact estimation of the amount of deoxyhemoglobin, and hence of percentage saturation, it is necessary to know the oxygen pressure in equilibrium with blood. The reason for this is that the introduction of a blood sample (typically 10 μl) into the cuvette shown in Figure 1 will displace a corresponding amount of buffer from the reaction chamber. The error which arises from neglecting blood p_{O_2} is given by the term $(\alpha p_{O_2} - \alpha' p_{O_2}')$ of equation 6. However, as Table 4 shows, the effect of this term on the calculated oxygen saturation of normal blood is quite small and varies between 1% in the physiological range of blood p_{O_2} to about 2% oxygen saturation when blood p_{O_2} is close to zero. The knowledge of blood p_{O_2} is not, in general, essential for most clinical studies, since an accuracy of $\pm 2\%$ for oxygen saturation is more than adequate. If, however, more

Table 4. Effect of Neglecting the Term $(\alpha p_{O_2} - \alpha' p_{O_2}')$ in Calculating Oxygen Saturation by Using Equation 6 [a]

S%	p_{O_2}	$(\alpha p_{O_2} - \alpha' p_{O_2}') \times 10^3$	Δ S%
10	10.3	0.209	2.09
20	15.4	0.202	2.02
30	19.2	0.197	1.97
40	22.8	0.191	1.91
50	26.6	0.186	1.86
60	31.2	0.180	1.80
70	36.9	0.172	1.72
80	44.5	0.161	1.61
90	57.8	0.142	1.42
95	74.1	0.120	1.20
96	81.0	0.110	1.10
97	91.6	0.095	0.95
98	111.0	0.068	0.68

[a] S% and p_{O_2} are percentage oxygen saturation and p_{O_2} of a normal blood sample at 37 °C, pH 7.4. p_{CO_2} = 40 mmHg; ΔS% is the decrease of oxygen saturation obtained from equation 6 by neglecting the second term on the right. The Hb$_t$ concentration was assumed equal to 10 × 10^{-3} equiv./liter and p_{O_2} = 152 mmHg.

accurate data are required as in physicochemical work, then a direct measurement of blood p_{O_2} may be necessary.

Another possible cause of error in the estimation of percentage oxygen saturation may be the effect of temperature. If a blood sample in equilibrium with oxygen at 37 °C is allowed to cool, a small change in oxygen saturation will occur due to the increase in oxygen affinity of hemoglobin. Because the concentration of total oxygen under anaerobic conditions is a constant, some physically dissolved oxygen will be bound by deoxyhemoglobin and change the oxygen saturation. This effect, for a moderate change in temperature, is also small and never clinically significant.

Table 2 gives an estimate of the accuracy and reproducibility of the method described here for measuring the oxygen saturation and the oxygen capacity of whole human blood. The reference method chosen to assess accuracy was the gasometric analysis of the CO combining capacity and oxygen saturation on 2 ml whole blood samples. The results reported in Table 2, which were obtained for blood samples whose hemoglobin concentrations varied from 4.2×10^{-3} to 12.3×10^{-3} equiv./liter, show that the maximum discrepancy between the gasometric method and the method of analysis described in this paper is, for the determination of oxygen capacity, not greater than 3×10^{-4} equiv./liter. This corresponds, for a blood sample of normal hemoglobin content, to about 3% of the total oxygen present in solution. Because this is also the estimated accuracy of the gasometric method for the determination of total oxygen content of whole blood [as thoroughly discussed by Roughton et al. (22)], it may be concluded that the accuracy of the micromethod proposed is, at least, of the same order as that of the standard Van Slyke method. The reproducibility of the oxygen capacity determina-

tions (Table 2) is about 1 to 2%. Similar reasoning applies to the determination of percentage oxygen saturation whole accuracy may be estimated as about ±1.5% saturation and whose reproducibility is about ±1% saturation.

The Determination of "Total" Hemoglobin

The accepted standard procedure to determine "total" hemoglobin ("total" hemoglobin here refers to the sum of Hb, HbO_2, HbCO, and Hb^+) is the Drabkin method, which is based on the irreversible transformation into cyanmethemoglobin of Hb, HbO_2, Hb^+, and of an undetermined amount of HbCO. There are two critical points in Drabkin's procedure: (a) the development of turbidity (23), possibly due to the precipitation of plasma proteins which is brought about by the acid pH of Drabkin's reagent, and (b) the slow transformation of HbCO. The presence of HbCO, whose concentration has been found to range from 0.2 to 13 g/100 g of hemoglobin in various individuals (24), represents by far the greatest source of error in the Drabkin procedure for estimating "total" hemoglobin. The method described here avoids such difficulties in that: (a) the combination of Sterox and of alkaline pH conditions effectively minimizes turbdity formation, as shown by the values of the absorption coefficients reported in Table 1 for purified hemoglobin solutions and for whole blood; and (b) it allows, due to the combination of wavelengths chosen (497 and 620 nm), the determination of the sum of HbCO + HbO_2, plus Hb^+ by observation at only two wavelengths. Because this method requires only 1 min, starting from whole blood, to obtain a stable absorbance reading, it is eminently suitable for the estimation of "total" hemoglobin in automatic analyzers.

The Determination of HbCO

The spectrophotometric procedures used to measure the concentration of carboxyhemoglobin in blood have been recently reviewed by Van Kampen and Zijlstra (3). Table 15 of their paper clearly shows that dilution of blood into a buffer in equilibrium with air causes a significant decrease of HbCO. Similar findings have been recently reported by Ramieri et al. (24).

Because the procedure described in the present paper for measuring the concentration of HbCO involves a 100-fold dilution of a blood sample into an oxygen-containing buffer, this dilution effect should be closely considered. Figure 8 shows that the analysis of a sample containing a known percentage of HbCO by the procedure described in this paper gives erroneous results only for HbCO saturations greater than 50 to 60%. The error increases exponentially with percentage HbCO, but it never reaches the high values shown by Van Kampen and Zijlstra (3). The reason for this difference will become apparent from the following analysis.

For a *closed* system containing HbO_2 and HbCO in equilibrium with dissolved CO and oxygen, the fol-

lowing mass conservation equations must hold.

$$[HbO_2] + [HbCO] = [Hb^t] \tag{14}$$

$$[HbCO] + [CO] = [CO^t] \tag{15}$$

$$[HbO_2] + [O_2] = [O_2^t] \tag{16}$$

where $[O_2^t]$ and $[CO^t]$ are the total concentration of O_2 and CO in mol/liter, and [CO] and $[O_2]$ are the concentrations of dissolved CO and O_2 in moles per liter.

By the Haldane relationship, at equilibrium

$$[HbCO]/[HbO_2] = M[CO]/[O_2] \tag{17}$$

The combination of equations 14 to 17 gives the second-order equation

$$[HbO_2]^2(M - 1) + [HbO_2](M[CO^t] + [Hb^t] + [O_2^t] - M[Hb^t]) - [O_2^t][Hb^t] = 0 \tag{18}$$

whence, from the initial values of the factor M (about 270), $[Hb^t]$, $[O_2^t]$, and $[CO^t]$, the final equilibrium concentrations of HbCO and HbO_2 can be calculated.

Results of computations according to equation 18 for solutions containing various initial hemoglobin concentrations, and which have various initial values of $[O_2^t]$ and $[CO^t]$, are shown in Figure 11. The crosses in the same figure refer to the experimental points shown in Figure 8. The agreement between the experimental determinations and the results of the theoretical analysis is well within 1 to 2% HbCO saturation, which is satisfactory considering the approximations made in deriving equation 18. Figure 11 clearly shows that no significant error due to the transformation of HbCO into HbO_2 upon dilution is observed if the initial HbCO concentration is less than 50%. Above such a value the error increases and becomes very significant for HbCO values near 100%, as indeed is required by the Haldane relationship. It thus seems that most of the errors in HbCO determinations reported by Van Kampen and Zijlstra (3) and Ramieri et al. (24) arose from the slow equilibration between the p_{CO} and p_{O_2} of their solutions
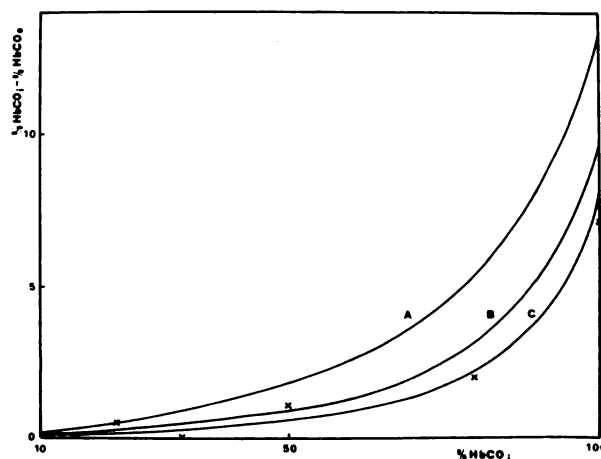

Fig. 11. Error in the determination of % HbCO vs. true % HbCO values

The curves show the results of theoretical calculations according to equation 18. A = 4.9, B = 9.9, and C = 14.9 × 10^{-3} equiv./liter total hemoglobin content. Crosses refer to the actual experimental points taken from Figure 8

with air. Below 50% HbCO saturation, the accuracy and the reproducibility of HbCO determinations by dual-wavelength spectrophotometry (see Figure 8) is about ±1.5%.

The Determination.of Total Oxygen Content

Equation 11 shows that the total oxygen content of a micro-scale sample of blood can be obtained from the experimental determination of [HbO$_2$] (by optical methods), p_{O_2}'', and p_{O_2} (by the p_{O_2} electrode). Equation 12 is a useful approximation to equation 11 if an error in the determination of total oxygen content of ±1 to 2% is allowed.

The Determination of Methemoglobin

Figure 10 shows that the reproducibility of the dual-wavelength method for estimating percentage Hb$^+$ is about ±1.5% Hb$^+$. This value, however, has been obtained on purified solutions of human hemoglobin. It should be mentioned that it was not convenient to prepare mixtures of oxyhemoglobin and methemoglobin by mixing whole blood samples of various Hb$^+$ content because of the rapid reduction of Hb$^+$ by the enzymatic system present in the erythrocytes. The dual-wavelength method for estimating methemoglobin can only be considered quantitative in the case of methemoglobinemia arising from (a) autoöxidation, (b) oxidation of the normal hemoglobin molecule by toxic agents, and (c) deficiency of the methemoglobin reductase system. In the case of congenital methemoglobinemia due to the presence in the erythrocyte of a pathological hemoglobin (e.g., methemoglobins M), it should be stressed that the absorption coefficient for Hb$^+$ varies according to the type of pathological hemoglobin being investigated. For such cases, the spectrophotometric method described can only give results of a semiquantitative nature.

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