JOURNAL OF APPLIED PHYSIOLOGY
Vol. 31, No. 1, July 1971. *Printed in U.S.A.*

A rapid method for simultaneous measurement of carboxy- and methemoglobin in blood

KENNETH A. SMALL, EDWARD P. RADFORD, JOHN M. FRAZIER, F. LEE RODKEY, AND HAROLD A. COLLISON
Department of Environmental Medicine, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore 21205; and Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland 20014

SMALL, KENNETH A., EDWARD P. RADFORD, JOHN M. FRAZIER, F. LEE RODKEY, AND HAROLD A. COLLISON. *A rapid method for simultaneous measurement of carboxy- and methemoglobin in blood.* J. Appl. Physiol. 31(1): 154–160. 1971.—A spectrophotometric method for rapid measurement of carboxyhemoglobin (CO Hb) and methemoglobin (Met Hb) simultaneously is described. The technique requires no more than 0.1 ml of blood and depends on measurements of absorbance of a dilute blood solution at four wavelengths in the near ultraviolet range. The method has an accuracy of ±0.6% CO Hb at low levels of CO Hb, and ±2.5% of the measured value at concentrations of CO Hb above 25%. The accuracy of the Met Hb determination is ±2% Met Hb for concentrations below 20%.

spectrophotometric method; blood carbon monoxide

THE MEASUREMENT OF SMALL AMOUNTS of carbon monoxide in blood has been a subject of increasing interest because of the exposure of large populations to CO in urban environments. The most accurate methods developed to date have generally been by gasometric techniques (4, 15), but these methods are relatively time consuming and may require large blood samples. A recent gas chromatographic method (5) gives good precision on blood samples of less than 1 ml with a sample handling time of about 20 min, but in addition each sample requires separate measurement of hemoglobin capacity. The need is for a method fast enough to allow routine analysis of small samples from human subjects and to monitor experiments in laboratory animals. Although spectrophotometric methods are simple and rapid, they have been in disrepute because of inaccuracy at low values of carboxyhemoglobin (3, 8, 9).

Improved modern equipment has permitted us to achieve good results by a spectrophotometric technique. With a Gilford model 240 spectrophotometer and model 110 digital absorbance meter providing direct readout, we have developed a method with which one technician can run as many as 60 blood samples/day. The blood sample size is 0.1 ml or less, and a measurement of methemoglobin is obtained along with that of carboxyhemoglobin, an important addition if one wishes to measure carboxyhemoglobin in blood obtained post mortem. Errors due to interfering pigments (methemoglobin, reduced hemoglobin, and bile salts) are minimal in our technique.

The method utilizes the difference in absorption spectra between oxyhemoglobin (O_2 Hb), carboxyhemoglobin (CO Hb) and methemoglobin (Met Hb) in the Soret region (390–440 nm). The absorbance of each blood sample is measured at four wavelengths which depend on the animal species (see Table 1). Based on these absorbance values we obtain measurements of both the percent carboxyhemoglobin (% CO Hb) and the percent methemoglobin (% Met Hb) by means of the Beer-Lambert Law. Reduced hemoglobin does not interfere with our measurements since it is oxygenated by excess O_2 available during sample preparation. Carboxyhemoglobin and methemoglobin are expressed as percent of total hemoglobin and the results are independent of the initial hemoglobin concentration.

The accuracy is adequate for most purposes and is especially good at low CO Hb saturations, namely, a standard deviation of ±0.6% CO Hb up to 25% saturation and ±2.5% of the measured percent saturation for saturations over 25%. The methemoglobin measurement has an accuracy of ±2% Met Hb for concentrations below 20%.

Several improvements in modern spectrophotometers have enabled us to achieve this accuracy. The linearity of the machine extends to greater than two absorbance units. The wavelength reading is reproducible to better than 0.02 nm, although the absolute wavelength setting is not that close. The range of available wavelengths allows use of the Soret band (390–440 nm) without instrument modifications. This spectral region has much better separation of the oxy- and carboxyhemoglobin spectra than the visible region; furthermore, conveniently located isosbestic points allow two measurements of CO Hb whose average is quite stable under such disturbing influences as wavelength calibration error, change in temperature of the sample, and the presence of methemoglobin or small amounts of reduced hemoglobin.

We have found the stability of our spectrophotometer sufficient to eliminate the necessity of oxygenating half of the sample for comparison, as was done by Commins and Lawther (6) in a method similar to ours. Additional factors in making the method fast are the direct digital readout in absorbance units obtained with our equipment, and use of a computer for the actual calculations from the four absorbance readings taken.

DESCRIPTION OF PROCEDURE

A heparinized whole blood sample, diluted about 1:70 with 0.04% ammonia solution, is placed in a 1-mm pathlength cuvette, and the absorbance is measured on a Gilford model 240 spectrophotometer at four wavelengths in the Soret band. Measurements are made twice to minimize reading and wavelength setting errors. To obtain absorbance readings in the optimum linear range of the spectrophotometer, which we consider to be 0.35–2.10 absorbance units, the dilution of whole blood must be about 1:70 for a 1-mm pathlength cuvette (1:100 for CO Hb saturations over 40% because of the high absorbance). Since the cuvettes hold 0.4 ml, as little as 10 µl of whole blood is sufficient after dilution to fill the cuvette. To increase ease of handling and to provide for flushing of the cuvette we use 0.05-ml samples for rat blood and 0.10 ml for dog and human blood. One-millimeter pathlength cuvette were chosen in order that the hemoglobin concentration of the sample solution be as great as possible without exceeding the

linear absorbance range of the spectrophotometer. The higher hemoglobin concentration is desirable to minimize the loss of CO into the diluent (see APPENDIX 2).

We have found that results are more consistent on postmortem human blood samples if the sample is centrifuged, and the cells washed and resuspended in an equal volume of normal saline solution prior to their dilution. In this way any blood already hemolyzed in the initial sample is eliminated.

To prevent contact of the diluted sample with air, the 1:70 dilution is made in a syringe containing a glass bead for mixing, and the sample is transferred to the cuvette through a 23-gauge needle; the cuvette is flushed from the bottom with four or more changes of the solution to ensure that air contact with the final sample is minimal. We found that keeping the solution at a pH of 10, and hence shifting the O_2 Hb dissociation curve toward a higher affinity for O_2, enabled us to keep the amount of reduced hemoglobin to less than 1%. In addition, a high pH slows the formation of methemoglobin. For these reasons we used a 0.04% NH_3 solution as a diluent, which has a pH of 10.2, changing to 10.0 when blood is dissolved in it. We found that neither NH_3 nor sodium heparin solutions have any absorbance at 390–440 nm, and the absorbance of potentially conflicting pigments such as bile salts is negligible.

The four wavelengths used for human, rat, and dog blood measurements are given in Table 1. Two of these wavelengths (approximately 413 and 431 nm, depending on the species) are isosbestic points for oxy- and carboxyhemoglobin spectra, one (421 nm) is the point of maximum difference between the two spectra, and the fourth (392 nm) is a point where the oxy- and methemoglobin absorbances are reversed compared with those at 421 nm. The CO Hb peak reading is divided by each of the isosbestic readings, and two ratios are found. By comparing these to standard ratios taken on pure pigments, we obtain two measurements of the percent carboxyhemoglobin. In addition, the ratio of absorbances at 392 and 413 is sensitive to both carboxy- and methemoglobin, and from this ratio a measure of the latter can be obtained. A study of the spectra shows that the presence of up to 10% Met Hb influences our CO Hb measurement by less than 0.5% CO Hb, but even that small influence can be corrected for quite easily. The complete calculations are given in APPENDIX 1.

The method is strongly dependent on the reproducibility of the wavelength setting, a source of error which probably accounts for the inaccuracy of previous attempts to measure small concentrations of carboxyhemoglobin by similar spectrophotometric methods. We have found that the monochromator in the Gilford apparatus shows continuous random drifting of a few tenths of a nanometer. It is possible to correct for this drift, as is shown in APPENDIX 1, when the drift is detected by repeated check of the wavelength setting of a holmium oxide filter with peak absorbance at 446.10 ± 0.20 nm. Such correction for wavelength setting errors complicates the computer program, and in practice this difficulty can be avoided by resetting the wavelength to correspond to the holmium peak repeatedly during the day. As long as the drift does not exceed 0.15 nm its effect can be ignored.

To minimize errors, complete data analysis must be carried out on a computer system capable of storing 24 constants and solving 2 simultaneous equations. We use a PDP-12 computer (4K storage) programmed in the FOCAL language. If a large computer of this type is not accessible the calculation can be simplified by neglecting the wavelength drift. In this case the program requires only 12 constants to be stored. These requirements are compatible with the Olivetti-Underwood Programma 101 desk computer with the program stored on magnetic cards. The computer program calculates both % CO Hb and % Met Hb.[1]

[1] Copies of the programs for these two computers can be obtained by writing E. P. Radford at The Johns Hopkins University.

TABLE 1. *Four wavelengths used for spectrophotometric determination of % CO Hb and % Met Hb in human, dog, and rat blood*

	Wavelength, nm			Band Width, nm
	Human blood	Dog blood	Rat† blood	
λ_I	392.0	392.0	392.0	1.1
λ_{II}*	413.2	413.3	413.1	1.0
λ_{III}	421.0	421.0	421.0	0.9
λ_{IV}*	431.3	431.7	430.7	0.9

* These wavelengths are isosbestic points for O_2 Hb and CO Hb.
† Long-Evans hooded rats

One requirement of the method is that any reduced hemoglobin must be oxygenated without altering the CO Hb saturation. We have simply used the oxygen dissolved in our diluent to combine with the reduced hemoglobin, both experiment and a simple calculation show that only a fraction of a percent of the hemoglobin remains unbound, provided that care is taken to ensure that the dilute ammonia solution is saturated with air. Suppose 0.1 ml of venous blood is dissolved in 7 ml of water. The water contains 0.044 ml of O_2 in equilibrium with air at room temperature. The blood has the capacity to combine with 0.02 ml of gas, but about 50% or more is already combined, and thus only about 0.010 ml of dissolved O_2 is bound. This reduces the Po_2 from 155 to 120 mm Hg, which results in well above 99% saturation at this pH and temperature.

Several authors have commented (10, 14) that percent CO Hb saturation decreases when blood is diluted, and in fact a simple calculation based on the relative affinities of hemoglobin for CO and O_2, done in APPENDIX 2, shows that some CO will be displaced from the hemoglobin by the dissolved oxygen. We correct for this loss in our computer calculation. The results of Paul and Theorell (10) at initial CO Hb saturation of 100% show somewhat more CO Hb dissociation than is predicted by this calculation. Our own measurements of this effect are consistent with the theoretical prediction of APPENDIX 2.

For normal blood the presence of reduced hemoglobin causes as much error as methemoglobin, and a correction for that pigment might seem logical also. Our procedure is designed, however, so that reduced hemoglobin cannot exceed 1%, but there may be cases when methemoglobin reaches values as high as 10 or 20%, and thus correction for methemoglobin is much more important quantitatively.

Several checks are made to verify the accuracy of crucial steps. Cuvettes can become unmatched due to scratches or inadequate cleaning, hence they are cleaned after every other measurement with a dilute solution of ethanol and HCl, and are filled with water and their absorbance checked frequently. The wavelength calibration of the spectrophotometer is measured on a built-in holmium oxide filter to within 0.02 nm before measurement of each sample, and it is reset if in error by more than 0.15 nm. A chamber temperature of 22 ± 1.5 C is maintained by pumping water from a thermostatically controlled bath through a jacket built into the spectrophotometer. As a check of stability of the instrument over time, standard whole blood samples are measured every few days. These standard samples are kept refrigerated for periods up to a few weeks.

Calibration of the method for a new species requires several days. The spectra of oxy-, carboxy-, met-, and reduced hemoglobin must be taken at known concentrations at pH 10. The procedure for O_2 Hb is described below. CO Hb was obtained by equilibrating diluted blood in a Farhi tonometer with 0.4% CO in N_2. Reduced hemoglobin was made from oxyhemoglobin by the addi-

TABLE 2. *Molar absorbances (a) and slopes of absorption spectra (σ) for pure species (O_2 Hb, CO Hb, and Met Hb) at standard wavelengths for human blood*

Wavelength, nm	O_2 Hb		CO Hb		Met Hb	
	a, $\times 10^5$ M^{-1} cm^{-1}	σ, $\times 10^5$ M^{-1} cm^{-1} nm^{-1}	a, $\times 10^5$ M^{-1} cm^{-1}	σ, $\times 10^5$ M^{-1} cm^{-1} nm^{-1}	a, $\times 10^5$ M^{-1} cm^{-1}	σ, $\times 10^5$ M^{-1} cm^{-1} nm^{-1}
392	0.465 ± 0.005	0.021 ± 0.001	0.276 ± 0.005	0.019 ± 0.001	0.611 ± 0.005	0.014 ± 0.001
413.2	1.288 ± 0.010	0.014 ± 0.002	1.288 ± 0.010	0.128 ± 0.005	1.000 ± 0.030	−0.004 ± 0.001
421	1.125 ± 0.010	−0.051 ± 0.001	1.896 ± 0.010	−0.040 ± 0.001	0.818 ± 0.020	−0.037 ± 0.001
431.3	0.529 ± 0.005	−0.044 ± 0.001	0.529 ± 0.005	−0.079 ± 0.001	0.411 ± 0.010	−0.028 ± 0.001

Values are means ± SD.

tion of sodium dithionite. Hemoglobin concentration was measured as cyanmethemoglobin, on the assumption of an (Fe) molar absorbance at 540 nm of 1.10×10^4. We used a modified form of Drabkin's reagent, described by Richterich (11).

The most critical constants are the pure oxyhemoglobin absorbances at the four wavelengths. These must be obtained from a solution as free as possible from methemoglobin, with a known percentage of CO Hb for which the absorbance can be corrected. The most consistent method for establishing O_2 Hb absorbances has been to determine them on routine samples on several normal individuals not exposed to special sources of CO. We assumed that normal laboratory rats and dogs kept indoors in a downtown area of Baltimore have an average of 1.0% CO Hb. For human blood, we assumed that normal nonsmoking subjects living in an urban area have an average of 0.9% CO Hb (4). The absorbances obtained in this manner were used for comparisons with the gas chromatographic method of Collison et al. (5). These comparisons indicated a slightly different zero level (about 0.5% CO Hb) so for all other measurements of human blood we adjusted the absorbance to set the zero level halfway between the two, i.e., to make 0.7% CO Hb the average value for nonsmokers. It is these adjusted absorbances which we report in Table 2. The samples also undoubtedly contained some methemoglobin, but fortunately, the effect of small amounts of this pigment on the CO Hb determination is negligible. For purposes of the methemoglobin determination we assumed that our samples from normal individuals contain 0.8% Met Hb as measured by the Evelyn-Malloy procedure (7).

The methemoglobin spectrum at pH 10 tends to be unstable, as pointed out by Austin and Drabkin (1). We found it is changed by the presence of glycine buffer, borate buffer, Sterox, and potassium ferricyanide, where in the latter two cases a gradual change continuing for at least several hours was observed. This was true even though ferricyanide was added at pH 7 and then the pH was adjusted to 10.

We were able to obtain a stable curve, with the same conditions under which our routine samples are prepared, by using $NaNO_2$ following the procedure for producing Met Hb presented by Van Assendelft and Zijlstra (13). Excess nitrite was added to whole blood, the solution was allowed to stand 15 min, then the cells were washed four times with normal saline to eliminate all excess nitrite. We then diluted the cells in 0.04% NH_3 solution. The curves obtained in this manner were used to determine the molar absorbances of Met Hb at the selected wavelengths, although the absolute values of the absorbances varied by ±5% of the values shown, probably because some conversion to hematin occurred. This uncertainty in the Met Hb spectra is the dominant source of error in the % Met Hb measurement.

RESULTS

The absorbance spectra of the four main forms of hemoglobin in the Soret range are shown in Fig. 1, for human, dog, and rat blood. Some peak wavelengths and molar absorbances are also listed in Table 3, and those for human blood are compared with findings of Van Kampen and Zijlstra (14) and Benesch et al. (2).

The method is based on the linearity of absorbance of a given pigment with its concentration. We tested this in two ways, first by varying the concentration of a pure oxyhemoglobin solution, and second by creating known mixtures of O_2 Hb and CO Hb. By the first procedure, we found our absorbance readings were linear with concentration of O_2 Hb up to 2.2 absorbance units within the pipetting error. The second test also gave a linear relationship between % CO Hb and absorbance for both dog and human blood except when % CO Hb approached 100%, where the absorbance was below that predicted. In this case we believe

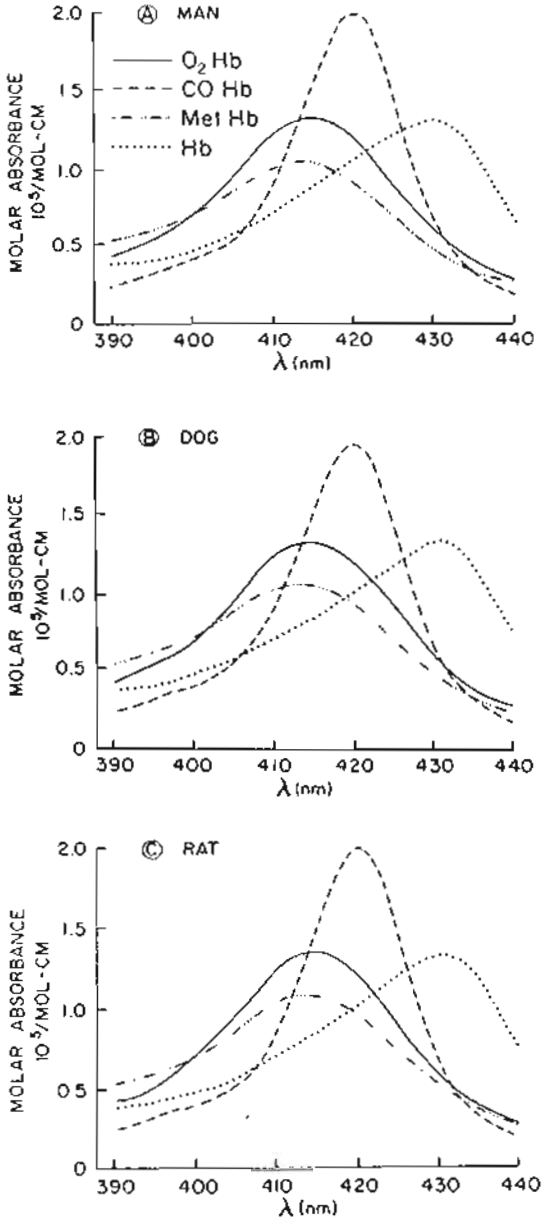

FIG. 1. Absorption spectra of O_2 Hb, CO Hb, Met Hb, and reduced Hb for (*A*) human, (*B*) dog, and (*C*) rat blood in the Soret region at pH 10.

that the Hb was not fully saturated with CO due to loss into the diluent (cf. APPENDIX 2).

Another check we have made is to store bottles of whole blood, after oxygenating them as fully as possible, and measure samples from these bottles periodically. This was done mainly to determine whether there is any systematic change in the zero CO Hb level by our measurement from day to day. No such change was found, and in fact, the variation was less than one would expect from random error as described in the next section.

As an independent check on our method, we have compared blood samples measured by our method and by the gas chromatographic method developed by Collison, Rodkey, and O'Neal (5). Some comparisons were made as whole blood, others as dilute solutions made by mixing dilute O_2 Hb and CO Hb solutions which had been equilibrated with appropriate gas concentrations. Whole blood samples were first exposed to various amounts of CO gas and were measured according to our routine procedure, including the correction for CO lost into solution described in APPENDIX 2. The O_2 Hb molar absorbances used for calculating percent saturation in all these samples were determined by setting the average CO Hb level for nonsmokers at 0.9% CO Hb, as described in the previous section. As a result of these studies, we subsequently changed this base-line value to 0.7% CO Hb.

The results are shown in Fig. 2. The agreement is, in most cases, within the experimental error predicted below. The diluted samples appear to read slightly lower than whole blood samples, probably due to slightly greater amounts of reduced hemoglobin in the former, due to the method of preparation.

We have also compared the % Met Hb as determined by our method with that given by separate measurements by the method of Evelyn and Malloy (7). The results are presented in Fig. 3 where we have indicated the errors associated with each method, ±2.0% for our method and ±0.5% for the Evelyn and Malloy method. There is excellent agreement at the low values of Met Hb (less than 20%) but the spectrophotometric procedure gives low values at concentrations above 20%. We have no explanation for the discrepancy, but as samples containing more than 20% Met Hb are extremely unlikely to occur, we consider this deviation of the results to be of little practical consequence.

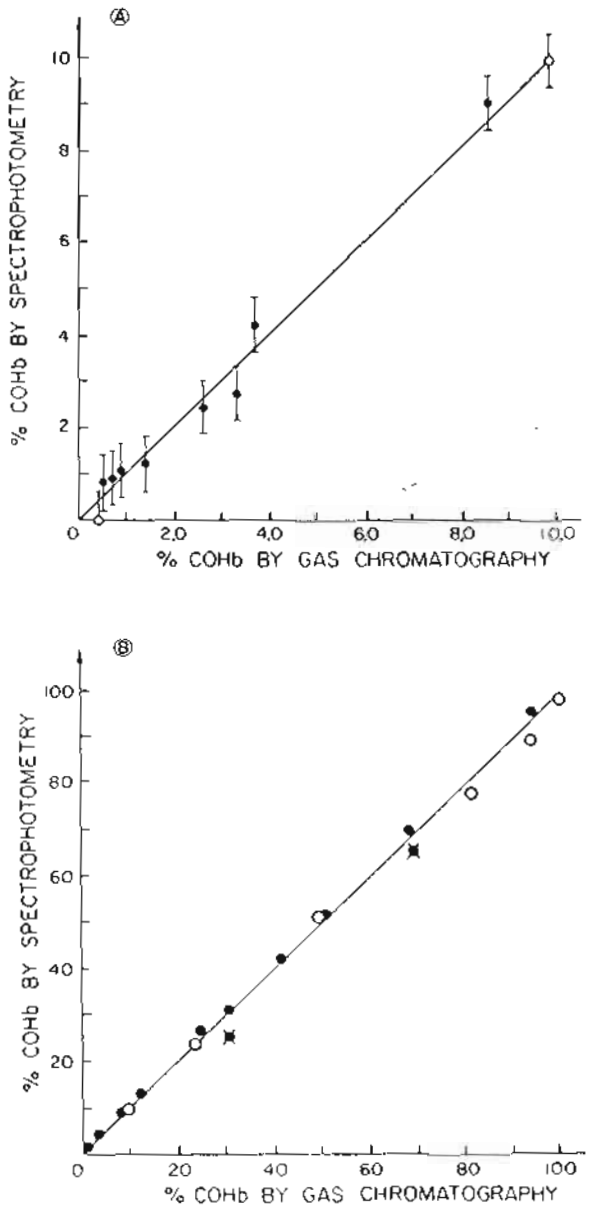

FIG. 2. Comparison of % CO Hb in human blood samples measured spectrophotometrically with measurements on the same samples by the gas chromatographic technique of Collison et al. (5). Closed circles: CO equilibrated with whole blood samples. Open circles: CO equilibrated with diluted blood samples. *A*: measurements at CO Hb levels less than 10% CO Hb. Identity line drawn at 45°, and vertical lines through points represent the estimated standard deviation of the error by the spectrophotometric method. *B*: comparison over the whole range of % CO Hb up to 100%. Low values at the two points denoted by crosses are probably due to difficulty in obtaining true total hemoglobin measurements in the gas chromatographic method. These two samples showed low hemoglobin values compared to the others done at the same time due evidently to incomplete mixing.

ANALYSIS OF ERRORS

Errors may arise from random factors, which affect the precision of our measurement, and from uncertainty in the constants used for the calculations, which affects the accuracy (this latter error we refer to as "systematic"). Random error is caused by machine variation in absorbance reading, the presence of unwanted pigments, temperature variation, dirty cuvettes, and drifting of the wavelength calibration. There are two major sources of systematic error in the molar absorbance measurement: *1*) uncertainty in the average CO Hb level of normal nonsmoking individuals, and *2*) errors associated with dispersion of the molar absorbance data due

TABLE 3. *Peak wavelength and peak molar absorbances for pure Hb pigments and various animal species at pH 10*

	Peak Wavelength, nm			± SD	Van Kampen & Zijlstra (13)	Benesch et al. (2)
	Man	Dog	Rat			
O_2 Hb	414.7	414.4	414.2	±0.5	414	415
CO Hb	420.1	419.6	420.1	±0.5	420	
Met Hb	413.2	412.7	413.5	±0.5		
Hb	431.2	430.5	430.5	±0.5	431	430
Molar absorbance, 10^5 $M^{-1}cm^{-1}$ (based on cyanmethemoglobin molar absorbance at 540 nm of 1.10×10^4 per iron)						
O_2 Hb	1.30	1.32	1.32	±0.01	1.31	1.26*
CO Hb	1.93	1.95	2.02	±0.01	1.92	
Met Hb	1.05	1.06	1.09	±0.03		
Hb	1.36	1.39	1.34	±0.02	1.40	1.34*

Data of Van Kampen and Zijlstra (13) and Benesch et al. (2) on human blood shown for comparison. * Data corrected by a factor (1.10/1.15) since these authors assumed a cyanmethemoglobin molar absorbance of 1.15×10^4 $M^{-1}cm^{-1}$ per iron.

The errors associated with dispersion of the molar absorbance data due

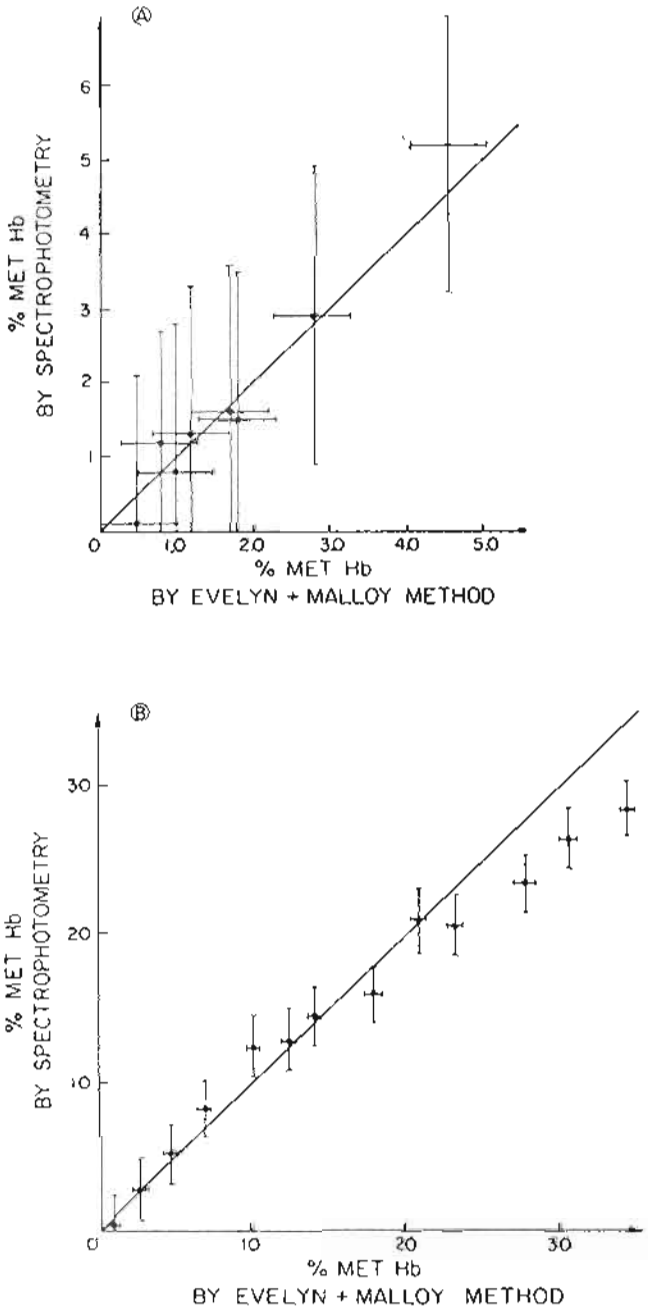

FIG. 3. Comparison of % Met Hb measured by the spectrophotometric method with the analysis by the Evelyn and Malloy technique (7). *A*: comparison for % Met Hb levels below 5%. *B*: comparison up to 30% Met Hb. Identity lines drawn at 45°, and vertical and horizontal lines indicate approximate errors of the two methods.

to variability in the spectra and uncertainty in total hemoglobin measurements. The first source of error is estimated to produce a base-line error of ±0.3% CO Hb. The second type of error results in a systematic error of ±2.5% of the measured CO Hb percent.

The specifications for our Gilford spectrophotometer give 0.002 absorbance units as the precision of the instrument. This accords with our own observations. The accuracy of the absorbance is given as 0.5% of the spectrophotometer reading, but presumably most of this error is proportional to the absorbance, which in our method just appears as a change in concentration, having no effect at all. A random error of 0.002 in each absorbance reading results in a random error of ±0.27% CO Hb in the final result, if each absorbance is measured twice.

Reduced hemoglobin, as pointed out earlier, will constitute less than 1% of the hemoglobin. It is the variation, rather than the actual amount of reduced hemoglobin, that concerns us, and we estimate the variation to be 0.15% of the total hemoglobin. In addition, we have found that an increase in temperature of the sample during measurement causes an increase in reduced hemoglobin. We limit the temperature variation to ±1.5 C, which causes variation of ±0.15% in the reduced hemoglobin. The combined effect is ±0.21% reduced. From the absorbance curves we have calculated that 1% reduced Hb causes an error of −1.0% in our measurement of CO Hb; thus the variation in amount of reduced Hb contributes ±0.21% to our error in % CO Hb. As for interference by methemoglobin, up to 5% Met Hb has a negligible effect on our CO Hb determination, and we can correct for it if it is present in larger amounts.

A wavelength shift of no more than 0.15 nm introduces an error of ±0.14% CO Hb. The effect of this error is minimized in our reported % CO Hb by averaging the two separate estimates of % CO Hb as a change in wavelength calibration raises one estimate while lowering the other. This cancellation of errors is not present in our % Met Hb estimate and therefore if high accuracy in the % Met Hb is desired, one must pay careful attention to wavelength calibration effects. We have found empirically that for a given wavelength shift at the holmium oxide peak there is an apparent wavelength shift of twice this amount at 392 nm due to nonlinearities in the optical system.

The combined effect of the sources of random variation is a standard deviation of ±0.49% CO Hb. Combined with the systematic error of ±0.3% CO Hb at CO Hb levels less than 10%, we have an overall uncertainty of ±0.55% CO Hb for low CO Hb levels.

The amount of random error actually found is somewhat less than ±0.45% CO Hb. On a large number of samples measured in duplicate, the difference between two samples is expected to have a root mean square value of $\sqrt{2}$ times the standard deviation for each measurement, or ±0.65% CO Hb. In fact, on typical runs we found ±0.35% on dogs, ±0.30% for rats, and ±0.46% for human subjects, the latter samples being done by an untrained technician. A more severe test was 8 determinations over a period of 16 days on a sample of oxygenated whole human blood. The standard deviation was ±0.28%. A similar test with dog blood gave ±0.48%. The results of our independent check with the gas chromatographic method indicate that our systematic error is indeed within ±0.3% CO Hb at low levels.

If the effect of each of the above sources of error on the methemoglobin reading is computed (except wavelength drift which may be accounted for in the calculation), the methemoglobin estimate is found to have a precision of ±2% Met Hb.

Although the errors in our spectrophotometric method are somewhat higher than in other methods, such as gas chromatography, the method is advantageous for many purposes. We have used it to monitor blood CO Hb repeatedly during experiments on small laboratory animals and for studies of human populations. The advantages of speed of analysis, small sample of blood needed, and simultaneous measurement of methemoglobin make it a desirable method for many applications.

APPENDIX 1

The basic simplicity of the method is seen if we first assume that there is no wavelength shift; $\Delta\lambda = 0$.

Let:

λ_I = 392 nm
λ_{II} = isosbestic point ~ 413 nm
λ_{III} = 421 nm
λ_{IV} = isosbestic point ~ 431 nm

The observed absorbances of the unknown sample at these wavelengths must be compared with the corresponding molar absorbances

of the pure hemoglobin species involved. Let A stand for the observed absorbance of an unknown sample and a represent the molar absorbances of pure pigments, then

$$a_{\mathrm{I\,O_2}},\ a_{\mathrm{II\,O_2}},\ a_{\mathrm{III\,O_2}},\ a_{\mathrm{IV\,O_2}} \text{ for oxyhemoglobin}$$

$$a_{\mathrm{I\,CO}},\ a_{\mathrm{II\,CO}},\ a_{\mathrm{III\,CO}},\ a_{\mathrm{IV\,CO}} \text{ for carboxyhemoglobin}$$

and

$$a_{\mathrm{I\,m}},\ a_{\mathrm{II\,m}},\ a_{\mathrm{III\,m}},\ a_{\mathrm{IV\,m}} \text{ for methemoglobin}$$

are the molar absorbances at the indicated wavelengths.

Note that at the isosbestic points:

$$a_{\mathrm{II\,O_2}} = a_{\mathrm{II\,CO}} \quad \text{and} \quad a_{\mathrm{IV\,O_2}} = a_{\mathrm{IV\,CO}}$$

According to the Beer-Lambert Law, the absorbance of each component in a mixture is proportional to its concentration. If x = fraction CO Hb and y = fraction Met Hb, then $1 - x - y$ is the fraction O_2 Hb (if no other species of hemoglobin are present), all fractions being of total pigment.

The observed absorbance is

$$A_{\mathrm{I}} = Kl[a_{\mathrm{I\,O_2}}(1 - x - y) + a_{\mathrm{I\,CO}}x + a_{\mathrm{I\,m}}y] \qquad (1)$$

or

$$A_{\mathrm{I}} = Kl[a_{\mathrm{I\,O_2}} + (a_{\mathrm{I\,CO}} - a_{\mathrm{I\,O_2}})x + (a_{\mathrm{I\,m}} - a_{\mathrm{I\,O_2}})y] \qquad (2)$$

where K is the molar hemoglobin concentration and l is the pathlength (in cm).

To eliminate the necessity of measuring hemoglobin concentration, we take the ratios of the observed absorbances:

$$R_1 = \frac{A_{\mathrm{II}}}{A_{\mathrm{I}}} = \frac{a_{\mathrm{II\,O_2}} + (a_{\mathrm{II\,CO}} - a_{\mathrm{II\,O_2}})x + (a_{\mathrm{II\,m}} - a_{\mathrm{II\,O_2}})y}{a_{\mathrm{I\,O_2}} + (a_{\mathrm{I\,CO}} - a_{\mathrm{I\,O_2}})x + (a_{\mathrm{I\,m}} - a_{\mathrm{I\,O_2}})y} \qquad (3)$$

$$R_2 = \frac{A_{\mathrm{III}}}{A_{\mathrm{II}}} = \frac{a_{\mathrm{III\,O_2}} + (a_{\mathrm{III\,CO}} - a_{\mathrm{III\,O_2}})x + (a_{\mathrm{III\,m}} - a_{\mathrm{III\,O_2}})y}{a_{\mathrm{II\,O_2}} + (a_{\mathrm{II\,CO}} - a_{\mathrm{II\,O_2}})x + (a_{\mathrm{II\,m}} - a_{\mathrm{II\,O_2}})y} \qquad (4)$$

and

$$R_3 = \frac{A_{\mathrm{III}}}{A_{\mathrm{IV}}} = \frac{a_{\mathrm{III\,O_2}} + (a_{\mathrm{III\,CO}} - a_{\mathrm{III\,O_2}})x + (a_{\mathrm{III\,m}} - a_{\mathrm{III\,O_2}})y}{a_{\mathrm{IV\,O_2}} + (a_{\mathrm{IV\,CO}} - a_{\mathrm{IV\,O_2}})x + (a_{\mathrm{IV\,m}} - a_{\mathrm{IV\,O_2}})y} \qquad (5)$$

Note there are only three independent ratios.

Our data analysis procedure is to solve *equations 3* and *5* simultaneously for x and y which we call x_1 and y_1. Next we substitute the % Met Hb estimate y_1 into *equation 4* and again solve for x giving x_2; x_1 and x_2 obtained in this manner are two independent estimates of the % CO Hb, we report their average as the % CO Hb saturation. Finally we resubstitute this average x into *equation 3* and solve for y giving our reported % Met Hb.

The rationale for this procedure is that R_2 and R_3 are most sensitive to CO Hb, hence our reported value of % CO Hb is essentially based on these two estimates. Furthermore, the averaging of x_1 and x_2 tends to cancel errors due to wavelength drift and the presence of reduced Hb. The reported value of % Met Hb is based on R_1 which is most sensitive to Met Hb and least sensitive to wavelength drift, an important point since we only have a single estimate for % Met Hb.

To take into consideration the wavelength shift, we merely replace the molar absorbances by:

$$a_\lambda \rightarrow a_\lambda + \sigma_\lambda \Delta\lambda$$

where σ_λ is the slope of the pure spectrum ($da/d\lambda$, see Table 2) at λ_1 and $\Delta\lambda$ is the wavelength shift as measured by the hohlraum peak at 446.10 nm. If additional accuracy (better than ±2%) of the % Met Hb measurement is desired, careful analysis of the wavelength drift effect must be considered. In our work we merely take the % Met Hb determination least sensitive to wavelength drift as our reported value.

APPENDIX 2

When whole blood is diluted with water containing dissolved air, the ratios of various forms of hemoglobin will adjust to be in equilibrium with the partial pressures of the dissolved gases. For instance, some dissolved oxygen will combine with reduced hemoglobin, as explained in the text. If carboxyhemoglobin is present in the blood, some carbon monoxide will dissociate and go into solution.

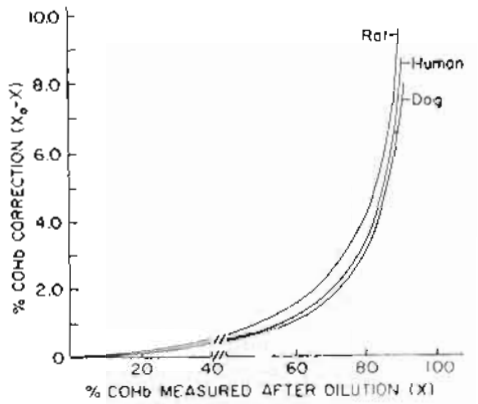

FIG. 4. Correction factor by which % CO Hb measured on diluted blood must be adjusted to take into account loss of CO on dilution. Curves show results for human, dog, and rat blood with Haldane constants given in text. O_2 and CO capacity, f, is assumed to be 0.2 ml STP/ml whole blood. At values of % CO Hb less than 40% the dilution factor n is 1:70, whereas at high values n is 1:100.

The latter process is described by Haldane's law, which states that the ratio of carboxy- to oxyhemoglobin is the same as the ratio of partial pressures of CO and O_2, multiplied by a constant m, which represents the relative affinities of blood for CO and O_2. Values of m are on the order of 230 in vivo, but have not been studied under our conditions, namely, dilute solutions at a pH of 10 and a temperature of 22 C. For this reason we made rough measurements ourselves on dog and rat blood and found a value of 305 for dog and 230 for rat blood in diluted samples. We assume that m for human blood under our conditions is approximately 280, as reported by Rodkey et al. (12) for concentrated solutions at 22 C and pH 8.8. In fact, the precise value of m affects this calculation relatively little, as can be seen from Fig. 4 where the correction described below has been plotted for rat, dog, and human blood.

We will assume that only two forms of hemoglobin are present, O_2 Hb, and CO Hb, and that the O_2 and CO capacity of the blood is f ml STP/ml blood. Up to 30% reduced hemoglobin in the initial sample leads to no significant difference in the result and the very small partial pressure of ammonia is negligible.

Let:

x = fraction CO Hb (after dilution and with equilibrium)
x_0 = fraction CO Hb initially before dilution
p = volume CO dissolved per volume of water (after reaching equilibrium)
q = volume O_2 dissolved per volume of water (after reaching equilibrium)
q_0 = volume O_2 dissolved per volume of water initially, before blood added

Note that:

$1 - x$ = fraction O_2 Hb after dilution
$1 - x_0$ = fraction O_2 Hb initially before dilution

The quantity we measure is x, and x_0 is what we want to determine; q_0 is known from the solubility of O_2, and f can either be assumed to be 0.2 or estimated from the isosbestic absorbances. We then have three unknowns (x_0, p, q) and can write three equations. The first is Haldane's law, the other two are simply statements that the total amount of each gas is the same after equilibrium is reached as before. If S_{CO} and S_{O_2} are the solubility of CO and O_2 respectively, in volumes of gas (STP) per volume water at 22 C, then Haldane's law is:

$$\frac{x}{1 - x} = m\frac{p/S_{CO}}{q/S_{O_2}}$$

For a dilution of one part blood to n parts water, the material balance equations for CO and O_2 per volume of blood are:

$$fx + np \quad fx_0$$

$$f(1 - x) + nq \quad f(1 - x_0) + nq_0$$

The elimination of p and q from these three equations gives

$$x_0 - x = \frac{nq_0}{f} \cdot \frac{1}{1 + m \dfrac{So_2}{Sco} \cdot \dfrac{1 - x}{x}}$$

The ratio of the solubilities at 22 C is $So_2/Sco = 1.33$. The amount of dissolved oxygen in the diluent q_0 is equal to $0.21 \times So_2 = 0.00625$ v/v when ambient O_2 is 0.21 atm. The equation for the change in the fraction CO Hb is then

$$x_0 - x = \frac{0.00625n}{f\left(1 + 1.33n \dfrac{1 - x}{x}\right)}$$

Ordinarily the dilution we use is 1:70, i.e., $n = 70$. However, for very high CO saturation we have to use a more dilute solution in order to keep the absorbance at 421 nm within the linear range of the spectrophotometer, so n may be as high as 100. The absorbances at the isosbestic points (413 and 431 nm) may be used to determine n/f, then the correction $(x_0 - x)$ can be calculated from the measured value of x. The magnitude of the correction for f 0.2 (normal blood capacity) is shown in Fig. 4; it is evident that the correction is relatively unimportant for CO Hb saturations less than 40% when the dilution is 1:70, and can be ignored when the percent CO is less than 10%.

We are greatly indebted to James Kaufman, Eve Kresin, and Robert Friedman, without whose technical assistance this work would not have been possible.

This work was supported in part from Grant ES-00454 from the National Institute of Environmental Health Sciences and Grant HE-10342 from the National Heart and Lung Institute.

The research was supported in part by the Bureau of Medicine and Surgery, Navy Department, Research Task M4300.02.40308. The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

Reprints may be obtained from E. P. Radford, Dept. of Environmental Medicine, The Johns Hopkins University, School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, Md. 21205.

Present address of K. A. Small: Dept. of Physics, University of California, Berkeley, Calif. 94720.

Received for publication 8 February 1971.

REFERENCES

1. Austin, J. H., and D. L. Drabkin. Spectrophotometric studies. III. Methemoglobin. *J. Biol. Chem.* 112: 67–88, 1935.
2. Benesch, R., R. E. Benesch, and G. MacDuff. Spectra of deoxygenated hemoglobin in the Soret region. *Science* 144: 68–69, 1964.
3. Blackmore, D. J. The determination of carbon monoxide in blood and tissue. *Analyst* 95: 439–458, 1970.
4. Coburn, R. F., G. K. Danielson, W. S. Blakemore, and R. E. Forster II. Carbon monoxide in blood: analytical method and sources of error. *J. Appl. Physiol.* 19: 510–515, 1964.
5. Collison, H. A., F. L. Rodkey, and J. D. O'Neal. Determination of carbon monoxide in blood by gas chromatography. *Clin. Chem.* 14: 162–171, 1968.
6. Commins, B. T., and P. J. Lawther. A sensitive method for the determination of carboxyhemoglobin in a finger prick sample of blood. *Brit. J. Ind. Med.* 22: 139–143, 1965.
7. Evelyn, K. A., and H. T. Malloy. Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. *J. Biol. Chem.* 126: 655–662, 1938.
8. Forster, R. E. Techniques for the analysis of carbon monoxide. In: *Effects of Chronic Exposure to Low Levels of Carbon Monoxide on Human Health, Behavior, and Performance*. Washington, D.C.: Natl. Acad. Sci.-Natl. Res. Council, 1969, p. 61–65.
9. Maas, A. H. J., M. L. Hamelink, and R. J. M. de Leeuw. An evaluation of the spectrophotometric determination of HbO_2, Hb CO, and Hb in blood with the CO oximeter IL 182. *Clin. Chim. Acta* 29: 303–309, 1970.
10. Paul, K., and H. Theorell. A colorimetric carbon-monoxide-hemoglobin method of determination for clinical use. *Acta Physiol. Scand.* 4: 285–292, 1942.
11. Richterich, R. *Clinical Chemistry, Theory and Practice*. Basel, Switzerland: Karger, 1969, p. 333–335.
12. Rodkey, F. L., J. D. O'Neal, and H. A. Collison. Oxygen and carbon monoxide equilibria of human adult hemoglobin at atmospheric and elevated pressure. *Blood* 33: 57–65, 1969.
13. Van Assendelft, O. W., and W. G. Zijlstra. The formation of haemiglobin using nitrites. *Clin. Chim. Acta* 11: 571–577, 1965.
14. Van Kampen, E. J., and W. G. Zijlstra. Determination of hemoglobin and its derivatives. *Advan. Clin. Chem.* 8: 141–187, 1965.
15. Van Slyke, D. D., and H. A. Salvesen. The determination of carbon monoxide in blood. *J. Biol. Chem.* 40: 103–125, 1919.