

AN ELECTROLYTIC METHOD FOR DETERMINING OXYGEN DISSOCIATION CURVES USING SMALL BLOOD SAMPLES: THE EFFECT OF TEMPERATURE ON TROUT AND HUMAN BLOOD

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SUMMARY

1. A detailed account is given of an electrolytic method for determining the oxygen dissociation curve of fish blood using a single sample of 50-100 μ l for the whole curve. The accuracy and some of the problems arising from its uses are discussed.

2. Oxygen dissociation curves have been determined for trout blood and human blood at temperatures of 15 and 37 °C. The relationship between P_{50} and temperature is similar to that obtained using other methods. Absolute values of P_{50} are generally lower than those obtained by other methods, especially in the case of fish blood.

3. The effect of P_{CO_2} and pH on the oxygen dissociation curve of trout blood is tested and it is shown that P_{CO_2} has a more marked effect than pH when the other factor is maintained at a constant level. The Bohr factor ($\Delta \log P_{50}/\Delta \text{pH}$) appears to be approximately the same and independent of the P_{CO_2} .

4. The P_{50} of ray blood determined from fish during and after an operation showed an increased Bohr factor.

INTRODUCTION

The transport of oxygen forms a vital link in the respiratory chain whereby oxygen is transferred from the water to the sites of exchange in the cells. The oxygen dissociation characteristics of blood show many adaptations related to the particular mode of life of an animal (Johansen & Lenfant, 1972; Bartels, 1972). This type of study has been carried out for many years, especially since the classical works of Barcroft (1925, 1934), on the function of haemoglobin in the animal kingdom and the adaptations of the blood of different mammals. More recently it has become apparent that there are a number of dangers in the basic methods which have often been adopted, sometimes by force of circumstances, but more usually because they are commonly accepted techniques of mammalian physiology. Notable among these problems are those found in

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the blood of non-mammalian vertebrates, where nucleated red cells appear to have a greater metabolic activity than the non-nucleated r.b.c.'s of mammals. Thus a blood sample soon after removal from animals may have significantly different properties to those which are actually determined hours or even days later. In the case of ray blood this was found to be the case (Hughes & Wood, 1974), and consequently each sample was not used for the determination of more than two points on the dissociation curve. In many methods blood is equilibrated in tonometers for longer periods and obviously there are many dangers in this context.

When working with the blood of fish and some other animals it is specially important to be able to make as many determinations as possible on a small sample. Such techniques would be particularly valuable in studies of small fish and the development of the oxygen carrying properties of the blood. A number of methods have been developed in recent years with these problems in mind and recently a comparison of four such methods was carried out with respect to trout blood (Hughes, Palacios & Palomeque, 1975). Of these methods the electrolytic method, developed as a modification of the technique introduced by Longmuir and his colleagues, has proved to be extremely useful as the whole dissociation curve may be determined on a single sample within 20–30 min of the blood having been taken from the fish.

This paper is concerned with the development of that method so that the volume of blood required is reduced to between 50 and 100 μ l together with a number of other improvements. In order to test the usefulness of the method, comparisons have been made with the bloods of other animals, and in particular the determination of the dissociation curve of human blood at temperatures comparable to the normal range of trout and vice versa, has provided an interesting comparison.

MATERIALS AND METHODS

The basic method is derived from that of Colman & Longmuir (1963), Longmuir & Chow (1970) and Lutz *et al.* (1973). The principle is a very simple one but some detailed aspects of the technique have not been described altogether and it is hoped that at this stage some summary of these would be helpful to other comparative physiologists, who may wish to use the method. The basic principle depends on the fact that when a single unit of current is passed between two electrodes, in an aqueous solution, one unit of oxygen and two units of hydrogen are liberated. The hydrogen can be absorbed by palladium to an extent of about 600 vols. %, further aided by a retaining collodion membrane, and consequently free oxygen is made available. This free oxygen is liberated into a blood/buffer mixture in a restricted volume and a recording is made of the increase in oxygen tension. Correction is made for the oxygen solubility properties of the buffer and consequently the blood dissociation curve can be derived. These processes take place within a specially constructed cuvette (Fig. 1). The cuvettes used in the present work had an adjustable internal volume of 1.5–2.5 ml. A comparison between this and that used by Lutz *et al.* (1973) with respect to volumes of mitochondrial extract, oxaloacetic acid, and blood used is given in Table 1.

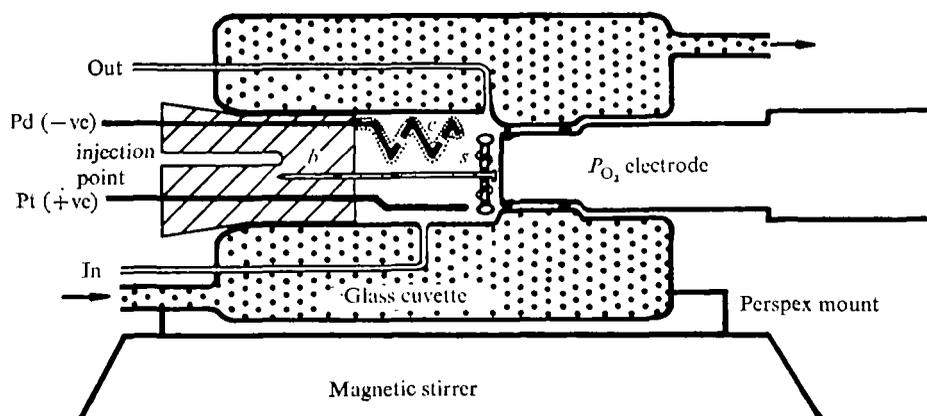


Fig. 1. Diagrammatic longitudinal section through the cuvette. A thermostatted water jacket (dotted) surrounds the reaction chamber and its inlet and outlet tubes. The coiled palladium cathode and platinum anode are inserted through a silicon rubber bung (*b*). The other end of the reaction chamber is filled by a P_{O_2} electrode, supported by two 'O' rings. Stirring of the contents is by a small magnet (*s*). Reagents are injected through a depression in the silicon rubber as indicated. The whole cuvette is supported on a perspex mount, which rests on a magnetic stirrer.

Table 1. *Volumes (ml) of reagents used with different sizes of cuvette*

Cuvette	Mitochondrial preparation	Oxaloacetic acid	Blood	Reference
10	0.2	0.3	0.5	Lutz <i>et al.</i> 1974
2.5	0.05	0.075	0.100	This study
10	to	to	to	
1.5	0.03	0.045	0.050	This study

The cuvette

The cuvette consists of a moulded open glass tube surrounded by a thermostatted water jacket, usually kept at 15 ± 0.5 °C for trout and ray blood. One end of the glass tube is filled by a P_{O_2} electrode (Radiometer type E5046) held in place by two 'O' rings. The other end is sealed by a silicon rubber bung (size 13) through which are inserted the palladium and platinum electrodes. Huber 23G, 1-in needles are also inserted through this silicon bung in order to inject reagents into the reaction chamber. The inlet and outlet tubes to the chamber consist of 19 G stainless steel needles, to which are attached Portex pp 120 tubing. The outlet from the chamber is positioned so that any air bubbles are automatically removed. The exact volume of this reaction chamber varies because of different sizes and positions of the silicon bung and should be determined for each experiment. Stirring of the chamber contents is ensured by means of an 8 mm ring magnet attached to a stainless steel needle. An uneven surface is produced on the PVC coating which must be well washed and soaked in distilled water.

Other equipment used in this work consists of a Beckmann 160 physiological gas analyser, Smith RE511 chart recorder with modified on/off switch (the layout of this equipment in relation to the other apparatus is shown in Fig. 2). Blood pH was determined with an Ingold blood pH micro-electrode assembly and a Pye Unicam 290 pH meter.

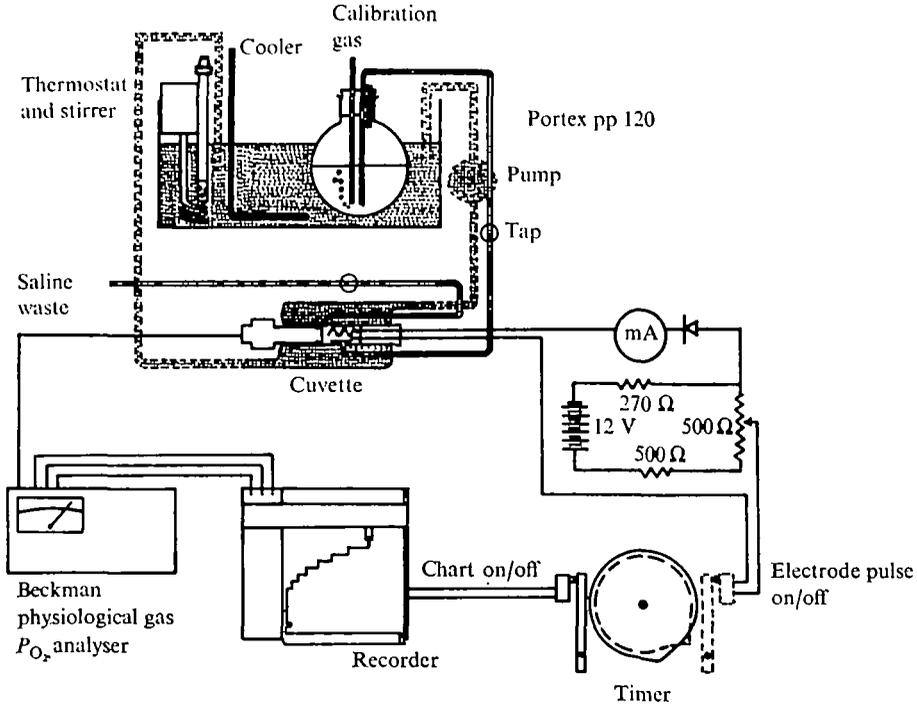


Fig. 2. Diagram showing the cuvette in relation to the apparatus used for temperature regulation, current production, timing device, P_{O_2} meter and recorder.

Electrolysis electrodes

Materials for the electrodes were obtained from Johnson Matthey (London) and consisted of:

(a) the platinum anode which was 1 mm diameter with a 1.5–2 cm loop in the cuvette. This electrode was unheated except for cleaning and sterilization by means of absolute alcohol and flaming.

(b) The palladium cathode was formed of 1 mm diameter wire with a total length at least twice that of the platinum electrode: 3–4 cm length was coiled over a 3 mm bar. The electrode length within the cuvette needs to be at least 1 cm for each mA current flow. A new palladium wire was heated white hot in a bunsen flame and then cooled and dipped in collodion. The heating need not be done on second and subsequent use of a given electrode, but its coating must be dissolved in a 50/50 mixture of ether and absolute alcohol before recoating. After four determinations the membrane of the palladium electrodes was dissolved and left to recover for at least 24 h. This could be accelerated by keeping in a warm oven at 37 °C. Without heating it would be preferable to keep it in air for a week. Thus a supply of several electrodes is advisable if regular determinations are made every day.

(c) *The coating and membrane* of the electrode was made of Necol collodion (BDH) using a 30% stock solution in a 50:50 ether/alcohol mixture. The coating takes 15 min to dry and should be done shortly before use. Several different types of membrane were tried, the non-flexible variety of collodion was best; the flexible form and collodion were not such efficient hydrogen retaining membranes.

Table 2. Composition of buffered salines g/l

A. 0.1 M phosphate saline (all trout and human blood samples)

Na ₂ HPO ₄	11.267	} pH buffer
NaH ₂ PO ₄	2.12	
NaHCO ₃	1.75	CO ₂ buffer
MgCl ₂	0.05	Activator
Na succinate	1.891	Substrate

(add at last minute) } for mitochondria

Made up to 1 l, pH adjusted to 7.5 with 5 M-NaOH or HCl.

B. 0.5 M elasmobranch saline (modified after Q. Bone (unpublished data))

NaCl	17.041	CaCl ₂ *	0.15
KCl	0.224	Na ₂ SO ₄	0.50
MgCl ₂	0.439	Na ₂ HPO ₄ *	11.267
Sucrose	25.0	NaH ₂ PO ₄ *	2.12
Urea	2.7 (add last)	Na succinate* (add last)	1.891
NaHCO ₃ *	1.30		

Made up to 1 l, pH adjusted to about 7.6. * Caution: effects of electrolysis in such a complex salt solution are unknown with respect to reaction products, poisoning of electrodes etc. The Na₂HPO₄/NaH₂PO₄ ratio is unaffected by electrolysis. Buffers should be made up just before they are required in order to avoid bacterial action. The buffer container, tubes and cuvette should be sterilized if possible. Half a litre of buffer is usually sufficient for determining at least five dissociation curves.

Buffered salines

Table 2 summarizes the composition of the salines used for the blood of different species. Buffers were made up very shortly before they were required in order to avoid bacterial action. The use of a sterile container for the buffer and sterile tubing and cuvettes is desirable. About 500 ml of buffer was made up on each occasion and used for the determination of five or more dissociation curves, depending on the amount required for washing the cuvette.

Calibration gases

These were made up by means of gas mixing pumps (Wosthof) and were chosen in relation to the desired final pH and P_{CO_2} required for the experiments. Atmospheric pressure was read before each experiment.

Mitochondria (heart muscle preparation)

These preparations were obtained from fresh ox heart prepared in a 0.1 M phosphate buffer at pH 7.8, using the method of Colman & Longmuir (1963). The mitochondrial preparations were kept in sealed 0.5 ml glass vials, frozen at -40 °C. These must be kept in small quantities as the preparation loses activity after a second refreezing. The removal of oxygen from the contents of the cuvette takes place by the oxidation of succinate without any accompanying evolution of CO₂. This requires an active preparation and a short period of action; thus the more concentrated the activity of the mitochondrial preparation, the better.

Metabolic inhibitor

When the P_{O_2} within the cuvette has fallen to zero, inhibition of the mitochondrial preparation and blood cell metabolism is carried out by injection of 0.5 M oxaloacetic acid (BDH, MW 132.08) made up in distilled water with its pH adjusted as required. This solution is made up fresh every two or three days and kept in a refrigerator. The solution is kept in a bottle fitted with a rubber seal so that air may be removed and replaced by N_2 by means of hypodermic needles. Samples of this solution were also removed by syringe in order to maintain anoxic conditions.

Sources of blood

Most experiments described here were done using rainbow trout [*Salmo gairdneri* (Richardson)] from the Nailsworth hatcheries. The dorsal aorta was cannulated in the usual way (Smith & Bell, 1964) using a Huber 21G needle and Portex pp 60 tubing. The anticoagulant used in these cannulae was 100 i.u. heparin/ml blood and samples were kept on ice following collection in polythene tubes. The tubes were occasionally turned over to prevent settling of the corpuscles. The percentage haematocrit and pH of the blood samples was determined immediately following withdrawal from the fish.

Cannulation of the ray (*Raia clavata* L.) was achieved via a branch of the coeliacomesenteric artery (Hughes & Wood, 1974).

Procedure

The general procedure involves filling the cuvette with the appropriate buffer solution, which is deoxygenated, and then the blood sample is injected.

Oxygen is removed by the mitochondrial preparation, which is then poisoned with the metabolic inhibitor. The generation of oxygen is carried out discontinuously by means of a timing device so that current passes between the electrodes for a certain period (10 s). This is followed by an equilibration period (40 s), then the chart recorder is switched on for 10 s to record the change in P_{O_2} . The recorder is switched off and the whole procedure starts once again. Thus a time/ P_{O_2} curve is obtained from which it is possible to derive the per cent saturation/ P_{O_2} curve of the blood, as discussed later. In order to clarify the sequence of events the procedure adopted in most experiments is given in further detail:

(1) *Zero P_{O_2} electrode* by injection of 50 μ l mitochondrial preparation, using a sterile Huber needle and syringe, into the cuvette containing buffer. Oxygen is removed in 3–5 min. This helps not only to record the zero of the P_{O_2} electrode, but also to check the seal of the bung and P_{O_2} electrode in the chamber.

(2) *Calibrate P_{O_2} electrode*. The buffer is equilibrated by passing a gas mixture containing a known P_{O_2} into the cuvette; this also ensures that all air bubbles are washed out. The buffer-inlet and buffer-outlet tubes are closed during the final calibration procedure.

(3) *Deoxygenation*. 50 μ l of mitochondrial preparation are injected, P_{O_2} falls to zero in about 3–5 min.

(4) *Blood sample* is injected (100 μ l). A certain time (2–3 min) is required to complete deoxygenation of the blood.

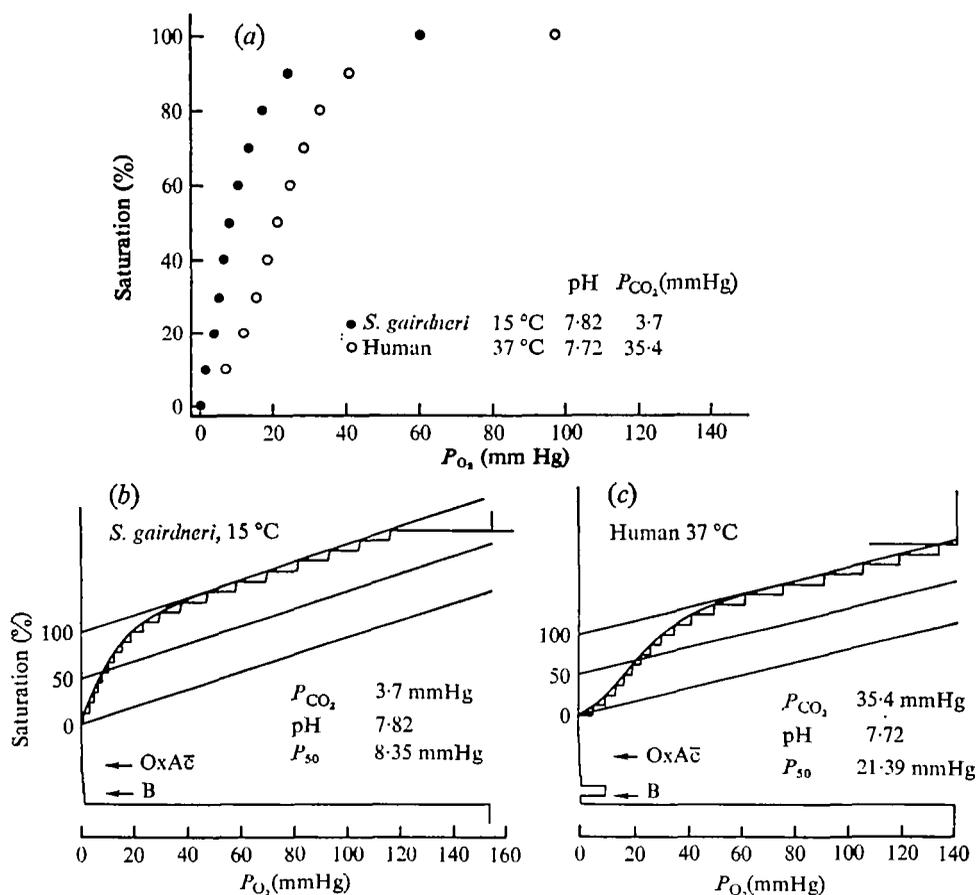


Fig. 3. (a) O_2 dissociation curves for rainbow trout and human blood at temperature, pH and P_{CO_2} within their normal range. In (b) and (c) are reproduced actual recordings obtained and indicate the times at which injection of the oxalacetic acid (OxAc) and blood sample (B) took place. The 100% saturation line is drawn along the points where the P_{O_2} changes become linear. The zero and 50% saturation lines are drawn parallel to this line.

(5) *Metabolic inhibitor.* 75 μ l oxaloacetic acid (0.5 M) are injected and left to act for at least 3 min. The action of both mitochondria and metabolic inhibitor is dependent on temperature, 1–2 min being required at 37 °C.

(6) *Current pulses* of 2.5–3.0 mA are now passed for 10 s periods, followed by 40 s equilibration, 10 s chart advance; the whole sequence being 1 min. It is important to monitor the current because it tends to fall off as the P_{O_2} increases. Ideally a constant current generator should be used.

(7) When the curve has been produced and its final slope is linear, a blood/buffer sample is removed with the Huber needle hypodermic syringe for pH measurement.

(8) *A second calibration* is carried out by passing equilibrated buffer into the cuvette to wash out the used blood/buffer solution and for calibrating the P_{O_2} electrode once more.

A number of minor problems have arisen during this procedure, notably the formation of 'strings' of protein hanging from the platinum electrode. This was probably

Table 3

Species	T (°C)	P_{O_2} (mmHg)	pH	P_{50}	Reference
<i>S. gairdneri</i>	15	1-2	—	18	Irving, Black & Safford (1941)
	10	3	—	18.5	Cameron (1971)
	15	3	—	20	Cameron (1971)
	20	3	—	27	Cameron (1971)
	6	3	7.2-8.4	19.1-25.5	Eddy & Hughes (1970)
	15	3	7.2-8.4	26.1-29.8	Eddy (1971)
	20	3	7.2-8.4	34.7	Eddy (1971)
	15	3.6	8.34	8.34	This study
	37	3.5	7.84	29.94	This study
	<i>R. clavata</i>	15	—	7.70	30.2
15		7.4	7.79	11.46	This study
<i>H. sapiens</i>	37	—	7.4	26.8	Bartels <i>et al.</i> (1961)
	37	—	7.4	24.3	Hilpert <i>et al.</i> (1963)
	38	—	7.4	29.7	Engel & Duc (1968)
	37	5.6%	7.4	25.87	Verarmold, Seifert & Riegel (1973)
	37	35.5	7.58	21.92	This study
	15	36.7	7.5	3.71	This study

due to incomplete purification of the mitochondrial preparation, possibly because of heart muscle ligaments, which are easily overlooked. Haemolysis of blood causes a pink gelatinous precipitate to form on the electrode, but this is only seen on rare occasions. The cuvette contents have been spun down to test for haemolysis with negative results, except in those cases where this is obvious from the electrode precipitate.

RESULTS

This method was mainly developed for studies on the blood of rainbow trout, and most of the results presented are from this fish. Trout blood has already been used to compare four methods (Hughes *et al.* 1975), including an electrolytic method. The more micro-electrolytic method described here has the advantage of using smaller blood samples. For comparison with rainbow trout, human blood samples were used as the dissociation curves are well established by many other methods. Experiments were carried out at temperatures of 15 and 37 °C for both bloods, being the normal body temperature for trout and man respectively. A brief account is given of some studies on the blood of thornback ray, a good cannulation method having been developed previously and several determinations being available for comparison using the mixing method (Hughes & Wood, 1974).

A. Rainbow trout

The curve shown in Fig. 3(a) illustrates the typical dissociation curve obtained at 15 °C and pH 7.82 and a P_{CO_2} of 3.7 mmHg. The chart record from which this curve was derived is also given (Fig. 3(b)). It is clear that the P_{50} can be determined very rapidly by a single line parallel to the 100% saturation, which is traced out when the change in P_{O_2} becomes linear as it follows the saturation line of the blood plasma and buffer solution.

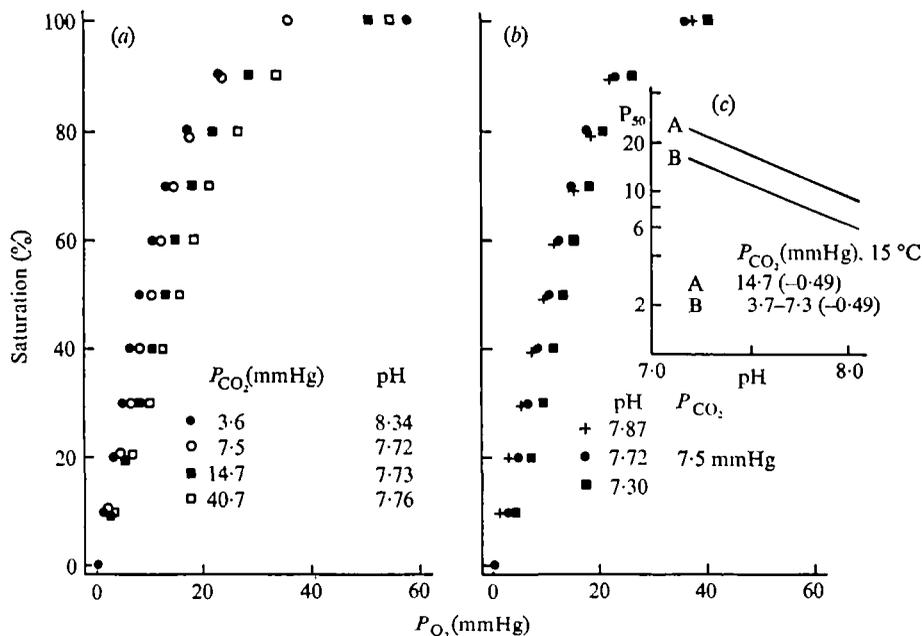


Fig. 4. Rainbow trout from Nailsworth. O₂ dissociation curves (15 °C) showing (a) the effect of changes in P_{CO₂} at constant pH, and (b) of changing pH at constant P_{CO₂}. (c) The Bohr shift is plotted for the relationship between P₅₀ and pH at constant P_{CO₂}, Bohr factor in brackets.

It soon became clear that the P₅₀'s obtained by this method were generally lower than those reported by other workers. Some of the values obtained are summarized in Table 3.

The effect of changes in pH was investigated and compared with those at constant pH but varying P_{CO₂}. The results are illustrated in Fig. 4, where a marked Bohr shift is apparent. The influence of P_{CO₂} is very marked whereas the actual P₅₀ changes resulting from decrease in pH do not seem as great as those indicated in the literature, where the distinction has not always been made between the effects of P_{CO₂} and pH. The Bohr factor ($\Delta \log P_{50}/\Delta \text{pH}$) appears to be approximately the same regardless of the P_{CO₂}. Differences were found when the Bohr factor for blood taken from the fish in water of the Water Research Laboratory, Stevenage, were compared with samples taken following a period in Bristol water. Most of the results discussed here are for blood from fish kept for about a week in Bristol after coming from the Nailsworth (Gloucestershire) hatchery. There are indications that the particular history of the specimens influences this aspect of the blood O₂ dissociation characteristics, but further work is necessary in order to elucidate its precise nature. The marked effect of temperature on the O₂ dissociation curve of fish blood is well known and is illustrated in Fig. 5, where the measurements were made on blood at 15 and 37 °C respectively. The complementary experiment, i.e. human blood at the same temperatures, is discussed below. The relationship between P₅₀ and temperature for rainbow trout blood is as follows:

$$P_{50} = 0.98T (\text{°C}) - 6.36.$$

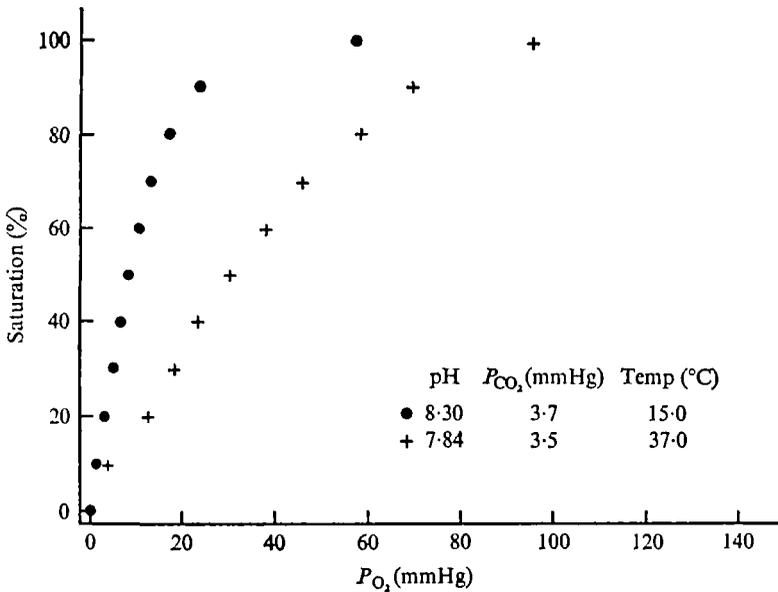


Fig. 5. Rainbow trout from Nailsworth. O_2 dissociation curves at P_{CO_2} about 3.6 mmHg. When determined at 37 °C the curve for blood obtained from fish whilst under MS 222 anaesthesia at 15 °C is also included; analysis indicates that the two curves are not significantly different.

B. Human blood

A typical dissociation curve for human blood at 37 °C and pH 7.7 and P_{CO_2} 35.4 mm is shown in Fig. 3(a), together with the actual recording from which this curve was derived (3(c)). The effect on this curve of pH and P_{CO_2} changes and of temperature were investigated and compared with published data. Once again a significant Bohr shift was obtained with changes in P_{CO_2} and constant pH. A marked effect of lowering body temperature was also found and the P_{50} of this blood was below 5 mmHg. Thus at the same temperature the two types of blood resemble each other much more closely. The relationship between P_{50} and temperature for human blood was as follows:

$$P_{50} = 0.83T (^\circ\text{C}) - 8.75.$$

C. Thornback ray (*Raia clavata*)

The conditions during cannulation made this suitable material for studying the effects of the operation by comparing blood taken immediately following cannulation with blood taken several days postoperatively. There was a significant difference between the dissociation curve in the two cases. In similar experiments with trout, no significant difference was discernible. In addition to the shift of the curve to the right at constant P_{CO_2} there was also an increase in the Bohr factor from -0.42 to -0.62 (Fig. 7).

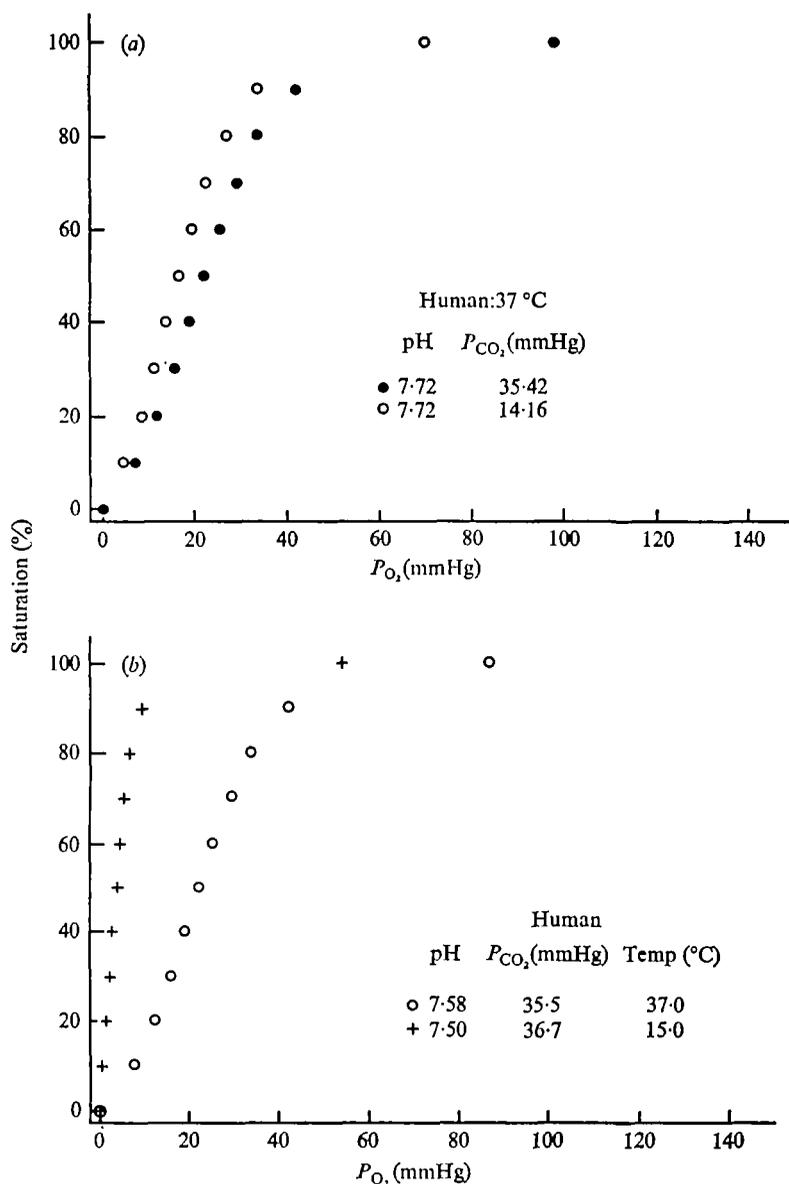


Fig. 6. Human blood. (a) O_2 dissociation curves at pH 7.72 and two different P_{CO_2} 's (35.42 and 14.16 mmHg). (b) The effect of temperature on the dissociation curve at pH 7.5 and P_{CO_2} of about 36 mmHg.

ACCURACY OF THE METHOD

The experiments discussed in this paper suggested some possible sources of error, and we made a number of tests to establish possible effects that may arise from the particular conditions under which the electrolysis was carried out. Runs were carried out to test the effect of oxygen generation in an electrolyte solution in the absence of a blood sample. Oxygen was generated with a constant current at 15 °C using the dis-

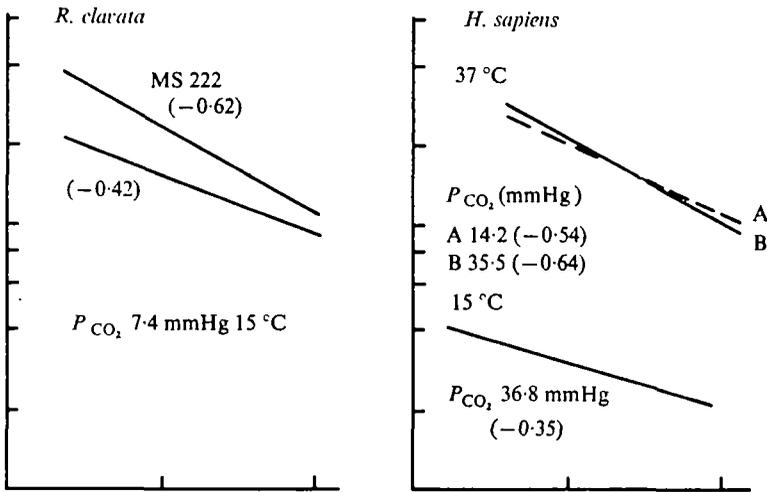


Fig. 7. Plots of the Bohr shift of the blood of the thornback ray and man. The ray curves determined at 15 °C show significant differences between blood obtained post-operatively under MS 222 anaesthesia and the normal curve. There is a significant difference in the slopes for human blood at the two temperatures, although the P_{CO_2} is fairly constant.

continuous method for registering the curve; it was found that the oxygen tension registered by the recorder during equal periods of electrolysis decreased with increasing P_{O_2} in the medium. A slightly but significantly non-linear curve results instead of the expected straight one (Fig. 8, curve Bc). This non-linearity is especially conspicuous above 80–100 mmHg. The curves were linear between P_{O_2} 's of 0 and 60 mm. This effect, although less severe, was also discernible when the oxygen was generated in solutions containing a blood sample, as was normally carried out when determining the whole blood O_2 -dissociation curve.

The phenomenon has been systematically investigated to try to find out the factors involved. First, the effectiveness of the oxaloacetic acid as a metabolic inhibitor and the thickness of the collodion membrane covering the palladium electrode were tested. Furthermore, the phosphate buffer was diluted 10-fold to 0.01 M (Longmuir & Chow, 1970). The effect of relative freshness of the buffer was also tested by using solutions immediately after they were made up or after being kept for several days. An effect of bacterial activity in the buffer was verified by comparing curves made under sterile conditions with those obtained under less stringent conditions. The curves obtained from these experiments still showed a non-linearity at the high oxygen partial pressures but it was less in fresh buffer solutions equilibrated with 1–2% CO_2 and air. The non-linearity was enhanced when the phosphate buffers of both strengths were used, if they were more than one day old (Fig. 8Ac).

Particular attention was paid to the properties of the palladium and its ability to absorb the hydrogen generated at 15 °C. It is known that the electrical resistance of palladium can almost double when it is saturated with hydrogen, produced by electrolysis or other means (Lewis, 1967). Consequently less current will flow following the absorption of appreciable amounts of hydrogen during the electrolytic process. However, according to Lewis (personal communication) this resistance effect is negligible

In the present situation because of the large diameter of the electrode wire used in the cuvette. Furthermore, the total amount of hydrogen absorbed during a single electrolytic run is very small compared with the volume of palladium. The electric circuit and resistances used for the generation of the electric pulse (Fig. 2) is such that very little current loss will occur, even if the resistance of the palladium electrode doubles during a single electrolytic run. However, if the palladium is constantly used without taking precautions to get rid of the accumulated hydrogen, the hydrogen will react with the oxygen during the electrolysis (Lewis & Ubbelohde, 1954; Longmuir & Chow, 1970; Barton, Leitch & Lewis, 1963). In view of this, it is important to maintain the palladium in as fresh a condition as possible. The procedure adopted after the palladium has become charged with hydrogen during a single electrolytic run is to remove the hydrogen according to the conditions described by Lewis & Ubbelohde (1954). With H/Pd greater than 0.6, hydrogen rapidly escapes *in vacuo* from the alloy even at room temperatures. With H/Pd equal or less than 0.6, hydrogen is held for an indefinite period (1 year) at room temperatures in air, but escapes on heating to 200 °C. Because the H/Pd ratio in the palladium electrode wires was not determined, each palladium electrode was de-gassed *in vacuo* at 200 °C for at least 12 h before use.

In spite of this treatment the expected linearity on the upper part of the curve, above 100 mmHg, was not achieved. There are, however, other possible factors concerned with the properties of the palladium electrode which may be involved in this non-linearity, e.g. formation of a poisonous layer of absorbed atoms on the palladium to prevent hydrogen diffusion through the metal (Lewis & Ubbelohde, 1954). Some of the following are particularly worthy of consideration:

(1) The small size of the cuvette necessitates the electrodes being close together and, with the vigorous stirring of the electrolyte, may increase the supposed reaction between oxygen and hydrogen. In this context, however, the P_{50} values obtained by Hughes *et al.* (1975) for rainbow trout blood (at the same pH of 7.8 and 15 °C) using a more conventional macro-cuvette did not differ from the values obtained with this micro-cuvette (Table 4).

(2) Lowering the temperature from 37 °C, as used in the original method, to 15 °C will increase the solubility of both oxygen and hydrogen in the electrolyte. This factor, together with the increased P_{O_2} at maximum electrolytic saturation at lower temperatures (80–100 mmHg at 37 °C, 140–160 mmHg at 15 °C) may play a key role in the supposed oxygen-hydrogen reaction.

Effect of an increase in P_{O_2} when the supporting electrolyte contains a blood sample (Fig. 8A)

When the O_2 dissociation curves are constructed for the blood sample the non-linearity at the upper part of the tracing is considerably reduced compared with curves made without blood. A much longer linear line could be superimposed on this curve (Fig. 8A*a*). When the curve is constructed without CO_2 in solution, and old buffer is used, a curve such as that shown by *Ab* may result.

Therefore, it seems probable that if the precautions described above are observed a linear line for determining the point of 100% saturation of the blood with oxygen can be drawn fairly accurately. Observance of the precautions is especially necessary where

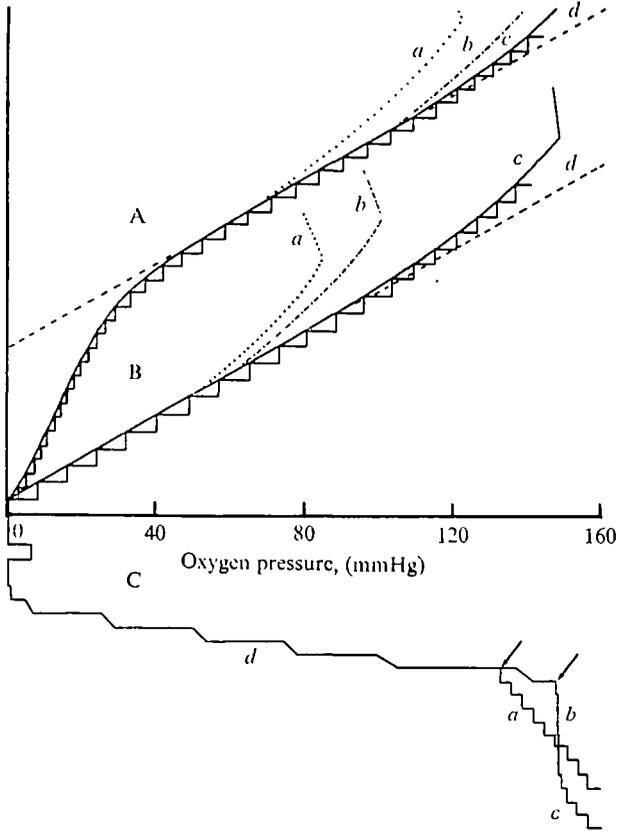


Fig. 8. Composite tracing of P_{O_2} against discontinuous time during the generation of oxygen by a constant current under different conditions.

A. Oxygen generation for electrolyte containing a blood sample

a, curve obtained with a palladium electrode containing hydrogen before electrolysis. *b*, palladium degassed at 200 °C, but electrolyte without bicarbonate and CO_2 gas. *c*, degassed (200 °C) palladium, electrolyte with bicarbonate and 1-2% CO_2 gas. *d*, straight line obtained from the linear part of curve *c*.

B. Oxygen generation without blood sample

a, palladium cathode substituted for a platinum cathode. *b*, palladium electrode containing hydrogen without degassing. *c*, curve constructed with degassed Pd but without CO_2 equilibration of electrolyte. *d*, straight line obtained from the linear part of curve *c*.

C. Part of initial recordings (steps are due to automatic stopping and starting of recorder)

a, the removal of oxygen when the palladium contains hydrogen. *b*, shows part of the initial recording; P_{O_2} decrease is minimal when the palladium electrode is thoroughly degassed. *c*, the oxygen electrode responds to temperature equilibration. Degassed palladium was used with constant stirring. *d*, removal of oxygen by the mitochondrial preparation. Arrows refer to time when mitochondrial preparation was applied. Recorder speed 30 mm/min.

Table 4. Summary of results

(Blood Dissociation Data obtained from whole blood samples of not more than 100 μ l using a micro-electrolytic cell of the Longmuir type. Bohr factors ($\Delta \log P_{50}/\Delta \text{pH}$) for blood sampled at the same time is also given.)

Species	Temp. (°C)	P_{O_2}	pH	$P_{50} \pm \text{s.e.}$	Bohr factor
<i>S. gairdneri</i> (from Nailsworth)	15.0	3.7	8.34	8.34 ± 0.04	-0.49
		7.5			
		7.5	7.87	9.83 ± 0.32	-0.5
		7.5	7.72	10.28 ± 0.91	—
		7.5	7.30	12.86 ± 0.68	-0.49
		14.7	7.73	12.84 ± 0.68	-0.49
		40.7	7.76	15.61	—
		37.0	3.5	7.84	29.94 ± 1.3
<i>R. clavata</i>	15.0	7.4	7.79	11.46 ± 0.28	-0.42
			7.81	13.66 ± 0.77	-0.62 (MS 222)
<i>H. sapiens</i>	37.0	14.1	7.66	16.92 ± 0.27	-0.54
		35.5	7.58	21.92 ± 1.44	-0.64
	15.0	36.8	7.50	3.71 ± 0.51	-0.35

bloods of low oxygen affinity or haemoglobin solutions are measured at low temperatures. Clearly the absolute values of P_{O_2} obtained can be significantly altered by the slope of the 100% saturation line.

DISCUSSION

The method described has already shown its usefulness in the form originally developed by Longmuir and his colleagues, especially in relation to the dissociation curve of bird blood (Lutz *et al.* 1973), where a reduction in time between blood sampling and determination of the dissociation curve indicated an affinity that was not significantly higher than that of mammalian blood. With fish blood there is the same advantage of reducing the time during which changes can occur in the blood characteristics. That these may be significant has been suggested by previous results using a mixing method (Hughes & Wood, 1974). Some advantages and disadvantages with respect to other methods frequently used with fish blood have already been discussed (Hughes *et al.* 1975), and this method is particularly advantageous when it is required to determine the whole curve. For speed of determination it was concluded that the mixing method is better for P_{50} alone. A common finding of previous workers and those recorded in this paper is of P_{50} values less than those obtained by most other methods. Although true for fish blood, the same tendency was also present in results obtained here for fresh human blood. Thus although a P_{50} of about 26 mmHg is generally taken as typical, figures recorded for normal man (Table 3) range as high as 29.7 so that the result obtained in the present experiments of about 22 is lower by about the same amount. With more detailed investigations, it has become clear that there are individual variations between humans and that the blood P_{50} may also vary for a given individual at different times. Such variations are commonly accepted by comparative physiologists and it is well known that the P_{50} can alter fairly rapidly. Some of the underlying mechanisms have been investigated more recently, for

example studies of fish under hypoxia have shown that intracellular organic phosphates are involved, but not necessarily DPG, which is the common modifying organic phosphate among higher vertebrates (Wood & Johansen, 1973).

The differences between results obtained with the electrolytic method and those of previous authors using the same species are quite definite, but only further work can distinguish between the extent to which these are real differences and the extent to which they are due to the methods. Furthermore, the extent to which procedures of a given method affect the dissociation curve will vary according to the species. In the present results divergence from previous data is greatest for *Raia* and least for man. Possible reasons for the difference between these results and those obtained using whole blood may be sought in two aspects of the method: (a) dilution with buffer and (b) inhibition of the red blood cell metabolism with oxaloacetic acid. Dilution in phosphate buffer might influence the dissociation curve of haemoglobin in so far as there is any exchange of materials across the RBC membrane and this would affect the intra-erythrocytic environment of the haemoglobins. It seems unlikely that dilution itself could have any marked effect. The method of analysing the recordings also renders this method independent of haematocrit, and the final curves plotted do not incorporate dissociation characteristics of the plasma or buffer. In this context the precise nature of the buffer solution is clearly important and the known role of phosphate in modifying the dissociation of haemoglobin makes a phosphate buffer not an ideal one. Evidence that inorganic phosphates may influence the affinity of fish blood cannot be ruled out, and the use of other buffers is at present being investigated. Limitations on the type of buffer are related to the ability of the solution to conduct sufficient current as well as providing a suitable environment for the red blood cells.

The use of any metabolic inhibitor must make the red blood cells different from those contained in whole blood in at least two ways: (a) oxygen consumption of the nucleated red cells will be substantially reduced, and (b) synthesis of certain substrates will be prevented and these may be important determinants for the haemoglobin dissociation curve. An example of the latter for human blood has been given by Engel & Duc (1968) with respect to iodoacetate as a metabolic inhibitor which was shown to lower the P_{50} . Incubation of blood samples with iodoacetate inhibited DPG production with a consequent increase in affinity of the haemoglobins. DPG itself is not so important in fish bloods but other organic phosphates play comparable roles. Exactly how the oxaloacetic acid metabolic inhibitor affects these remains to be elucidated. In the experiments of Engel & Duc, however, the blood and iodoacetate were incubated for 6 h at 38 °C, whereas in most experiments described here the oxaloacetic acid and blood were at 15 °C for about 30 min. Nevertheless it remains possible that the depletion of organic phosphates within the nucleated red blood cells is a factor contributing to the low P_{50} 's obtained with this method.

If these changes are significant then clearly they will have least effect shortly after injection of the oxaloacetic acid into the buffer containing the blood and consequently the lower part of the dissociation curve would be least influenced. This part of the curve is also more accurate than the upper part because the method used for drawing the 100% saturation line will have least effect. Thus the electrolytic method has undoubted advantages for determining the lower part of the curve and this is especially true for bloods of high affinity. The careful zeroing of the P_{O_2} electrode before each

Determination, which begins at zero oxygen tension, must increase accuracy over many other methods.

Apart from the lower P_{50} values, many of the results obtained by this method with respect to important blood characteristics, such as the Bohr Shift and temperature effects, are comparable with those obtained previously. Thus as a method for comparing the dissociation characteristics of samples from different individuals or under different conditions there seems little doubt that the present one has great advantages, especially as the whole dissociation curve can now be obtained on such small samples and so soon after sampling. It is particularly convenient for determining the dissociation curve under different conditions such as pH, P_{CO_2} and temperature. For example, this study has shown that the curve for human blood measured at 15 °C has a P_{50} which is even lower than that of trout blood at the same temperature. Correspondingly the trout blood dissociation curve determined at 37 °C has a P_{50} in the same range as that for man. Studies with trout blood have been especially interesting in relation to the effect of pH and P_{CO_2} . Few previous workers have carried out experiments studying the effect of P_{CO_2} and pH independently. From these results it is clear that a change in P_{CO_2} has a greater effect *per se* than that resulting from any consequent pH change. Experiments of this kind are conveniently carried out using the electrolytic method because controllable buffer solutions are normally used. Results obtained with elasmobranch blood suggest that the method needs to be further tested with these animals. The relatively low P_{50} emphasizes still further the observations of Hughes & Wood (1974) on the same species. Its convenience and sensitivity has shown how differences can be detected during operative procedures using MS 222. Again it is of interest that such effects were not present in trout blood during comparable experiments.

In a way, the preparation used in the present work for determining the oxygen dissociation curve is intermediate between whole blood and haemoglobin solutions. Consequently the absolute values obtained cannot, as yet, be compared strictly with those obtained by the other methods. Electrolytic methods can be applied to haemoglobin solutions, however, and might be especially valuable for investigating different haemoglobin fractions in fish and other animals.

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