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THE *IN VITRO* RESPIRATORY PROPERTIES OF BLOOD OF THE PRIMITIVE FISH *POLYPTERUS SENEGALUS*

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INTRODUCTION

It can be assumed that the respiratory properties of blood reflect, to a certain degree, the adaptative changes of the gas-transport system due to both the phylogenetic development and the conditions of habitat in which the species survived. While their interpretation cannot be simple, the respiratory properties of blood are yet of interest from the point of view of comparative physiology (Prosser, 1961). Of special interest are the changes in the respiratory properties of blood connected with the evolution from water to land, i.e. with the replacement of the gill by the lung as respiratory organ. The lung fish and the primitive fish with accessory organs of air-breathing provide one of the connecting links between the purely aquatic and terrestrial mode of respiration.

It has already been shown that the swim-bladders of the primitive fish *Polypterus senegalus* function as accessory respiratory organs (Abdel Magid, Vokac & Nasr El Din Ahmed, 1970). The present investigation describes the respiratory properties of blood of this species, and the results are compared with the properties of blood of lung fishes and of mammalian blood as represented by human blood.

MATERIALS AND METHODS

As in our previous study (Abdel Magid *et al.* 1970) the fish used in this investigation were caught in the White Nile in the vicinity of Khartoum or else in the Jebel Awlia area some 30 miles to the south. The blood of 46 specimens of *Polypterus senegalus* (weighing 85–320 g) was investigated either separately or pooled. The fish were anaesthetized prior to sampling by being placed in 0·2% aqueous tricaine methane sulphonate (MS 222, Sandoz). The caudal peduncle was severed and the dripping blood was drawn into a syringe which contained 0·5 ml of mercury to ensure proper mixing of the contents. Approximately 1–1·5 ml of blood per 100 g of body weight was obtained in this way and 0·1 ml of heparin solution per 5 ml of blood was used to prevent coagulation. The syringes containing blood were kept in an ice-water bath and all analyses were started within 4 h of blood withdrawal.

Haemoglobin concentration was routinely determined photometrically in duplicate as that of cyanmethaemoglobin (haemoglobincyanide, HICN), using commercial cyanmethaemoglobin standards (Hycel, U.S.A.) for calibration (Ziljstra & van

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Kampen, 1960). A Unicam spectrophotometer SP. 500 was used for determination of extinction curves of cyanmethaemoglobin and oxyhaemoglobin solutions in the 460–590 mμ range (slit 0·03 mm). Packed cell volume was estimated in duplicate using a microhaematocrit centrifuge (7000 rev/min for 5 min). No correction for trapped plasma was applied. Erythrocyte count was carried out using Neubauer slide and Hayem solution for dilution of the blood. Mean corpuscular haemoglobin concentration and mean volume of erythrocytes were calculated from the primary values. The sizes of 100 erythrocytes and of their nuclei were determined with the aid of ocular micrometer in blood smears stained with Giemsa dye solution.

Equilibration of blood with known gas mixtures was carried out in a thermostated tonometer (Laué, 1951). The composition of the gas mixtures was controlled before each equilibration by duplicate analyses using a Scholander micro-analyser (Scholander, 1947). All equilibrations were carried out at 30 °C with the exception of the experiments in which the temperature shift of the O_2-dissociation curve was investigated. Oxygen and carbon dioxide content in 0·5 ml samples of the equilibrated blood were determined by combined analysis using a Van Slyke manometric apparatus and following the modifications of the original procedures as recommended by Bartels *et al.* (1963) and Consolazio, Johnson & Pecora (1963). The amount of dissolved oxygen in the blood at 30 °C was calculated by extrapolation as 0·34 ml/100 ml at P_{O_2} 100 mmHg (Bartels *et al.* 1963). pH was measured with an Astrup micro-electrode (E 5021) and pH meter (PHM 27, Radiometer, Copenhagen) thermostatically maintained at the temperature of the equilibrated blood.

RESULTS

Haematological observations

The haematological investigation performed in nine specimens chosen at random revealed a high inter-individual variation of the assessed values (Table 1). However, a distinct pattern can be traced in the results. The parallel decrease of haemoglobin concentration, haematocrit and erythrocyte count, connected with the increase of mean erythrocyte volume, indicates the presence of various degrees of anaemia of macrocytic type in at least one-third of the investigated specimens. The most constant value was that of the mean corpuscular haemoglobin concentration (approximately 32 g/100 ml), which decreased markedly only in specimen No. 9, suffering from the highest degree of anaemia.

The average size of 100 erythrocytes in smears of blood of specimen No. 3 (Table 1) was $17{\cdot}7 \pm 1{\cdot}2 \times 10{\cdot}3 \pm 0{\cdot}9$ μm. The average size of the nuclei of the measured cells was $7{\cdot}1 \pm 0{\cdot}4 \times 4{\cdot}1 \pm 0{\cdot}7$ μm.

Observed through the microscope the nucleus seems to occupy a large proportion of the cell area. But when the average volumes of both the cell and the nucleus were calculated, in the first approximation, as ellipsoids using the assessed mean diameters, the volume of the nucleus (18 μm^3) amounted to only 6–7% of the volume of the cell (275 μm^3). Since the nucleus does not contain haemoglobin, the true intracellular concentration of haemoglobin in the erythrocyte cytoplasm of *Polypterus* can be assumed as approximately 34 g/100 ml.

Table 1. *Haemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular haemoglobin concentration (MCHC), red blood cell count (RBC) and mean erythrocyte volume (MEV) in nine specimens of* Polypterus

No.	Hb (g/100 ml)	PCV (vol. %)	MCHC (g/100 ml)	RBC (10^6/mm^3)	MEV (μm^3)
1	14·0	43	32·6	—	—
2	12·4	39	31·8	1·01	386
3	10·9	34	32·1	1·15	296
4	10·8	33	32·7	0·76	434
5	10·5	34	30·9	0·71	479
6	9·7	29	33·4	—	—
7	8·5	27	31·5	0·50	540
8	6·2	21	29·5	0·33	636
9	4·3	17	25·3	—	—

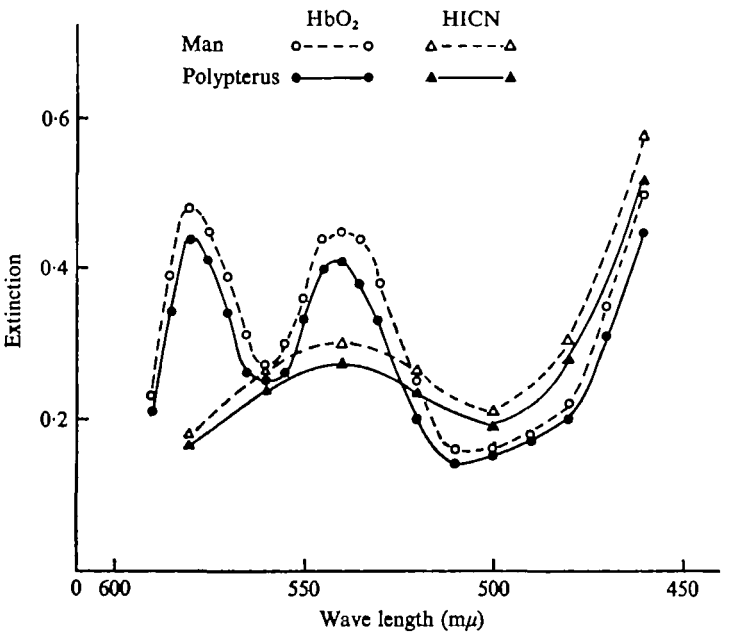

Fig. 1. Comparison of extinction curves of solutions of oxyhaemoglobin (HbO_2) and cyanmethaemoglobin (HICN) (Hb ~ 0·03 mM/l) for human blood and for *Polypterus* blood.

Transport of oxygen

With the exception of a small, dissolved proportion, the oxygen is transported by blood bound to haemoglobin. In order to find out whether the conventional photocolorimetric methods were suitable for the determination of haemoglobin concentration in the blood of *Polypterus*, the extinction curves of oxyhaemoglobin and cyanmethaemoglobin solutions were compared with those of human haemoglobin. Fig. 1 shows that the maxima were found at the same wavelengths as in solutions of human haemoglobin (Ziljstra, 1953) and that the course of the extinction curves of the solutions of *Polypterus* haemoglobin was strictly parallel to the course of the human curves.

Table 2. *Root effect. Calculated oxygen capacity (O_2 cap.) and estimated oxygen content (C_{O_2}) of blood of various pH equilibrated at P_{O_2} 200–220 mmHg*

N	Hb (g/100 ml)	pH	P_{CO_2} (mmHg)	$C_{HCO_3^-}$ (m-equiv/l)	O_2 cap (ml/100 ml)	C_{O_2} (ml/100 ml)	S_{O_2} (%)	C_{O_2}/Hb (ml/g)
5	10·3	7·74	5	7·0*	13·8	13·7	99·3	1·33
3	10·8	7·70	6	7·7*	14·5	14·6	100·7	1·35
5	10·3	7·65	8	9·2*	13·8	13·7	99·3	1·33
5	10·3	7·50	16	13·0*	13·8	13·8	100·0	1·34
5	10·4	7·49	6	5·1	13·9	13·7	98·6	1·32
4	10·0	7·47	6	4·4	13·4	13·3	99·3	1·33
2	10·4	7·35	30	17·2*	13·9	13·4	96·4	1·29
5	10·3	7·32	30	16·1*	13·8	13·6	98·6	1·32
5	10·4	7·16	31	11·2	13·9	13·0	93·5	1·25
1	14·0	7·13	31	10·7*	18·8	17·4	92·6	1·24
4	10·0	7·07	30	8·7	13·6	12·6	94·0	1·26
2	11·5	7·00	30	7·7*	15·4	14·4	93·5	1·25

* Calculated.

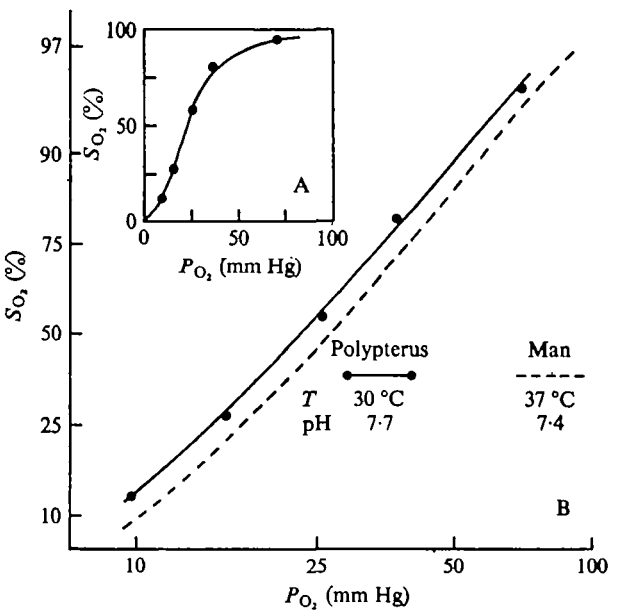

Fig. 2. (A) Oxygen dissociation curve of *Polypterus* blood. Hb 10·8 g/100 ml, PCV 35 vol. %. (B) The same curve, and oxygen dissociation curve of human blood in bi-logarithmic co-ordinates. Ordinate: % HbO_2 as log 100 Hb/HbO_2. Abscissa: P_{O_2} as log P_{O_2} mm Hg.

Table 2 enables a comparison of the calculated oxygen capacity (O_2 cap.) of *Polypterus* blood, arrived at as haemoglobin concentration (Hb) multiplied by Hüfner's coefficient 1·34, and the estimated amount of oxygen (C_{O_2}) bound to haemoglobin in samples of blood equilibrated in various experiments at P_{O_2} 200–220 mmHg. The samples usually contained pooled blood of 2–5 specimens (N) and their haemoglobin concentrations were well within the range found in the individual specimens (Table 1). The pH at the end of the equilibration depended both on the partial pressure of carbon dioxide in the equilibrating gas (P_{CO_2}) and on the amount of metabolic acids in th

Table 3. *Respiratory properties of blood of* Polypterus, *lungfishes and man*

	Polypterus	*Protopterus*	*Lepidosiren*	*Neoceratodus*	Man
Temperature (°C)	30	25	23	18	37
Hb (g/100 ml)	10–14	6·2	?	?	15
PCV (vol. %)	30–43	25	14–19	24–36	44
MCHC (g/100 ml)	31–33*	24·8	?	?	34
O capacity (ml/100 ml)	13·5–18·5	6·8	4·9–6·8	6·0–9·0	20
P_{50} (mmHg at P_{CO_2} 6 mmHg)	23·5	12	11	15	26·6
$\Delta \log P_{50}/\Delta$ pH (Bohr effect)	−0·43	−0·47	−0·24	−0·62	−0·48
$\Delta \log P50/\Delta T$ (temperature shift)	0·022	0·028	?	0·013	0·024
Root effect	±	?	?	0	0
$C_{HCO_3}-/\Delta$ pH (buffering capacity, m-equiv/l)	−15·4	−15·2	−14·9	−13·3	−31
C_{CO_2}/O_2 cap (Haldane effect mM/l)	−0·10	0	−0·12	−0·25	−0·13

* 'True' MCHC ~ 34 g/100 ml.

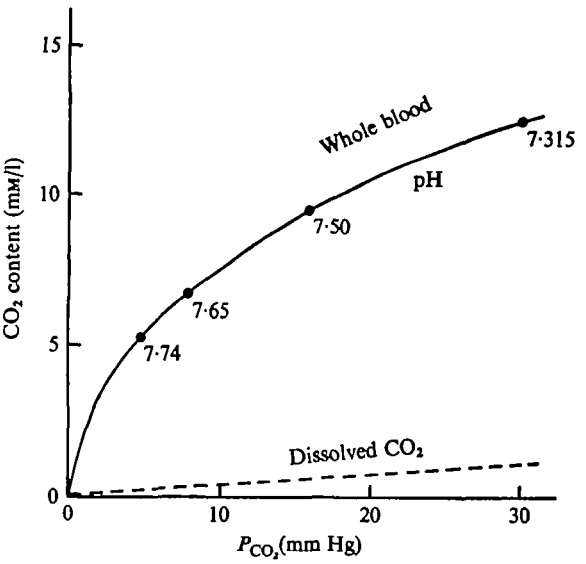

Fig. 3. CO_2 combining power in oxygenated blood of *Polypterus*. Hb 10·3 g/100 ml, PCV 34 vol. %.

drawn blood as indicated by the different concentrations of plasma bicarbonate (C_{HCO_3-p}) at the same P_{CO_2}.

The oxygen saturation (S_{O_2}) was calculated as the ratio of the measured amount of oxygen bound to haemoglobin (C_{O_2}) i.e. of the oxygen content of blood minus the amount of dissolved oxygen, and the calculated oxygen capacity (O_2 cap.). A complete saturation was found at pH 7·47–7·74 confirming that under these conditions approximately 1·34 ml of oxygen was bound to 1 g of haemoglobin (C_{O_2}/Hb). The saturation decreased slightly in the lower range of pH (7·00–7·16), showing a small Root effect in the blood of *Polypterus* (Root, 1931).

The oxygen dissociation curve determined in pooled blood of three specimens (Hb 10·8 g/100 ml, PCV 35 vol. %) showed a typical sigmoid shape (Fig. 2A). Drawn on logarithmic coordinates (Fig. 2B), the dissociation curve becomes almost linear

and the P_{O_2} at 50% saturation ($P50$) as well as the changes of $P50$ due to the temperature and pH shift of the curve can be read more accurately than when the usual linear coordinates are used (Dill *et al.* 1940). $P50$ of the investigated *Polypterus* blood at 30 °C and pH 7·7 was 23·5 mmHg as compared with $P50$ 26·6 mmHg of the standard dissociation curve in man (Severinghaus, 1968). The curves were parallel.

The oxygen affinity may be affected by change of pH, and the Bohr effect in *Polypterus* blood between pH 7·3 and 7·6 at 30 °C corresponded to $\Delta \log P50 = -0{\cdot}43\Delta$ pH. The effect of temperature on the position of the dissociation curve was investigated between 20 and 30 °C. and can be expressed as $\Delta \log P50 = 0{\cdot}022\Delta T$.

Transport of carbon dioxide

Fig. 3 shows the CO_2 combining power in oxygenated blood pooled from five specimens of *Polypterus* (Hb 10·3 g/100 ml, PCV 34 vol. %). The CO_2 dissociation curve had a typical steep initial portion and its slope even at P_{CO_2} 30 mmHg was markedly steeper than that for the dissolved carbon dioxide. The solubility of carbon dioxide at 30 °C in this blood of approximately 14 vol. % O_2 capacity was assumed, by extrapolation, as 0·0369 mM/l per mmHg P_{CO_2} (Bartels *et al.* 1963). Using the parameters of Fig. 3 the buffering capacity in the whole blood was calculated as $\Delta C_{HCO_3^-}/\Delta$ pH $= -15{\cdot}4$ mM/l. The relationship between pH and $C_{HCO_3^-}$ was linear. An apparent increase in the buffering capacity caused by increased ability of the blood to take up CO_2 with progressive reduction in haemoglobin oxygen saturation (Haldane effect) could be seen when the oxygen dissociation curve was construed. Using oxygen capacity as the measure of the concentration of active haemoglobin, the Haldane effect in the blood of *Polypterus* can be expressed as $\Delta C_{CO_2}/O_2$ cap. $= -0{\cdot}10$.

The dissociation constant of plasma carbonic acid (pK′) was determined by measuring the CO_2 content and pH in separated plasma of six samples of blood equilibrated at various values of P_{CO_2} and assuming the solubility of CO_2 in plasma at 30 °C. as 0·0362 mM/l per mmHg P_{CO_2} (Severinghaus, Stupfel & Bradley, 1956*a*). The range of pH of the samples was 7·07–7·55 and the calculated pK' of *Polypterus* plasma was found to be, on the average, 0·03 higher than pK' of human plasma at corresponding pH and temperature 30 °C (Severinghaus *et al.* 1956*b*). The pK' arrived at in this way was used for calculation of plasma bicarbonate concentration ($C_{HCO_3^-p}$) in Table 2.

Finally, Table 3 presents a comparison of parameters assessed in the blood of *Polypterus* with corresponding parameters in the blood of the lungfishes, *Protopterus aethiopicus* (Lenfant & Johansen, 1968), *Lepidosiren paradoxa* (Johansen & Lenfant, 1967), *Neoceratodus forsteri* (Lenfant, Johansen & Grigg 1966/7) and man (Bartels *et al.* 1963; Sigaard-Andersen, 1963; Severinghaus, 1968).

DISCUSSION

Respiratory properties of blood belong to the various respiratory functions which enable the necessary exchange of oxygen and carbon dioxide between the environment and the tissues, and which contribute, at the same time, to the maintenance of acid-base equilibrium. An effective oxygen transport is, in the first instance, a function of oxygen-binding pigments.

Several properties of the haemoglobin of *Polypterus* were found to be similar to those of mammalian haemoglobin, as represented by human haemoglobin. The absorption spectra of *Polypterus* oxyhaemoglobin and cyanmethaemoglobin solutions were identical with those of human haemoglobin solutions (Fig. 1). This allowed measurement of the haemoglobin concentration by conventional photocolorimetric methods.

A comparison of the measured oxygen capacity of blood and haemoglobin concentration yielded, at pH 7·3–7·7, approximately the same haemoglobin binding capacity of 1·34 ml O_2/g Hb as that of human blood. Only at pH lower than 7·2, a slightly less than complete saturation (Root effect), typical for blood of some fishes (Root, 1931), was found (Table 2). *In vivo*, the small Root effect is probably of no consequence for the efficiency of oxygen transport in *Polypterus*.

Disregarding the findings in species suffering from an apparent anaemia, the normal haemoglobin concentration in *Polypterus* appears to be rather high, 10–14 g per 100 ml blood (Table 1). Haemoglobin is contained in the erythrocytes, and an interesting observation was made concerning its corpuscular concentration. The actual concentration of haemoglobin in the cytoplasm of nucleated erythrocytes is always higher than that calculated from the haematocrit value because the nucleus occupies a part of the erythrocyte volume which does not contain haemoglobin. The 'true' mean corpuscular haemoglobin concentration in *Polypterus* was found to be, on the average, approximately 34 g/100 ml erythrocyte cytoplasm. It was the same haemoglobin concentration as that assessed in non-nucleated mammalian erythrocytes, where it seems to be, for physico-chemical reasons, the maximal attainable concentration (Wintrobe, 1967). Hypoxia can be considered as one of the strongest impulses which induce erythropoiesis and haemoglobin formation, but the mean corpuscular haemoglobin concentration in Peruvian natives living permanently at an altitude of 4500 m (P_{O_2} of the air 94 mmHg instead of 159 mmHg at sea level) was found to be unchanged in spite of their markedly increased haemoglobin concentration (Hurtado, 1945).

Compared to the relatively high oxygen affinity of haemoglobin in lungfishes, the oxygen affinity in *Polypterus* is low (Table 3). *In vivo*, the position of the oxygen dissociation curve is altered by changes in both the temperature and pH (Bohr effect). The comparatively high value of the temperature shift of the dissociation curve, similar to that of *Protopterus* (Lenfant & Johansen, 1968) and mammalian blood, is compatible with the rather uniform annual temperature of Nile water. The Bohr effect is not as conspicuously high as that in *Neoceratodus* (Lenfant, Johansen & Grigg, 1966), but it may be of importance as to the actual position of the oxygen dissociation curve in various parts of the circulatory system because the buffering capacity of *Polypterus* blood is rather low – approximately one-half of that of mammalian blood. The maintenance of acid-base equilibrium during respiration is helped by the Haldane effect, which, in the blood of *Polypterus* is of the same order of magnitude as in *Lepidosiren* (Johansen & Lenfant, 1967) and man.

SUMMARY

1. Respiratory properties of blood of *Polypterus senegalus* were studied *in vitro* at 30 °C.

2. The following values were found in species which did not suffer from an apparent anaemia: erythrocyte count 0·7–1·15 × 10^6/mm^3, haemoglobin concentration 10–14 g/100 ml, haematocrit 33–43 vol. %, mean erythrocyte volume 300–480 μm^3, and mean erythrocyte size 18 × 10 μm. Subtracting the calculated volume of the nucleus, the 'true' mean corpuscular haemoglobin concentration in the erythrocyte cytoplasm was found to be 34 g/100 ml, which corresponds to the haemoglobin concentration in the non-nucleated mammalian erythrocytes.

3. Transport of oxygen: The oxygen affinity of haemoglobin was found to be moderate, $P50$ being 23·5 mmHg at P_{CO_2} 6 mmHg and pH 7·7. The O_2 dissociation curve had a typical sigmoid shape. The oxygen capacity of the investigated samples was 13·5–18·5 ml/100 ml, showing a binding capacity of approximately 1·34 ml O_2 per 1 g Hb at pH > 7·3. A slight Root effect was observed at pH lower than 7·2. The coefficients of the Bohr effect ($\Delta \log P\,50/\Delta$ pH) and of the temperature shift of the oxygen dissociation curve ($\Delta \log P50/\Delta T$) were −0·43 and 0·022, respectively.

4. Transport of carbon dioxide: the CO_2 combining power in oxygenated blood was 12·5 mM/l at P_{CO_2} 30 mmHg and pH 7·3 and the CO_2 dissociation curve showed the typical steep initial portion. The buffering capacity of blood ($C_{HCO_3^-}/\Delta$ pH) was moderate, the coefficient being −15·4. The Haldane effect was also found to be moderate, C_{CO_2}/O_2 cap. being −0·10.

The dissociation constant of plasma carbonic acid (pK′) was assessed to be 0·03 higher than that of human plasma at corresponding pH and temperature 30 °C.

5. The position of the absorption maxima of *Polypterus* oxyhaemoglobin and cyanmethaemoglobin solutions was the same as that of human haemoglobin solutions.

6. The results are compared with the respiratory properties of lungfish and human blood.

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