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A Comparison of Some Methods for Determining Oxygen Dissociation Curves of Fish Blood

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A brief description is given of four different methods for determining the oxygen dissociation curve of trout blood at *p*H 7.8 and 15° C. A comparison of the results obtained indicates that the methods give similar results, especially when used by experienced workers. The relative advantages and disadvantages of the methods for determination of some O_2-carrying parameters of fish blood are discussed.

During recent years great interest in the comparative study of the respiratory properties of vertebrate blood (1, 6, 7) has led to the development of methods which are particularly suited to overcome special problems found in organisms which are generally smaller than man. Similar requirements are attached to the study of developing mammals (10) and such problems are further accentuated in studies during the development of fish.

The present study compares four methods which have been used frequently with fish blood. For such studies the amount of blood available is usually small and the presence of nucleated erythrocytes with relatively high metabolic rate even at low temperatures have led to a number of problems, particularly because of possible changes in the properties of blood following its withdrawal from the animal (5). Fish blood often clots more readily than most mammalian blood (4). P_{50}'s are usually lower that those found in birds and mammals and the Bohr shift is generally much greater. In addition, the O_2 capacity of fully oxygenated blood is reduced at low *p*H (Root effect).

Each of the methods described in this paper has advantages and disadvantages but in the hands of an experienced user all four give sufficiently consistent results. Complete consistency is not expected to be as great as in many higher vertebrates because of variation between different individuals of a species and indeed there is evidence for variations in a given individual at different times.

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Materials and Methods

Blood samples were taken from rainbow trout *(Salmo gairdneri)* of about 100 g, obtained from the hatchery at Nailsworth, Glos., and kept in laboratory holding tanks at a temperature of 15±1° C. For all the methods blood was obtained in the same way using cannulae in the dorsal aorta (11). Following cannulation, fish were allowed to recover for at least 24 hours before samples of up to 2.5 ml blood were withdrawn. The cannulae and syringes used in sampling were heparinized. Blood was immediately placed in tonometers for equilibration with different gas mixtures. Where it was necessary to store the blood for short periods, this was in a refrigerator at 4° C, in sealed syringes. It was found that gradual acidification of the sample occurred and this was probably related to anaerobic metabolism of the erythrocyte.

For two of the methods, it was necessary to equilibrate the blood in microtonometers with known gas mixtures before determinations of either the O_2 content (Tucker method) or the percentage oxy-haemoglobin (spectrophotometric method). For the other two methods, measurements were made of the P_{O_2} in blood samples of known O_2 saturation. All measurements were made at 15° C and the data corrected to *p*H 7.8.

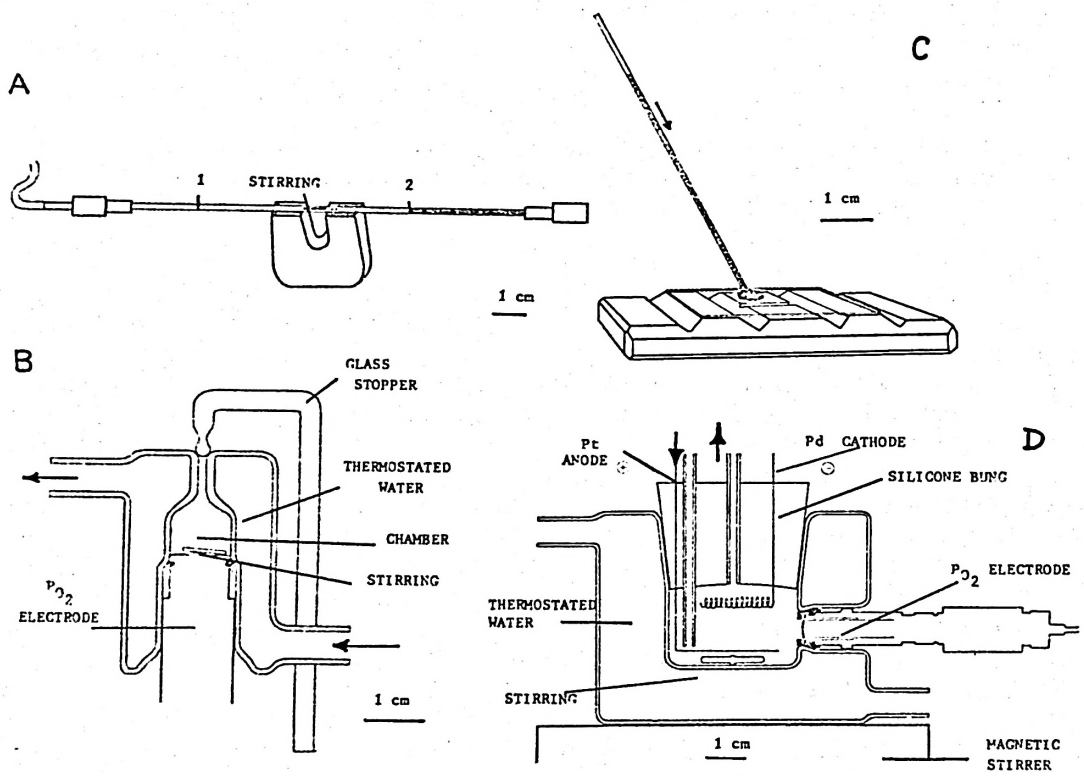

Fig. 1. *Diagrams indicating the basic equipment used in the four methods.* (A) A capillary tube which has been marked (1) to show 10 cm length and (2) 4 cm length in order that a P_{40} can be determined. (B) Chamber used for measurement of O_2 content of a 10 μl blood sample pipetted into the central chamber. (C) Cuvette for the oximeter. (D) Chamber used for electrolytic method showing the position of O_2 electrode in the side.

It should be emphasized that no claim to originality of the four methods used is made, although some small practical details doubtless varied from those originally described. However, there seems to have been no previous comparison of these methods by the same investigators using the same animal species.

Method A. Mixing method. Two samples of blood were equilibrated in small tonometers at 15° C with air and nitrogen (100 %); these gases were saturated with water vapour in humidifiers before entering the tonometers. Equilibration time for obtaining fully oxygenated and reduced blood was about 1/2 h, but in certain instances with small volumes of blood this could be as short as 15 min. Samples from each of the two tonometers were drawn into small glass capillaries (about 100 microlitres) which were marked on the side of the tube (fig. 1a). Thus if P_{40} is required, 6 parts of deoxygenated blood would be drawn into the capillary followed by 4 parts of oxygenated blood. Mixing within the capillary is carried out by vigorous movement along the capillary of a small piece of steel using a magnet to give a homogenous mixture. This process is achieved quite rapidly and the mixture is then injected into the cuvette of a P_{O_2} electrode. The *p*H of the mixture is determined either simultaneously or following its withdrawal from the P_{O_2} cuvette. Because of differences in the *p*H of the different mixtures (due to different P_{CO_2} of air and N_2 used), some correction had to be made for the Bohr effect. The Bohr factor was calculated by determining the P_{50} using deoxygenated blood equilibrated with N_2, followed by the use of a mixture containing 10 % CO_2 and 90 % N_2. P_{50} and *p*H were measured and Bohr factor ($= \Delta \log P_{50}/\Delta\, p\mathrm{H}$) obtained.

Method B. Tucker method. The procedure has been described in detail by TUCKER (12). Blood samples are equilibrated in tonometers using gas mixtures containing different proportions of oxygen and nitrogen. These can be either from standard cylinders or from gas mixing pumps, etc. The composition of the mixtures was checked with a Scholander 0.5 ml gas analyser. If possible, P_{CO_2} is kept constant. All samples were equilibrated for about 1/2 h at 15° C and the gas mixtures were previously saturated with water vapour.

10 μl samples of this blood were taken and inserted through the small opening at the top of the chamber (fig. 1b), which contains a de-aerated solution of potassium ferricyanide (6 + 3 g Saponin/l H_2O). Stirring of the blood immediately releases O_2 which raises the P_{O_2} within the chamber and this was recorded using a Beckman P_{O_2} electrode. It is necessary to measure accurately the volume of the chamber in order to carry out the calculations. Other samples were also taken from the tonometer at the same time to measure *p*H and to check how close the P_{O_2} was to that expected for complete equilibration with the gas mixture. Oxygen content was calculated using the following relationship:

$$O_2 \text{ content (ml } O_2/100 \text{ ml blood)} = \frac{P_{O_2} \text{ (mmHg)} \times \alpha \times \text{vol. of the chamber } (\mu l) \times 100}{760 \times \text{vol. of sample } (\mu l)}$$

where α = the coefficient of solubility of O_2 in ferricyanide solution at 15° C expressed as ml/l/760/mmHg.

Method C. Spectrophotometric method. The same procedure as in Method B was used to obtain equilibrated samples with different gas mixtures. Blood was drawn into glass capillaries and haemolysed using Triton X-100. The haemolysed blood was transferred to a special cuvette and

the optical density of this sample was measured at two different wavelengths (505 nm, 598 nm) in an Oxygen Saturation Meter type OSM1 (Radiometer). The % saturation of the haemoglobin in the blood samples was calculated from measurements obtained using full oxygenated and fully reduced blood according to the relationship:

$$O_2 \text{ saturation } \% = A - B \times \frac{E_R - E_{H_2OR}}{E_G - E_{H_2OG}}$$

where A and B are apparatus parameters, that were calculated using fully oxygenated and fully reduced blood:

E_{H_2OR} = water optical density using the longer wavelength and

E_{H_2OG} = the water optical density at the smaller wavelength;

E_R = the optical density of the sample at the longer wavelength;

E_G = the optical density of the sample at the smaller wavelength.

Method D. Electrolytic method. This method makes use of a sample of blood being placed in a buffer solution of constant P_{CO_2} from which all oxygen has been removed by the activity of a respiring mitochondrial preparation. This preparation is then poisoned and oxygen is generated electrolytically within the chamber in known amounts and the rise in P_{O_2} is recorded. Consequently successive points on the curve are recorded as the haemoglobin becomes more saturated. The difference between the rate of rise of the P_{O_2} relative to that which would occur in a haemoglobin-free solution gives the dissociation curve. This method is particularly suitable for determining the shape of the curve at low P_{O_2}'s which is extremely difficult using other methods, especially as the P_{50} may be less than 10 mmHg as in the blood of some fish.

Results

Table I indicates that the P_{50}'s were close to one another in the range 16-21 mmHg, with the exception of method (9-10 mm).

Table II shows that sample results obtained using the four methods are quite similar, and in figure 2 curves for each of the methods are superimposed.

It is clear that in spite of methodological differences, all curves give a fair indication of the way the blood dissociates during its function in gas transport. In order to help other investigators to decide which of the methods might be most useful, some account of the advantages and disadvantages of each of the methods will now be given.

Method A. Advantages of this method include the following: It may be done relatively rapidly and this is especially true where the P_{50} is to be determined.

Different points on the dissociation curve are easily obtained and equilibration with only two gas mixtures is required i.e. one containing no O_2 and the other containing sufficient O_2 for full saturation. Ideally the two mixtures should have the same P_{CO_2}.

Table I. *Estimation of the minimum volume of blood required to make sufficient measurements for determination of P_{50}, Bohr effect and O_2 dissociation curve.*

Figures in parentheses show total number of samples required, assuming single measurements. In fact, triplicate measurements were made with the Tucker method and duplicates for the oximeter. Amounts expressed in μl.

	P_{50}	Bohr factor	Dissociation curve
A) Mixing method	80 (1)	160 (2)	560 (7)
B) Tucker method	70 (7)	140 (14)	70 (7)
C) Spectrophotometric method	175 (7)	350 (14)	175 (7)
D) Electrolytic method	500 (1)	1,000 (2)	500 (1)

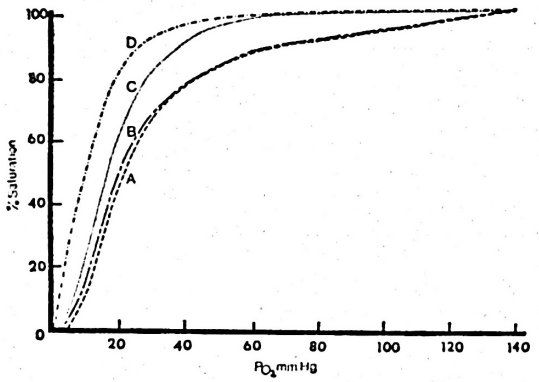

Fig. 2. *Superimposed O_2 dissociation curves obtained by the four methods, in rainbow trout.*

The Bohr effect can easily be determined if a N_2/CO_2 or Air/CO_2 mixture containing a different proportion of carbon dioxide is available. If the resulting *p*H's are sufficiently wide apart the straight line relationship can be drawn; for more detailed measurements several P_{CO_2}'s can be used.

This method is particularly good for the middle range of the dissociation curve.

Disadvantages include (1) the fact that the mixing method is not so accurate at the two ends of the dissociation and for fish blood this may be serious at low P_{O_2}'s.

It is difficult to obtain fully reduced samples and for very accurate determinations at the lower end of the curve it is necessary to determine the O_2 content of the reduced sample.

Precautions should be taken to ensure that contamination of the reduced blood samples does not occur while the mixing is being carried out. As mentioned previously it is necessary to make a correction for the varying *p*H of the different mixtures unless gases containing equal P_{CO_2}'s are used to equilibrate the oxygenated and deoxygenated samples.

It is necessary to make allowance for the O_2 dissolved in the plasma if the haemoglobin dissociation curve is required.

The O_2 capacity of the blood must be determined separately.

Finally, for the determination of sufficient points for the whole dissociation curve a relatively large sample is required as compared with some of the other methods (table I).

One improvement of the method is for the capillary used for mixing to be filled with nitrogen before drawing deoxygenated blood into the capillary and this can also be done by injecting nitrogen into the P_{O_2} cuvette especially when the low P_{O_2}'s are to be measured.

Method B. One of the main advantages of this method is the relatively small total volume of blood which is required to determine all the points required to construct a given O_2 dissociation curve. Determinations for each point can often be carried out in duplicate or triplicate. Total O_2 capacity of blood and the Root effect are readily determined. This method gives a plot of P_{O_2} against O_2 content which is readily converted to % saturation. (In Method A some separate method is required for determining the O_2 content of the fully saturated blood.)

The main disadvantage of the Tucker method is the amount of time required for each equilibration, as it is necessary to equilibrate many blood samples for determination of the whole curve. Hence much longer time is required than for the mixing method.

Because of the time elapsed between the first and last samples, a fall in *p*H of the blood is quite measurable. This may indicate not only metabolism of the red cells but some other changes might also be occurring, such as the danger of haemolysis.

Because of the need for equilibration with many different gas mixtures a whole battery of cylinders or a good mixing

Table II. *Comparison of mean values obtained for P_{50} of trout blood (15° C, pH 7,8), using four differents methods.*

Method	Individuals [a]					95 % Confidence limits [b]
	1	2	3	4	5	
Mixing	20.6	19.20	21.5	19.9	25.5 [c]	20.30 ± 1.5591
Tucker	18	18.54	19.52	20.5	19.4	19.19 ± 1.3324
Spectrophotometric	15.8	16.20				16.00 ±
Electrolytic	9.63	9.27	8.92	9.13		9.24 ± 0.4773

[a] The individuals analyzed in each method are different.
[b] $X \pm ts_{\bar{x}}$ (t corresponds to P = 0.05).
[c] This measurement has been discarded to obtain the confidence limits after testing the homogeneity of the data (P = 0.05).

pump is required. Once again it may be necessary to make a correction for *p*H unless care is taken to maintain the same P_{CO_2} for the different gas mixtures. Allowances must also be made in this method for the O_2 dissolved in the plasma if the Hb dissociation curve is required.

Determination of P_{50} by this method is therefore relatively long because it is not certain until the whole dissociation curve has been constructed or at least a large part of its middle range. As with other methods depending on equilibration with gas mixtures, there is always the danger of incomplete equilibration.

An improvement in the general technique, specially in relation to this last point, is to check the P_{O_2} of the blood sample following its equilibration with a given mixture. This allows the plotting of the dissociation curve even without perfect equilibration with the gas mixture and/or it checks how good the equilibration has been.

Method C. This is also very rapid and requires very small blood samples. It gives a direct determination of saturation of haemoglobin and does not require correction for dissolved O_2.

Some of the disadvantages are similar to those applying to the Tucker method, such as *a*) the time required for equilibrating many blood samples with different gas mixtures; *b*) the temperature should be regulated (the apparatus used, OSM1, is not thermostatically controlled) perhaps this is best done in a constant temperature room; *c*) the use of detergents to haemolyse the blood and particularly the presence of a nucleus within the erythrocytes, causes turbidity which interferes with transparence spectrophotometry (reflexion spectrophotometric determinations are less accurate); *d*) if whole blood is used instead of centrifuged blood cells, the dilution of haemoglobin by the plasma changes the O_2 dissociation characteristics because some of the factors controlling it e.g. intracorpuscular ATP, are diluted. Haemolysis also results in *p*H changes. The use of centrifuged samples of blood cells reduces this problem.

Method D. This method has the advantage of enabling a complete dissociation curve to be made from a single blood sample, the whole curve being determined about 25 minutes after sampling from the living fish. In the method used, a sample of about 0.5 ml of blood was required and is slightly greater than that for some of the other methods. In more recent studies the method has been modified and requires smaller volumes. As mentioned earlier, one great advantage of this method is that it starts at zero P_{O_2}, and is particularly good at the lower end of the dissociation curve, but accuracy decreases as the blood becomes more saturated. The

spread of results obtained by this method has been generally less than that of the others.

Some of the disadvantages are similar to those with the oximeter method with regard to the condition of the red blood cells. In this work the blood was diluted by a relatively large volume of buffer, and the effect upon the red cells is not certain. The method necessitates the injection of a respiratory inhibitor into the solution and the addition of other living material, the mitochondrial preparation, and either or both of these might influence the dissociation characteristics.

The method has been successfully applied to bird blood (9), and gave particularly interesting results which, as in the present case, suggested that the P_{50} was lower than that obatined by other methods. In bird blood, the speed of the measurements was especially important because of the high metabolic rate of the red nucleated cells. Clearly this is not so important at lower temperatures used here. But even so this is eliminated by the action of the respiratory inhibitor.

Details of this method as applied to smaller samples and to fish blood are in course of investigation.

Discussion

The dissociation curves obtained using the mixing and Tucker methods are whole blood curves (no correction has been made for O_2 dissolved in the blood). The oximeter curve is a dissociation curve of the haemoglobin because of this, the curves obtained by the first two methods differ from the latter and the asymptote of this is horizontal whereas the asymptote for the whole blood curves continues to show a slope as a consequence of the relationship between dissolved oxygen in the blood and the partial pressure of oxygen.

The mixing and Tucker methods gave similar results but the P_{50} using the mixing method is slightly higher. Perhaps the difference is a result of the difficulty of obtaining fully reduced blood in the mixing method (no correction was made for oxygen content in the reduced sample). There is also a great danger of air contamination using the mixing method which would also tend to produce a higher P_{50}. P_{50} found using the oximeter is lower than the P_{50} by both the Tucker and mixing methods. Perhaps this is due to an increase in *p*H, which in this case is the *p*H of the haemoglobin solution of haemolysed blood and exceeds that within the erythrocytes of whole blood, and consequently there would be a shift of the dissociation curve to the left. Furthermore, haemolysis would also result in the dilution of any factors controlling the position of the dissociation curve (e.g. ATP), and this must also shift the curve to the left.

Of all the methods, the electrolytic method has given the lowest P_{50}'s. As mentioned above, this has been a common finding with this method, which has been particularly valuable for avian blood. The precise condition of the haemogoblin and erythrocytes during this method needs to be examined further before a complete interpretation can be obtained. But clearly this method has many advantages especially with the development of micromethods. Such methods also have the advantage of using higher concentrations of blood in the surrounding medium.

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