

# A Simple Rapid Method for Determining Oxyhemoglobin Affinity: Illustration Using Blood from the Rhesus Monkey\*

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The oxyhemoglobin affinity curve, also known as the "oxygen dissociation curve," "oxyhemoglobin equilibrium curve," or "oxygen-hemoglobin dissociation curve," describes the qualitative relation between oxygen pressure and oxygen uptake or release by hemoglobin. If oxygen pressure ( $P_{O_2}$ ) is measured in mm Hg, and oxygen uptake is measured as percent saturation of hemoglobin with oxygen, an oxyhemoglobin affinity curve can be constructed by varying only the  $P_{O_2}$ . Of course, other factors which affect the affinity of hemoglobin for oxygen, such as salt ionic strength, pH, carbon dioxide tension, and temperature, must be kept constant. The curve is obtained by plotting the percent saturation of hemoglobin with oxygen (ordinate) against the oxygen pressure (abscissa), and drawing a best-fit line through the points measured. A preliminary report on the spectrophotometric method to be described was made by Burke and Powell (1962), and the procedure was mentioned in a recent paper (Burke, 1965). Although the procedure has also been used in the teaching laboratory for first-year medical students at the Medical College of Virginia, it has not been published in detail. Adaptations and modifications have been made from methods described pre-

viously by Hall (1935), Riggs (1951), Redmond (1955), Rossi-Fanelli and Antonini (1958), and in personal communication with Dr. Clyde Manwell at the University of Illinois, Urbana. As many as eight points on the curve can be determined in less than two hours.

## Description of Apparatus

The set-up (*see* fig. 1) is as follows: a three-way stopcock is connected to a one-meter manometer (filled with mercury to the 500 mm mark), and a vacuum pump with a five-gallon carboy is placed between the pump and stopcock as a compensator-trap. Optical density readings are made in specially constructed tonometers in a spectrophotometer such as the Coleman, Jr. The tonometer is a 125 ml separatory funnel connected to a cuvette by a rubber stopper. When in use the stopper is affixed to the open end of the funnel which has a light coating of high-pressure vacuum grease on the outer rim only. An epoxy resin is used to seal the glass cuvette to the rubber stopper. With the stopcock on the funnel closed and the tonometer in place on one opening of the three-way stopcock, a partial vacuum can be pulled on the tonometer, and the oxygen pressure can be measured by reading the manometer. With the apparatus in position, an oxyhemoglobin affinity can now be determined.

## Procedure

### *Preparation of Hemoglobin Solution*

1. Centrifuge 5 to 10 ml of heparinized blood in a 15 ml centrifuge tube for five minutes at 3,000 rpm. Aspirate the plasma without disturbing the cells. Now add cold, physiological saline solution to the 15 ml mark on the tube, and mix gently. Place the tube in the centrifuge, and spin it for five minutes at 3,000 rpm. Repeat washing twice.

2. Hemolyze the washed cells by adding two parts cold distilled water, and 1 ml of toluene. Stopper the tube, and mix gently for several minutes. Now place the tube with the hemolysate in it in the centrifuge and spin at 5,000 rpm for five minutes. Aspirate the clear and buffy layers above the red hemolysate, and filter the hemolysate. Centrifuge the filtered hemolysate for one hour in a refrigerated centrifuge at  $20,000 \times g$ . If a refrigerated centrifuge is not available, use the highest speed possible on the available centrifuge; do not centrifuge for a prolonged period, however, or the hemoglobin will be denatured. Keep the hemolysate refrigerated until it is used. Hemoglobin prepared in this manner may also be used to "spot" in electrophoresis as well as in determining an oxyhemoglobin affinity curve.

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*Oxyhemoglobin Affinity Curve*

1. Place 1.0 ml of the hemoglobin (Hb) hemolysate (1 to 2 g% Hb) in a 15 ml centrifuge tube. Add 3.0 ml of phosphate buffer:

a. Buffered sample of 6.8 pH and ionic strength ( $\mu$ ) of 0.3: Add 24.98 g potassium phosphate, monobasic ( $\text{KH}_2\text{HPO}_4$ ), to 12.56 g potassium phosphate, dibasic ( $\text{K}_2\text{HPO}_4$ ), and make up to one liter with water. Mix hemolysate 1:3 (v/v) with buffer.

b. Buffered sample of 7.4 pH and ionic strength ( $\mu$ ) of 0.3: Add 9.58 g potassium phosphate, monobasic, to 19.13 g potassium phosphate, dibasic, and make up to one liter with water. Mix hemolysate 1:3 (v/v) with buffer.

2. Pipette 4.0 ml of the buffered hemolysate into a 125 ml separatory funnel (with stopcock closed). Attach the stopper which was affixed to the spectrophotometric cuvette to the open end of the funnel.

3. Open the stopcock on the tonometer to room air, or 100% oxygen (water-washed). Equilibrate the buffered hemoglobin solution for five minutes with gentle shaking in a water bath at the desired temperature, or use a platform shaker without a water bath for room temperature determinations. Allow the solution to flow back carefully into the cuvette, close the stopcock, and read the optical density (absorbency =  $A$ ) on the spectrophotometer at a wave-length of 640  $\text{m}\mu$ . (This wave-length setting is obtained by previously making an absorbency plot for oxyhemoglobin, and one for reduced hemoglobin solution, and then determining by inspection where a large difference occurs between the two hemoglobin plots at a point on the graph. For mammal blood this is usually at a wave-length of 640  $\text{m}\mu$ ). The first reading is taken as 100% oxygenation and is represented as  $A_o$ . The tonometer must

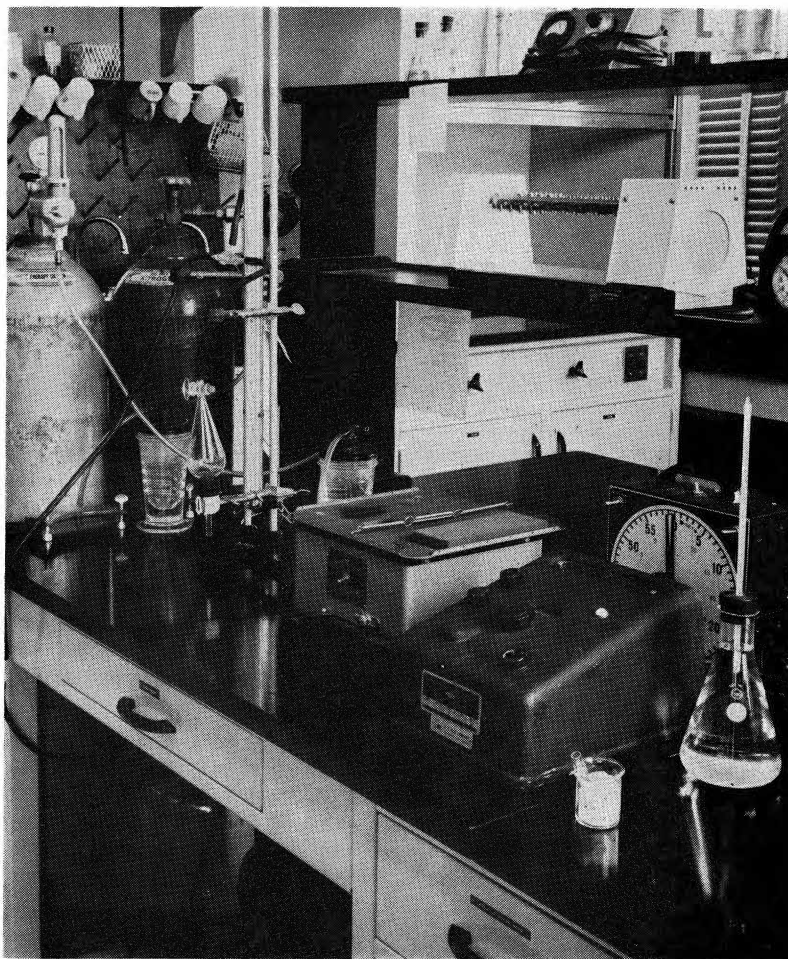


Fig. 1—Apparatus for determining the affinity of oxygen for hemoglobin at different oxygen pressures. From left to right are tanks of oxygen and nitrogen, connected individually to a gas-washing bottle; mercury manometer attached to three-way stopcock, leading to tonometer and vacuum pump via five-gallon carboy seen below the table; platform shaker and timer; spectrophotometer; and temperature-equilibrating bath which holds the tonometer prior to a spectrophotometric reading.

be oriented in the holder of the spectrophotometer in the same manner for each subsequent reading; e.g., if the stopcock was aligned to the right, it should be read each time in that position. Do not leave the tonometer in the spectrophotometer any longer than it takes to record a reading, since heat too will denature hemoglobin. In taking readings, it may be more convenient to read percent transmittance ( $\%T$ ) and convert it to optical density in the calculations.

4. Evacuation of the tonometer is done with the pump and mercury

manometer apparatus described above. Connect the manometer to the tonometer with the stopcock closed; slowly open the stopcock on the tonometer until the left side of the manometer reads about 650 mm Hg. This corresponds to a  $P_{\text{O}_2}$  approximately 85 to 90 mm Hg. Close the stopcock of the tonometer, place it on the platform shaker and equilibrate it for five minutes. Now put it in the spectrophotometer and read the optical density as  $A_s$ . Remove the tonometer, open the stopcock to room air, and then connect it to the manometer for

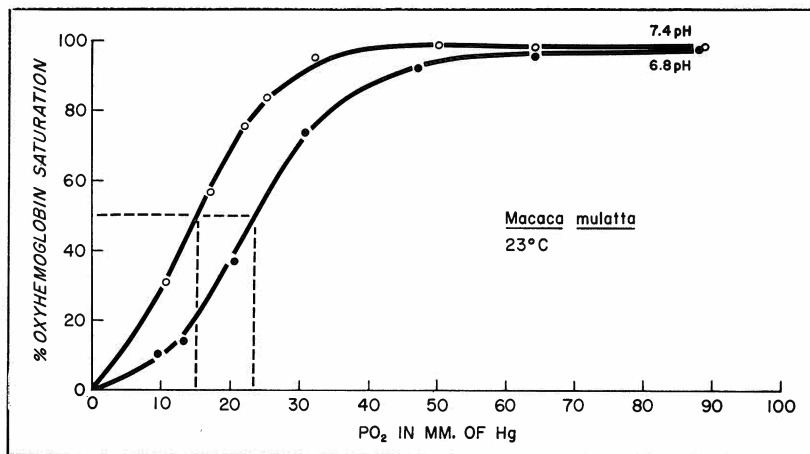


Fig. 2—Oxyhemoglobin affinity at pH 7.4 and 6.8 for the Rhesus monkey (*Macaca mulatta*). Data for both curves were obtained at 23 C. The P<sub>50</sub> is 15 mm Hg at pH 7.4, and 23 mm Hg at pH 6.8.

another evacuation at a lower P<sub>O<sub>2</sub></sub>.

5. Repeat the above procedure for P<sub>O<sub>2</sub></sub>'s of approximately one-half for each successive reading, e.g., 40, 20, 10, 5, and 2 mm Hg in order that the points may be spread over the curve.

6. After the last P<sub>O<sub>2</sub></sub> value has been obtained, the pigment should be deoxygenated (reduced) completely. Close the three-way stopcock, and evacuate the manometer to the full extent of the pump. Now connect the tonometer to the three-way stopcock, and evacuate the tonometer. Be cautious here because prolonged evacuation might lead to loss of water with consequent increase in Hb concentration. Disconnect the tonometer, equilibrate for five minutes, and read the optical density as *A<sub>r</sub>*.

7. The percent saturation of hemoglobin with oxygen (*S*) is calculated as:  $S/100 = (A_r - A_s) / (A_r - A_o)$ , where *A<sub>r</sub>*, *A<sub>s</sub>*, and *A<sub>o</sub>* represent, respectively, deoxygenated (reduced), partially oxygenated, and fully oxygenated hemoglobin.

8. The P<sub>O<sub>2</sub></sub> (oxygen pressure in mm Hg) is calculated as:  $P_{O_2} = (\text{barometer reading} - \text{manometer reading} - \text{vapor pressure of water}) \times 0.2094$ . (Note: the manometer reading is made by subtracting the

reading of the right arm from the left arm.)

Data are presented in fig. 2 for oxyhemoglobin affinity of the Rhesus monkey (*Macaca mulatta*) as an example of applying the procedure described above. The curves were derived from determinations made at pH 7.4 and 6.8 at 23 C. The oxygen pressure at 50% saturation of hemoglobin with oxygen (P<sub>50</sub>) was 15 mm Hg at the higher pH, and 23 mm Hg at the lower pH. Samples were run simultaneously in two different tonometers. The total time for making the determinations and plotting the curves was less than three hours.

### Summary

Blood was obtained by cardiac puncture from the Rhesus monkey (*Macaca mulatta*). Hemolysates were prepared by filtration and centrifugation at 20,000 × g at 4 C. Oxyhemoglobin affinity curves were determined by a simplified spectrophotometric procedure on hemolysates diluted with phosphate buffers at pH of 7.4 and 6.8, and at an ionic strength of 0.3. The spectrophotometric readings were made at a wavelength of 640 mμ and at a temperature of 23 C. The oxygen pressure at one-half saturation

(P<sub>50</sub>) for Rhesus hemoglobin at a pH of 7.4 was 15 mm Hg, and at the more acid pH of 6.8, 23 mm Hg. Using this spectrophotometric method, as many as eight points can be obtained and plotted, so that an oxyhemoglobin affinity curve can be constructed, in less than three hours.

### References

- BURKE, J. D. Oxygen affinities and electrophoretic patterns of hemoglobins in trout and basses from Virginia. *Med. Coll. of Virginia Quart.* 1 (1): 16–21, 1965.
- BURKE, J. D., AND W. A. POWELL. A spectrophotometric procedure for the determination of oxyhemoglobin affinity curves. *Virginia J. Sci.* 13: 243, 1962 (abstr).
- HALL, F. G. A spectro-comparator for the study of hemoglobin. *J. Elisha Mitchell Sci. Soc.* 51: 289–293, 1935.
- REDMOND, J. R. The respiratory function of hemocyanin in crustacea. *J. Cellular Comp. Physiol.* 46: 209–247, 1955.
- RIGGS, A. The metamorphosis of hemoglobin in the bullfrog. *J. Gen. Physiol.* 35: 23–40, 1951.
- ROSSI-FANELLI, A., AND E. ANTONINI. Studies on the oxygen and carbon monoxide equilibria of human myoglobin. *Arch. Biochem. Biophys.* 77: 478–492, 1958.