# Effects of buffer composition, pH and temperature on oxygen binding by planorbid snail haemoglobins

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The oxygen-binding properties of the haemoglobins of four species of freshwater planorbid snails *Bulinus africanus*, *Biomphalaria glabrata*, *Bulinus tropicus* and *Heliosoma trivolvis* were examined. Samples chromatographed on Sephadex G75 in CO-saturated buffer (0,1 mol $dm^{-3}$ $MgCl_2$, 0,05 mol $dm^{-3}$ Tris-HCl, 0,5 mmol $dm^{-3}$ phenylmethyl sulfonyl fluoride (PMSF), 1,0 mmol $dm^{-3}$ Dithioerytritol, ionic strength 0,3) gave the best stability against methaemoglobin formation. The highest haemoglobin oxygen affinity ($P_{50}$ = 0,35 mmHg) was measured with $MgCl_2$-buffer with a Hill coefficient, $n_{max}$, of 1,16. An $n_{max}$ value of 2,2 was obtained with snail buffer (4 mmol $dm^{-3}$ KCl, 50 mmol $dm^{-3}$ NaCl, 1 mmol $dm^{-3}$ $MgCl_2$, 6 mmol $dm^{-3}$ $CaCl_2$, 50 mmol $dm^{-3}$ Tris-HCl, 0,5 mmol $dm^{-3}$ PMSF, ionic strength 0,075, pH 8,0) that gives a $P_{50}$ value of 1,58 mmHg. A Bohr effect of between –0,44 and –0,11 was measured in the physiological range of pH 8,0–7,1 in snail buffer for the three species, *B. glabrata*, *B. africanus* and *B. tropicus*. For these planorbids the heat of oxygenation of the haemoglobins, ΔH, was –95,3 kJ/mol and –68,1 kJ/mol at pH 8,0 and pH 7,1 respectively.

Die suurstofbinding van die hemoglobien van vier varswaterslakspesies, *Bulinus africanus*, *Biomphalaria glabrata*, *Bulinus tropicus* en *Heliosoma trivolvis*, is ondersoek. Monsters wat gechromatografeer is met Sephadex G75 in CO-versadigde buffer (0,1 mol $dm^{-3}$ $MgCl_2$, 0,05 mol $dm^{-3}$ Tris-HCl, 0,5 mmol $dm^{-3}$ feniel-metielsulfonielfluoried (PMSF), 1,0 mmol $dm^{-3}$ dithioeritritol, ioonsterkte 0,3) gee die beste stabiliteit teen methemoglobienvorming. Die hoogste waarde van hemoglobien-suurstofaffiniteit ($P_{50}$ = 0,35 mmHg) is met $MgCl_2$-buffer gemeet met 'n Hill-koëffisiënt $n_{maks}$, van 1,16. Die waarde $n_{maks}$ van 2,2 is met slakbuffer verkry (4 mmol $dm^{-3}$ KCl, 50 mmol $dm^{-3}$ NaCl, 1 mmol $dm^{-3}$ $MgCl_2$, 6 mmol $dm^{-3}$ $CaCl_2$, 50 mmol $dm^{-3}$ Tris-HCl, 0,5 mmol $dm^{-3}$ PMSF, ioonsterkte 0,075, pH 8,0) wat 'n $P_{50}$ waarde van 1,58 mmHg gee. 'n Bohr-effek tussen –0,44 en –0,11 is gemeet by 'n fisiologiese pH omvang van pH 8,0 en pH p7,1 in slakbuffer vir die drie spesies *B. glabrata*, *B. africanus* en *B. tropicus*. Vir hierdie spesies is die oksigeneringswarmte van die hemoglobiene (ΔH) –95,3 kJ/mol en –68,1 kJ/mol by pH 8,0 en 7,1 respektiewelik.

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There are several reasons why planorbid haemoglobin function has received much attention from respiratory physiologists. Firstly, planorbid snails are particularly well adapted to sustain large colonies in extreme ecological conditions of low oxygen and highly variable temperatures in the tropical parts of the world. The shallow ponds and streams where most of these snail species reproduce and grow are extremely unstable regarding their capacity to hold water or to keep the temperature, pH and the oxygen concentration at physiologically acceptable levels (Sturrock & Sturrock 1972; Combrinck & Van Eeden 1975; Brown 1980). Secondly, the extracellular haemoglobins carried by molluscs display a large variation in molecular size, ranging from monomeric molecules to highly aggregated molecules comparable in size to intracellular organelles (Vinogradov 1985). The smaller molecules are of great interest because they provide useful models of co-operative systems. The polymeric extracellular haemoglobins have been insufficiently studied in terms of quaternary protein structure and haemoglobin synthesis (Terwilliger & Terwilliger 1985). Lastly the functional properties of the haemoglobins in aquatic animals usually reflect the ecophysiology of the animals where they live. An example of such an adaptation is the synthesis of at least two kinds of haemoglobins in many Amazonian fishes to meet both the environmental limitations and metabolic needs (Riggs 1979). Determinations of the molecular masses of planorbid haemoglobins (Figueiredo, Gomez, Heneine, Santos & Hargreaves 1973; Almeida & Neves 1974; Wood & Mosby 1975; Afonso, Arrieta & Neves 1976; Nacimento, Daniel & Heneine 1982) show a sedimentation coefficient, $S_{20,w}$, between 34,8 and 35,0 and a molecular mass of $1,65 \times 10^6 \pm 0,04$. Functional studies on the haemoglobins of planorbid snails with the aid of tonometric devices (Jones 1964; Figueiredo *et al.* 1973; Wood & Mosby 1975; Terwilliger, Terwilliger, Bonaventura & Bonaventura 1977; Van Aardt & Frey 1981; Nacimento *et al.* 1982; Terwilliger & Terwilliger 1982) show that the Bohr-effect is absent or weakly developed with a high affinity of the molecule for oxygen and a value for the Hill coefficient, $n$, of between 1,08 and 3,4. Lapennas, Colacino & Bonaventura (1981) pointed out that thin-layer methods for the study of haemoglobin-oxygen binding offer many advantages over classic tonometric methods — the most important being speed, small sample size and gentle treatment of the sample. Particular care should be taken to avoid undue mechanical stress to giant haemoglobins during tonometric measurement and not to promote its oxidation to methaemoglobin during sample preparation (Bonaventura & Bonaventura 1981).

The thin-layer method used here is that of Dolman & Gill (1978). This technique does not suffer from the disadvantages of some other thin-layer techniques because non-equilibrium between sample and $O_2$

electrode is not a problem (Lapennas *et al.* 1981; Gill 1981).

In this study the oxygenation properties of the haemoglobin of four species of planorbid pulmonate snails have been compared as a function of both temperature and pH. We have developed a buffer system in which the formation of methaemoglobin is minimized.

## Materials and Methods

The freshwater snail species were reared and maintained in the laboratory as described by De Kock & Van Eeden (1980). Haemolymph was sampled by mechanical stimulation of the foot surface, which yields up to 100 µl haemolymph from each snail (snail mass 0,4 g – 0,7 g). The sample is forced out, probably through the haemal pore (Lever & Bekius 1965). The haemolymph was pipetted from the bottom of the penultimate whorl with a glass pipette. Exactly 100 µl of haemolymph were diluted with 100 µl of snail buffer (buffer no. 10, Table 1) at 0°C. The haemolymph–buffer mixture was centrifuged for 5 min at 10 000g. The supernatant was pipetted off with a wide orifice Transfer-pipettor (Brand, West Germany) and transferred into a 150 µl centrifuge tube. The samples were centrifuged for 30 min at 175 000g. with a Beckman Airfuge (Beckman, U.S.A.). The temperature of the sample was kept below 20°C by cooling the pressurized air in the cylinder used to drive the small Airfuge rotor to 15°C. The supernatant was pipetted off with the aid of a thin polyethylene tube fixed to a syringe needle. Usually the compacted haemoglobin was diluted in a ratio of 1 : 1 with the appropriate buffer (Table 1). The percentage methaemoglobin formation was determined (see later) for each buffer tested. Eventually only three buffer types namely no. 8, 10 and 11 were used for the construction of oxygen equilibrium curves (Table 2). With these three buffers the following techniques were used in order to avoid methaemoglobin formation and to 'clean' the sample (Bonaventura & Bonaventura 1981):

(i) After collection the haemolymph was gently filtered through a 0,45 µm mesh filter (Milex, HV4) with the aid of a 1,0 ml syringe with a plastic Luer-coupling. Filtering of the haemocytes from the haemolymph was done to prevent proteolytic enzymes, released by lysis from these haemocytes to digest the haemoglobin molecule. After filtration the haemolymph was diluted 1 : 1 with buffer (buffer no. 8, Table 1) pelleted by the Airfuge and the compacted haemoglobin diluted 1 : 1 with buffer no. 8 (Table 1).

(ii) After extraction and concentration the pelleted haemoglobin (diluted 1 : 1 with buffer no. 8, Table 1 ) was dialysed in batches of 42 µl within specially made polyethylene dialysis buttons. These buttons, 20 mm in diameter, are constructed in three parts. The two large perforated lids each fitted on the inside with a 13,5 mm diameter membrane, screw tightly onto a frame in order to create a 42 µl reservoir. Six of these dialysis buttons were clamped vertically on a horizontal disc provided with a centrally located Perspex shaft that could be turned at a rate of 20 rpm. The dialysis of haemolymph was carried out in the presence of buffer no. 8 at a dilution of 1 : 50 000 for 12 h at 4°C.

(iii) Collected haemolymph was centrifuged for 15 min at

**Table 1** Summary of the buffer systems tested at 25°C in stabilizing methaemoglobin formation during measurements of haemoglobin-oxygen binding by a thin-layer method; unstable xxxx indicates more than 10% methaemoglobin formation per hour; stable xxxx indicates less than 1% methaemoglobin formation per hour; EDTA, Etylenediaminetetra-acetic acid; TES, 2-{[tris-(hydroxymethyl)methyl]amino} ethane-sulfonic acid; PMSF, phenylmethylsulfonylfluoride; DITHIO, dithioerythritol; TRIS-HCl, [tris-(hydroxymethyl)-aminomethane, hydrochloride]. Buffer concentrations are given in mol $dm^{-3}$. The different buffers were tested with preparations from *B. glabrata* haemolymph

| Buffer system | Ionic strength | $Na_2HPO_4$ mol $dm^{-3}$ | $K_2HPO_4$ mol $dm^{-3}$ | NaCl mol $dm^{-3}$ | $MgCl_2$ mol $dm^{-3}$ | $CaCl_2$ mol $dm^{-3}$ | Tris-HCl mol $dm^{-3}$ | EDTA mol $dm^{-3}$ | TES mol $dm^{-3}$ | PMSF mol $dm^{-3}$ | DITHIO mol $dm^{-3}$ | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0,70 | 0,1 | | 0,4 | | | | | | | | stable x |
| 2 | 0,10 | | | 0,1 | | | 0,05 | 0,001 | | | | unstable x |
| 3 | 0,103 | | | 0,1 | 0,01 | | 0,1 | | | | | stable x |
| 4 | 0,133 | | | 0,1 | 0,001 | 0,01 | 0,05 | | | 0,0005 | 0,001 | stable |
| 5 | 0,1003 | | | 0,1 | | | | | 0,05 | 0,0005 | 0,001 | unstable |
| 6 | 0,300 | | 0,1 | | | | | 0,001 | | | | stable |
| 7 | 0,400 | 0,1 ($NH_4$ acet.) | | | | 0,01 | | | | 0,001 | | unstable xxxx |
| 8* ● ○ | 0,300 | | | | 0,1 | | 0,05 | | | 0,0005 | 0,001 | stable xxxx |
| 9 | 0,200 | 0,2 (KCl) | | | | | 0,05 | | | | | unstable xx |
| 10+ (snail) | 0,075 | 0,004 (KCl) | | 0,05 | 0,001 | 0,006 | 0,05 | | | 0,0005 | | stable xxx for *B. tropicus* |
| 11 (NaCl) | 0,200 | | | 0,2 | | | 0,2 | | | | | stable x for *H. trivolvis* |

* Buffer no. 8 with CO treatment: stable xxx; ●, with CO and Sephadex treatment: stable xxxx; ○, with CO and dialysis: unstable xxxx

\+ Buffer no. 10 with CO treatment: stable xx

**Table 2** Haemoglobin-oxygen-binding data derived from Hill-plots obtained from four planorbid snail species with different buffers, pH and temperatures. ΔH was calculated from data obtained from Hill-plots made at 25°C (e.g. Hill-plot no. C) against Hill-plot data from 7°C (e.g. Hill-plot no. A). The Bohr-effect was calculated from Hill-plot data (e.g. Hill-plot F against Hill-plot G) for different pH conditions, a–d data from Hill-plots not presented in Figures 1–5. In total 114 Hill-plots were made (*n* = number of determinations). The haemoglobin samples were not subjected to $CO_2$ either in gas form or as a bicarbonate in the buffer systems used. * Haemoglobin samples chromatographed on Sephadex-G75

| Planorbid snail | Hill plot no. (Figure 1–5) | °C | $P_{50}$ (mmHg) | log $P_{50}$ | $n_{max}$ | Buffer system no. | pH | Bohr-effect ($\Delta\log P_{50} / \Delta pH$) | ΔH (Heat of oxygenation) (kJ $mol^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| *B. glabrata* | A* (*n* = 2) | 7 | 0,35 | –0,45 | 1,16 | 8 | 7,5 | | |
| | B* (*n* = 3) | 15 | 0,59 | –0,22 | 1,22 | 8 | 7,5 | | C : A = – 64,4 |
| | C* (*n* = 3) | 25 | 1,88 | 0,27 | 1,26 | 8 | 7,5 | | |
| | D (*n* = 2) | 7 | 1,05 | 0,02 | 1,19 | 8 | 7,5 | | F : D = – 60,2 |
| | E (*n* = 3) | 15 | 2,51 | 0,40 | 1,29 | 8 | 7,5 | | |
| | F (*n* = 2) | 25 | 5,01 | 0,70 | 1,40 | 8 | 7,5 | F : G = –0,44 | |
| | G (*n* = 2) | 25 | 8,31 | 0,92 | 1,30 | 8 | 7,0 | | |
| | H (*n* = 3) | 15 | 8,70 | 0,94 | 1,30 | 8 | 6,5 | | |
| *B. glabrata* | I (*n* = 2) | 7 | 0,66 | –0,17 | 1,50 | 10 | 8,0 | | K : I = –86,9 |
| | J (*n* = 5) | 15 | 1,58 | 0,20 | 2,20 | 10 | 8,0 | | |
| | K (*n* = 6) | 25 | 6,30 | 0,80 | 1,80 | 10 | 8,0 | | N : L = –76,6 |
| | L (*n* = 2) | 7 | 1,58 | 0,20 | 1,40 | 10 | 7,1 | K : N = –0,29 | |
| | M (*n* = 3) | 15 | 4,46 | 0,65 | 1,40 | 10 | 7,1 | | |
| | N (*n* = 5) | 25 | 11,54 | 1,06 | 1,20 | 10 | 7,1 | | |
| | O (*n* = 2) | 15 | 1,88 | 0,27 | 1,40 | 11 | 8,0 | | |
| *B. africanus* | P (*n* = 3) | 7 | 0,26 | –0,57 | 1,60 | 10 | 8,0 | | |
| | Q (*n* = 4) | 15 | 1,15 | 0,06 | 1,70 | 10 | 8,0 | | R : P = –116,0 |
| | R (*n* = 5) | 25 | 5,30 | 0,72 | 1,80 | 10 | 8,0 | | |
| | S (*n* = 2) | 7 | 1,15 | 0,06 | 1,50 | 10 | 7,1 | R : U = –0,11 | |
| | T (*n* = 3) | 15 | 3,34 | 0,52 | 1,50 | 10 | 7,1 | | U : S = – 67,8 |
| | U (*n* = 6) | 25 | 6,68 | 0,82 | 1,60 | 10 | 7,1 | | |
| *B. tropicus* | V (*n* = 3) | 7 | 0,66 | –0,17 | 1,80 | 10 | 8,0 | | |
| | W (*n* = 4) | 15 | 1,15 | 0,06 | 1,90 | 10 | 8,0 | | |
| | X (*n* = 5) | 25 | 5,62 | 0,75 | 2,10 | 10 | 8,0 | | X : V = – 82,4 |
| | Y (*n* = 3) | 7 | 0,89 | 0,05 | 0,93 | 10 | 7,1 | X : $Z^1$ = –0,24 | |
| | Z (*n* = 3) | 15 | 4,21 | 0,62 | 1,20 | 10 | 7,1 | | |
| | $Z^1$ (*n* = 5) | 25 | 9,44 | 0,97 | 1,10 | 10 | 7,1 | | $Z^1$ : Y = – 90,0 |
| *H. trivolvis* | $Z^2$ (*n* = 3) | 15 | 0,68 | 0,16 | 1,50 | 10 | 8,0 | | |
| | $Z^3$ (*n* = 3) | 15 | 2,23 | 0,35 | 1,50 | 11 | 8,0 | | |
| *B. glabrata* | a (*n* = 8) | 25 | 4,57 | 0,66 | 1,50 | 8 | 8,0 | a : b = –0,23 | |
| | b (*n* = 2) | 25 | 7,41 | 0,87 | 1,36 | 8 | 7,1 | | |
| | c (*n* = 3) | 15 | 6,67 | 0,82 | 1,40 | 8 | 6,5 | | |
| | d (*n* = 4) | 25 | 10,59 | 1,02 | 1,30 | 8 | 6,5 | a : d = –0,32 | |

50g. to remove the haemocytes (Bonaventura & Bonaventura 1981). After 1 : 1 dilution with buffer no. 8 (Table 1) the haemoglobin was pelleted with the aid of the Airfuge and the supernatant removed. The haemolymph was diluted 1 : 1 with buffer no. 8.

(iv) After collection and concentration the haemolymph sample was chromatographed at 4°C with Sephadex-75. The column (15 × 0,9 cm; Pharmacia, Sweden) with a bed volume of 7 ml was prepared according to Read & Terwilliger (1973). Fifty millilitres of ice cold buffer no. 8 saturated with CO (Read & Terwilliger 1973) at pH 8 was used to equilibrate the column at a flow rate of 8 ml/h. Usually, 100 µl of sample would be eluted from the Sephadex column at 8 ml/h at 4°C. The haemoglobin band (1,5–2,0 ml in volume) collected after 30–40 min was again concentrated with the Airfuge and pelleted twice with ice cold CO-saturated buffer no. 8. The CO was removed from the haemoglobin with light photons just before the start of the oxygen-binding measurements and just after preparation as a thin layer in the optical cell (see later). To convert CO-haemoglobin to the oxygenated form the haemoglobin layer in the optical cell was subjected to pure oxygen while flashing with a high intensity light source at 5-s intervals for 5 min. The light was guided as 'cold light' to the outer quartz window of the optical cell with the aid of an

optical fibre cluster (Schott Mainz, Model KL, West Germany).

Oxygen-binding equilibria of the snail haemoglobins were measured using a thin-layer technique (Dolman & Gill 1978). A Clark-type oxygen-electrode (Model E 5046, Radiometer, Denmark) was installed next to the optical cell containing the haemoglobin layer. The electrode was used as a gross monitor of $PO_2$. Changes in the partial pressure of $O_2$ in the chamber were achieved with a precision valve for gas dilution mounted next to the optical cell. The temperature of the cell holder, cell and valve was thermostatically maintained at ± 0,3°C by water circulating through copper tubes. The thin-layer apparatus was mounted inside the measuring chamber of a computerized spectrophotometer (Philips, Pye-Unicam, Model PU 8800). The formation of methaemoglobin from haemoglobin was monitored at 412 nm for at least 30 min and quantified by a method described by Fushitani, Imai & Riggs (1986). Oxygen-binding equilibria were measured at 428 nm. Leakages of oxygen from the surrounding air into the system during binding measurements of high oxygen affinity ($P_{50}$ = 0,35 mmHg) haemoglobins were checked before and after each measurement by purging high purity $N_2$ through the system and recording the spectrum of the deoxyhaemoglobin. The results were analysed as described by Dolman & Gill (1978) with the aid of a microcomputer (IBM-PC/XT, IBM, U.S.A.) in conjunction with a special software PASCAL program (Tiaan van Aardt, pers. comm.). At least two and up to eight equilibrium curves were constructed for each pH, temperature or buffer system as indicated in Table 2. The Hill-plot data for each measurement (Table 2) were superimposed on each other only if graphs showed $P_{50}$ values that did not differ by more than 1,0 mmHg. Furthermore, if the lower, middle and upper parts of the graphs (2 to 8 graphs were superimposed) on Hill-plots had the same slope the graphs were redrawn to present one Hill-plot. In total 114 Hill-plots were made in this study, as represented in Figures 1–5. When very high haemoglobin oxygen affinities ($P_{50}$ < 5 mmHg) were encountered, the first five to six absorbance values (measured after starting the dilution steps at a $PO_2$ of 131 mmHg, Potchefstroom, altitude 1288 m) were similar. This is so because at these first dilution steps 100% oxygen saturation of the haemoglobin molecules is possible for the high affinity haemoglobin. Therefore the first five absorbance values out of 10–12 forming the upper asymptote of the Hill-plot graph do not represent actual absorbance values. These absorbance values are therefore meaningless and were discarded when the slope of the upper asymptote was compiled in the final drawing.

## Results

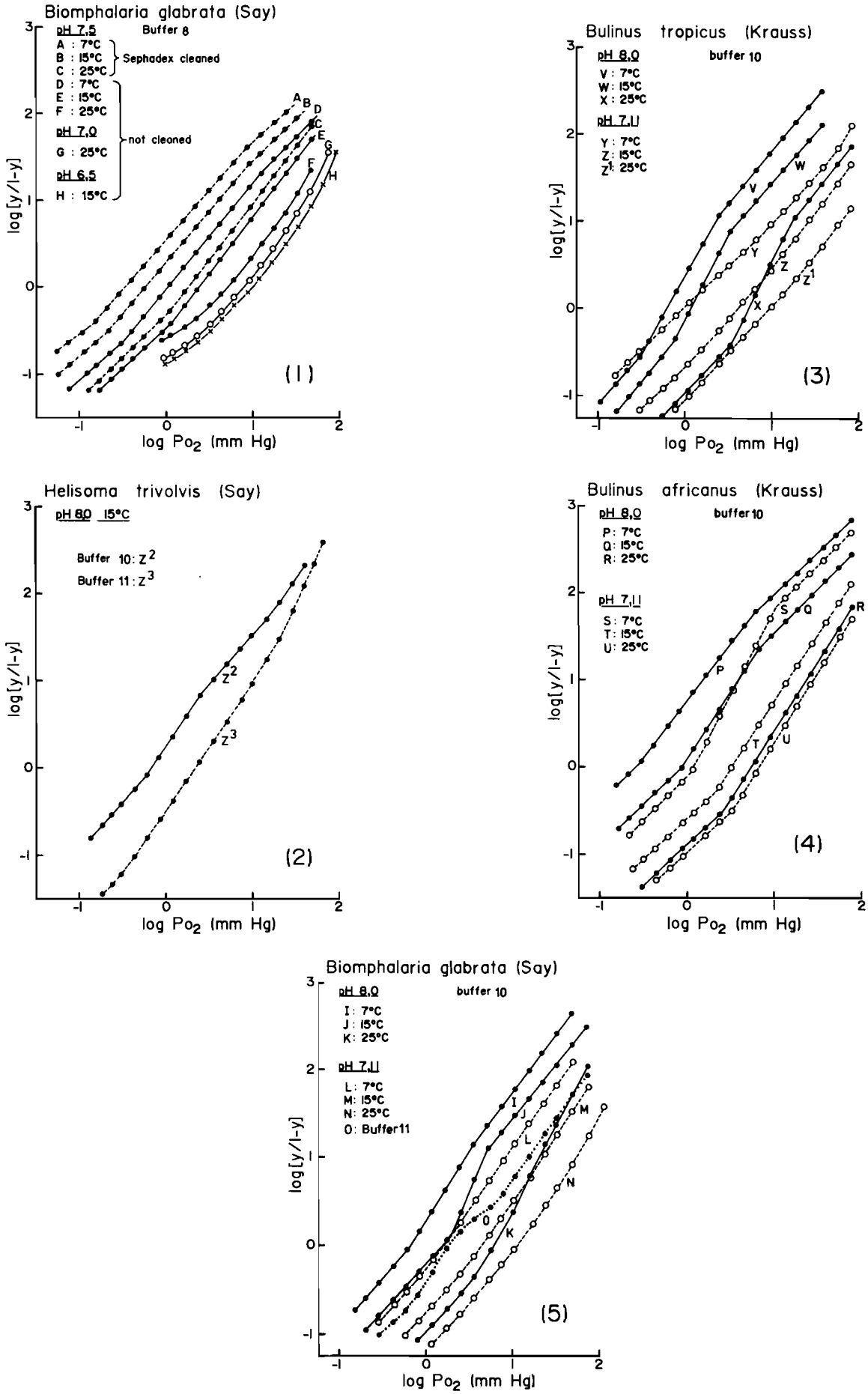
From the results in Table 1 it can be concluded that buffer no. 8 gives the best stabilization of the haemoglobin preparation against methaemoglobin formation. Ammonium acetate in combination with $CaCl_2$ (buffer 7) is the least effective in preventing the oxidation of snail haemoglobin to methaemoglobin. This is in accordance with the report by Bonaventura & Bonaventura (1981 ) that metal ions such as calcium may be responsible for the catalysis of the oxidation. The snail buffer, buffer no. 10, represents the major electrolyte composition found in the haemolymph of the four snail species studied. This buffer system was fairly successful in stabilizing *B. tropicus* and *B. africanus* haemoglobin but not *B. glabrata* haemoglobin (Table 1). Dialysis with buffer no. 8 caused considerable methaemoglobin formation (Table 1) while CO treatment with Sephadex 75 in no. 8 buffer gives the highest stability. It is interesting to note that in this laboratory practically no methaemoglobin from haemoglobin was formed when haemoglobins from a freshwater fish *(Labeo capensis)* and a bullfrog *(Pixicephalus adspersa)* were prepared with the same chemicals, buffers and distilled water (unpublished data). This is an indication of the ease of oxidation of the haemoglobin molecule of planorbid snails. Generally high ionic strength buffers containing NaCl and $MgCl_2$ seem to stabilize the haemoglobin better than low ionic strength buffers (Table 1). Contrary to the observations of Bonaventura & Bonaventura (1981) the high molecular mass haemoglobins of the four planorbid snails examined are quite resistant to dissociation during the extraction and purification steps. With low ionic strength buffers (no. 10, Table 1) the haemoglobin solution could easily be pelleted down within 15 min with the Airfuge without any trace of haemoglobin in solution in the supernatant when spectrophotometrically examined. This was also true when haemolymph was diluted 1 : 2 with distilled water (personal observations).

From Table 2 it is evident that a Bohr-effect, albeit small, is present for the three species examined. A value of –0,44 was found for *B. glabratus* at 25°C when the pH changed from 7,5 to 7,0 in $MgCl_2$ buffer. The smallest Bohr-effect, namely, –0,11 occurred for *B. africanus* with the snail buffer also at 25°C but with a pH change from 8,0 to 7,1.

The degree of haemoglobin co-operativity, $n_{max}$, was the highest in snail buffer with values ranging between 1,8 and 2,2 at 25°C and a pH of 8,0 (Table 2). Lower pH values decreased the co-operativity in snail buffer. Higher ionic strength buffers such as NaCl and $MgCl_2$ based buffers (Table 1) also decreased the $n_{max}$ value. Very low co-operativity was found for *B. glabrata* haemoglobin after Sephadex 75 chromatography with a $MgCl_2$ based buffer (Table 2).

Between a pH of 6,5 and 8,0, using the three buffer systems the haemoglobin oxygen affinity for the four planorbid species varied between 0,35 mmHg and 11,54 mmHg. Compared to other invertebrate haemoglobins (Weber 1980) our planorbid snails may be regarded as possessing high oxygen affinity haemoglobins. Sephadex 75-cleaned haemoglobin from *B. glabrata* (Table 2 A*, B* and C*) increased the oxygen affinity about fourfold compared with non-chromatographed samples. This is an indication of an unknown co-factor modulation of haemoglobin affinity in *B. glabrata*.

The effect of temperature on the oxygen affinity of the haemoglobins in the species investigated was higher than the effect of either the pH or ionic strength of the buffers




**Figures 1–5** Hill-plots of oxygen binding by haemoglobins of *Biomphalaria glabrata, Heliosoma trivolvis, Bulinus tropicus* and *Bulinus africanus* in different buffers and at different pH and temperatures. P, partial pressure of oxygen; Y, fractional degree of oxygenation.

used (Table 2). A rise in temperature of 18°C decreased the haemoglobin oxygen affinity nearly tenfold for *B. glabrata*, 10,6 times for *B. tropicus* and 20,3 times for *B. africanus* (Table 2). The heat of oxygenation, ΔH, was calculated with respect to the temperature change at constant pH using the equation:

$$\Delta H = -2{,}303\ R\Delta \log P_{50} / \Delta(1/T)\ \text{kJ mol}^{-1},$$

where R is the gas constant and T the absolute temperature.

From these calculations (Table 2) a mean ΔH value of –95,1 kJ $mol^{-1}$ was found for *B. glabrata, B. africanus* and *B. tropicus* at a pH of 8 and –78,4 kJ $mol^{-1}$ at a pH of 7,1 when using snail buffer no. 10. For *B. glabrata* in $MgCl_2$ based buffer at pH 7,5, ΔH was –64,4 kJ $mol^{-1}$ after Sephadex 75-cleaning and –60,2 kJ $mol^{-1}$ before cleaning (Table 2).

## Discussion

One of the functional characteristics of the planorbid haemoglobins found in this study is the weak co-operativity of its functional groups with oxygen compared with most vertebrate haemoglobins. This property, however, was also found with other gastropod haemoglobins (Bonaventura & Bonaventura, 1983) but generally not for most of the haemocyanin-bearing gastropods with $n_{max}$ values between 2 and 3 (Bonaventura & Bonaventura 1983). The near abolition of haem–haem interaction in planorbids is further enhanced by an unphysiologically low pH (6,5) and high ionic strength buffers. Another feature is the small Bohr-effect and relatively high oxygen affinity found for these haemoglobins. These three properties of planorbid snail haemoglobin may be related to the environment where these snails live. The snail buffer used for most of the experiments closely resembles the haemolymph of snails regarding the ionic strength and electrolyte composition (Van Aardt & Coertze 1981). When using this buffer the $P_{50}$ value at 25°C and at a pH of 8,0, which is the normal pH of the haemolymph (Van Aardt & Frey 1981) is between 5,3 and 6,3 mmHg for each of the three species namely *B. glabrata, B. africanus* and *B. tropicus* (Table 2). These planorbid snails prefer an alkaline water habitat with a pH 7,8 to 9,5 and a diurnal fluctuation of approximately one pH unit (Combrinck & Van Eeden 1975). Furthermore, dissolved oxygen in small bodies of water may approach $PO_2$ values near zero mmHg during the night with concomitantly high $PCO_2$ values (> 18 mmHg) when aquatic plants are present (Brown 1980; Dejours 1981). A high oxygen affinity haemoglobin with a small Bohr-effect and low haem-haem co-operativity would be an ideal respiratory pigment to bind small amounts of oxygen (at high temperature and pH) at the gas exchange surfaces for transportation to the cells and tissues.

On morphological grounds it is interesting to note that all members of the Planorbidae are haemoglobin carriers and possess a conspicuous and well-developed pseudobranch. This highly vascularized secondary gill (Van Aardt & Van Eeden 1969; Baker 1945) evolved when planorbids from a divergent stock of land pulmonates established themselves in temporary freshwater bodies low in oxygen content (Baker 1945). Furthermore it was found (Van Aardt & Frey 1979) that the oxygen depletion rate of a body of water by *B. africanus* is linear against time up to a partial oxygen pressure of 42 mmHg at 26°C. This $PO_2$ value, the critical oxygen pressure, Pc, (Herreid 1980) is usually determined by the limitations of the oxygen-carrying capacities of the haemolymph, particularly the haemoglobin part (McMahon 1984). From Figure 4 (graph R) it can be concluded that, at 25°C with $P_{50}$ = 5,30 and $n$ = 1,8, more than 95% oxygenation of the haemoglobin can be achieved at a $PO_2$ of 33 mmHg for *B. africanus*. Thus low ambient $PO_2$ values can nearly saturate the haemoglobin with $O_2$, with the effect that aerobic but not anaerobic respiratory processes would be functioning for this snail.

When constructing the oxygen dissociation curves (Figures 1–5) the samples were not subjected to $CO_2$ either in gas form or as a bicarbonate in the buffer systems used. It therefore can be safely concluded that the Bohr-effect found in these planorbid snails is caused by changes of the hydrogen ion concentration and not by $CO_2$. Weber (1980) and Jones (1972) also found that many aquatic invertebrates do not have a Bohr-effect caused by $CO_2$. Recently Abe & Meirelles (1985) also substantiated this finding for the haemoglobin of the earthworm *Glossoscolex paulistus*. Slow-moving animals in sluggish or stagnant water show small Bohr-effects (Weber 1980) while haemoglobin from trout in trout water show a large Bohr-effect upon small changes of $PCO_2$ (Hughes, O'Neill & Van Aardt 1975).

Early work on *Planorbis corneus* (Zaaijer & Wolvekamp 1958) could not demonstrate that increasing temperatures above 20°C decrease oxygen affinity. However, data from Weber (1980) on *P. corneus* and our results on tropical planorbids indicate that oxygen affinity decreases linearly with increasing temperatures. The temperate planorbid dweller *Planorbis corneus*, has a heat of oxygenation, ΔH, of 59,3 kJ (Weber 1980) which is lower compared to that of the planorbid snails that inhabit tropical regions. At a pH of 8,0 *B. glabrata, B. tropicus* and *B. africanus* haemoglobin gives ΔH values of –136,9 kJ $mol^{-1}$ , –82,4 kJ $mol^{-1}$ and –116 kJ $mol^{-1}$ respectively. This temperature effect on the oxygen binding of the haemoglobins of the snails studied is of ecophysiological significance. These snails are mainly found in shallow, sun exposed, temporary water bodies in the tropics. For such habitats the temperature may increase from a few degrees above freezing to about 30°C in a single day (Combrinck & Van Eeden 1975). A shift to the right of the oxygen dissociation curve will help in unloading oxygen to the tissues when it is most needed during increased metabolism at high temperatures. The shift to the right of the dissociation curve during hypoxic conditions will only be practical if a high affinity haemoglobin together with a small Bohr-effect is in operation. In this way tissue hypoxia will be kept under control with low acid metabolite production resulting in a co-operativity value of above 2 (Table 2).

This high co-operativity will keep the oxygen transport system functional during periods of severe oxygen depletion.

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