A Modification of the Spectrophotometric Method for Determining Oxyhemoglobin Affinity*

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The role of hemoglobin as a carrier of oxygen in the blood was established in 1859 by Hoppe-Seyler (12). Bert (3, 4), using whole blood, furnished experimental data whereby an oxyhemoglobin affinity curve could be plotted. In 1886, C. Bohr published data on purified hemoglobin for the second curve of oxyhemoglobin affinity (23). By 1903, Bohr had established that the oxyhemoglobin affinity curve was S-shaped for both whole blood and hemoglobin in solution. Since that time, various methods have been reported, or modifications thereof, to estimate oxyhemoglobin affinity (2, 14, 15, 16, 18, 19, 24, 25, 26, 27).

The curve resulting from a plot of the degree of hemoglobin saturation as a function of ambient oxygen pressure is conventionally described in terms of the empirical equation derived by Hill (17),

$$\frac{y}{100} = \frac{KP^n}{1 + KP^n}$$

where y is the percent saturation of hemoglobin, P is the partial pressure of oxygen, and K and n are constants. But as noted by Adair (1), this equation does not hold for all values of y. Deviation from the Hill approximation occurs when there are negative interactions between hemes and during progressive oxygenation of the molecule leading to a marked change in the strength of interaction, (21). The interactions of the hemes account for the sigmoid oxyhemoglobin affinity curve.

There are two shape parameters defined by the Hill equation, n and k. Called the sigmoid coefficient (11), because it denotes the degree of departure of the curve from a rectangular hyperbola, n has been shown from thermodynamic studies to be closely related to the average free energy of interaction of the oxygen-binding sites on the same molecule (29). The value of n can be estimated from a logarithmic plot of the Hill equation. From the equation it can be shown that

$$\log \frac{y}{100 - y} = \log K + n \log P$$

so that a plot of

$$\log \frac{y}{100 - y}$$

as a function of

$$\log P$$

yields a straight line in the range over which the equation holds; the slope of the line at the point of half-saturation (where $y = 50\% = P_{50}$), is n.

If $n = 1$, the sites are independent of each other; if $n > 1$, positive interactions between binding sites are indicated; if $n < 1$, the interactions are negative and the Hill equation fits the data poorly (22).

Frequently called the equilibrium constant, K can be shown by algebraic rearrangement of the Hill equation to be equal to $(1/P_{50})^n$ where P_{50} is the oxygen tension at which the hemoglobin is 50% saturated. The P_{50} is usually reported instead of K because it can be easily estimated from the plot of experimental values of oxygen tension and percentage hemoglobin saturation. A P_{50} value has physiological significance (20) as the unloading tension occurring in blood capillaries when oxygen is unloaded to tissue cells. The P_{50} value is also used to indicate the relative affinity of hemoglobin for oxygen. Hemoglobin which remains highly saturated or does not give up its oxygen at low oxygen tensions (a low P_{50}) has a high affinity for oxygen; the converse is also true. Therefore, the oxygen affinity of hemoglobin, or another respiratory pig-

* Supported by National Institute of Health Grant HE 08774.

ment, can under many conditions, be quite accurately represented by the Hill approximation as

$$\frac{y}{100} = \frac{\left(\frac{P}{P_{50}}\right)^n}{1 + \left(\frac{P}{P_{50}}\right)^n}$$

Description of the spectrophotometric method and modification. I would like to describe a modification (9) of the spectrophotometric method (7, 10) for determining an oxyhemoglobin affinity curve on hemoglobin solutions. The essential point in this method is that a buffered hemoglobin solution is prepared (6), placed in a tonometer (separatory funnel) attached to a cuvette via a rubber stopper, equilibrated with 100% oxygen, and placed in a spectrophotometer for an optical density reading. Subsequently, the tonometer is evacuated at intervals. At each interval, after an equilibration, an O. D. reading is made. Ultimately, the hemoglobin solution is reduced with sodium hydrosulfite or by nitrogen equilibration, and the final O. D. reading is made. Oxyhemoglobin saturation and PO_2 are then calculated (7). Other factors which affect oxyhemoglobin affinity, such as ionic strength of salt solutions, pH, carbon dioxide tension and temperature, must be kept constant. In this way, points for the construction of an oxyhemoglobin affinity curve can be obtained by varying only the PO_2.

The major difference between the spectrophotometric method and the modification described below is that a hemoglobin sample is removed at intervals from the tonometer and placed in the microgasometer for a BOC determination instead of putting the sample in the spectrophotometer to measure the percentage of oxyhemoglobin saturation.

There was some difficulty in transferring a hemoglobin solution from the tonometer, at intervals of evacuation, to the microgasometer. This difficulty was overcome by the construction and use of the adapter chamber illustrated in figure 1. The adapter consists of two 2-way stopcocks fused together to form a chamber between them to hold about 0.5 to 1 ml of solution.

The procedure in the modified method is as follows. The prepared hemoglobin solution is placed in the tonometer exactly as described for the spectrophotometric method. The adapter (fig. 1) is attached via its rubber stopper to the tonometer in place of the cuvette. Both stopcocks are closed. After equilibration 39.3 cmm (13) of solution, under oil, is removed from the tonometer with a micropipette. To do this, the lower stopcock is

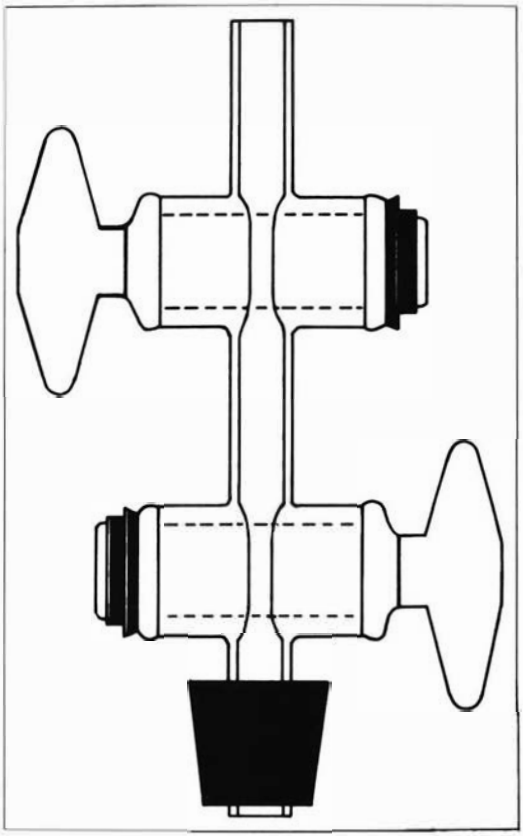

Fig. 1—The adapter is shown to consist of 2 two-way stopcocks fused together so that a chamber is formed between them having a capacity of 0.5 to 1 ml of hemoglobin solution.

opened and the tonometer is inverted gently to allow the solution to fill the chamber. The lower stopcock is then closed, and the upper stopcock is opened for pipette access to the chamber. After the sample is removed, the upper stopcock is closed and the lower one opened to allow the hemoglobin solution to flow back into the tonometer. The lower stopcock is closed in preparation for the next evacuation and equilibration. The hemoglobin solution in the micropipette is transferred, under oil, to the capillary cup of a microgasometer (28) for a BOC determination. The BOC analysis is as easy to make for hemoglobin solution as it is for the blood of various vertebrates (8), and the time for an analysis is shortened from 12 to about 7 minutes since equilibration is done in the tonometer.

Results. Hemoglobin solutions for oxyhemoglobin analysis were prepared from pooled blood samples which were collected in the MCV Clinic

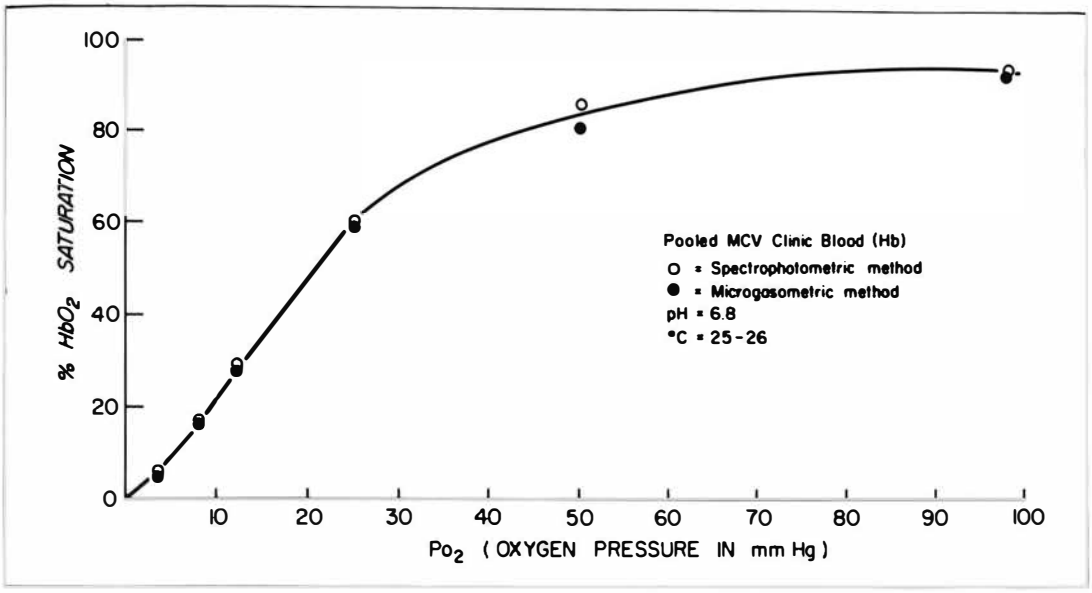

Fig. 2—An oxyhemoglobin affinity curve is shown as determined by both the spectrophotometric and the microgasometric methods. The data plotted were obtained on a 3% human hemoglobin solution. The analyses were made on aliquots of the same sample.

and kindly furnished by Dr. Lyman Fisher. The oxyhemoglobin affinity curve plotted in figure 2 represents a typical analysis by each of the two methods, determined simultaneously. The data were treated statistically for a closeness of fit. The coefficient of correlation (r) for the two methods is 0.99 ($P < 0.001$), and the slope of the line (b) is 0.95.

From these data it may be concluded that the microgasometric method yields results which are as accurate and precise as those obtained using the spectrophotometric method. In addition, there is a reduction of time and cost factors when the inexpensive microgasometer is substituted for the spectrophotometer in measuring oxyhemoglobin affinity at various PO_2's. The substitution is feasible when the adapter described is used in the transfer of the hemoglobin solution from the tonometer to the microgasometer.

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