

## NITRITE AND RED CELL FUNCTION IN CARP: CONTROL FACTORS FOR NITRITE ENTRY, MEMBRANE POTASSIUM ION PERMEATION, OXYGEN AFFINITY AND METHAEMOGLOBIN FORMATION

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### Summary

Red cell function was studied in carp by a combination of *in vivo* and *in vitro* experiments with nitrite as the perturbing agent. *In vivo* accumulation of nitrite caused a marked increase in the red cell methaemoglobin content, and reduced the mean cellular volume. The oxygen affinity of unoxidized haemoglobin was strongly decreased, partly as result of the elevated concentration of cellular nucleoside triphosphates and haemoglobin associated with red cell shrinkage. Red cell pH was unchanged compared to controls, but reduced when referred to constant extracellular pH and O<sub>2</sub> saturation. The mean cellular K<sup>+</sup> content decreased, reflecting a K<sup>+</sup> loss from the red cells during their shrinkage. This K<sup>+</sup> loss contributed significantly to the large plasma hyperkalaemia during nitrite exposure. *In vitro* experiments revealed that nitrite influx into deoxygenated red cells was much larger than into oxygenated red cells. Nitrite permeation of the red cell membrane was not inhibited by DIDS and did not change extracellular pH. Methaemoglobin (MetHb) formation was more pronounced in deoxygenated blood than in oxygenated blood, but quasi-steady states were reached, reflecting a balance between nitrite-induced MetHb formation and the action of MetHb reductase. Red cells incubated in the oxygenated state released K<sup>+</sup>, whereas a net K<sup>+</sup> uptake occurred in deoxygenated cells. Nitrite did not change the K<sup>+</sup> loss from oxygenated cells, but shifted the K<sup>+</sup> uptake in deoxygenated cells to a pronounced K<sup>+</sup> release by the time high MetHb levels were reached. Both types of red cell K<sup>+</sup> release were inhibited by DIDS and appeared to occur *via* a route involving Band 3. The data are consistent with the hypothesis that a significant DIDS-sensitive K<sup>+</sup> efflux from the red cells occurs whenever a large fraction of the haemoglobin molecules assumes an R-like quaternary structure.

### Introduction

Freshwater fish accumulate nitrite in their blood plasma when exposed to this ion in the environment (Eddy and Williams, 1987). The continuous uptake of

Key words: red cell K<sup>+</sup> release, red cell pH, oxygen affinity, methaemoglobin, nitrite, oxygenation-dependent ion transport.

nitrite is believed to result from an effective competition of nitrite with chloride for the active chloride uptake mechanism in the gills (Williams and Eddy, 1986). The principal effect of such nitrite loading is a progressive oxidation of the haemoglobin (Hb) to methaemoglobin (MetHb), but several other physiological changes occur. These include changes in acid–base and electrolyte status and in blood respiratory properties (Jensen *et al.* 1987). In carp, the MetHb-induced oxygen shortage eventually elevates plasma lactate to very high concentrations. Blood pH is, however, not decreased. This is partly due to hyperventilation (i.e. decreased  $P_{\text{CO}_2}$ ), but the main reason appears to be that excess hydrogen ions from lactic acid production are effectively transferred to the environment (Jensen *et al.* 1987). The large increase in plasma nitrite and lactate, together with changes in the bicarbonate concentration, are balanced by a large decrease in the chloride concentration. Also, a rise in plasma potassium is paralleled by a decrease in sodium concentration, so that the sum of anion and cation concentrations remains constant, as does osmolality (Jensen *et al.* 1987). The large changes in the concentrations of individual ions are, however, of physiological importance. Notably, the large increase in plasma potassium may be a critical event, as it may interfere with tissue excitability and muscle (e.g. heart) contraction. It is therefore important to establish the cause of this plasma hyperkalaemia.

At the red cell level, nitrite appears to induce changes other than that of oxidizing the haemoglobin. A large increase in the red cell concentrations of haemoglobin and nucleoside triphosphates suggests that the red cells shrink, and a reduced  $\text{O}_2$ -saturation of the functional haemoglobin in the face of an increased arterial  $P_{\text{O}_2}$  suggests that oxygen affinity is reduced (Jensen *et al.* 1987). This effect on oxygen affinity in carp attracts interest in the light of the opposite response (i.e. an increased  $\text{O}_2$  affinity) reported for nitrite-treated mammalian (dog and man) blood (Darling and Roughton, 1942). One purpose of the present study was to obtain further evidence and insight into the change in oxygen affinity during nitrite exposure in carp. However, knowledge of many other aspects of the physiological effects of nitrite at the red cell level is also lacking. Therefore, further aims were (1) to obtain information about factors that govern the entry of nitrite into the red cells and methaemoglobin formation; (2) to study the effects of nitrite on red cell acid–base status; and (3) to investigate red cell potassium ion changes, including a possible mechanism of membrane permeation and the extent to which the red cells contribute to the extracellular hyperkalaemia during nitrite exposure.

## Materials and methods

### *Experimental animals*

Carp (*Cyprinus carpio*; mass  $1.1 \pm 0.3$  kg,  $N=19$ ) were obtained from lakes at Gissselfeld, Zealand, Denmark. Prior to experimentation the fish were maintained in 500 l holding tanks with running, aerated ( $P_{\text{O}_2} > 17.3$  kPa; 1 kPa = 7.5 mmHg) tap water at 15°C and subjected to a 12 h light: 12 h dark rhythm.

## In vivo experiments

## Experimental protocol

To enable later sampling of arterial blood without disturbance to the fish, the carp were anaesthetized in MS 222 (3-aminobenzoic acid ethyl ester, Sigma) and the dorsal aorta was cannulated with PE 60 tubing (Portex) according to the method of Soivio *et al.* (1975). Following surgery, the fish were allowed to recover for 48 h in individual aquaria with 60 l of aerated ( $P_{O_2} > 17.3$  kPa) water at 15°C. The water was renewed daily and contained (in  $\text{mmol l}^{-1}$ ):  $[\text{Cl}^-]$ , 1;  $[\text{Na}^+]$ , 1.2;  $[\text{Ca}^{2+}]$ , 3.5; total  $\text{CO}_2$ , 5.

Blood samples (2 ml) were obtained through the catheters in the control situation and after 48 h of exposure to nitrite-containing water. Nitrite was added as dissolved  $\text{NaNO}_2$  (Merck, analytical grade) to a concentration of  $1.8 \text{ mmol l}^{-1}$ . A higher nitrite concentration than in our previous study (i.e.  $1 \text{ mmol l}^{-1}$ , Jensen *et al.* 1987) was chosen in order to counteract the protective effect on nitrite accumulation of the higher water  $[\text{Cl}^-]$  in this study (1 vs  $0.3 \text{ mmol l}^{-1}$ ). Reasonably elevated plasma nitrite concentrations were attained within 48 h.

## Measurements

Plasma and water nitrite concentrations were determined spectrophotometrically after the reaction of nitrite with sulphanilamide to yield a diazo compound which couples with *N*-1-naphthyl-ethylenediamide-dihydrochloride to give a red azo dye.

The fraction of methaemoglobin (MetHb) in the blood was assessed according to the method of Benesch *et al.* (1973), and the total haemoglobin (Hb) concentration in the blood was measured following conversion of all Hb to cyanmethaemoglobin, using a millimolar extinction coefficient of 11 at 540 nm. Haematocrit (Hct) was determined by centrifugation (3 min at  $12\,000 \text{ rev min}^{-1}$ ) in glass capillaries. Red blood cell (RBC) counts ( $N_{\text{RBC}}, \mu\text{l}^{-1}$ ) were performed using a Bürger-Türk counting chamber and a Reichert (type 300108) microscope. Mean cellular volume (MCV) was calculated from  $\text{Hct}/N_{\text{RBC}}$ , and the mean cellular haemoglobin content (MCH) from  $[\text{Hb}]_{\text{blood}}/N_{\text{RBC}}$ . The red cell Hb concentration was assessed from  $[\text{Hb}]_{\text{blood}}/\text{Hct}$ .

Arterial blood pH (pHe) and oxygen tension ( $P_{O_2}$ ) were measured with Radiometer (Copenhagen, Denmark) electrodes (BMS 3 systems) with signals displayed on PHM 73 blood gas monitors and REC 80 recorders. The plasma total  $\text{CO}_2$  content ( $C_T$ ) was measured by the method of Cameron (1971), and arterial  $P_{\text{CO}_2}$  was calculated as:

$$P_{\text{CO}_2} = C_T / \alpha_{\text{CO}_2} (10^{(\text{pH} - \text{pK})} + 1), \quad (1)$$

using values of pK and the plasma  $\text{CO}_2$  solubility coefficient predicted from the formulae of Heisler (1984). Plasma bicarbonate concentrations were determined as  $C_T - \alpha_{\text{CO}_2} P_{\text{CO}_2}$ .

The plasma lactate concentration was assayed using the Boehringer-Mannheim

lactate dehydrogenase method. Plasma chloride concentration was measured by coulometric titration (Radiometer CMT 10), and plasma potassium, calcium and magnesium concentrations by atomic absorption spectrophotometry (Perkin-Elmer 2380). Plasma sodium was measured with a flame photometer (Instrumentation Laboratory 243).

The oxygen affinity of the blood was measured by the mixing method (Haab *et al.* 1960). About 500  $\mu\text{l}$  of freshly drawn blood was divided into two portions and transferred to an Eschweiler (Kiel, FRG) tonometer system. One sample was equilibrated with 0.4%  $\text{CO}_2/99.6\%$   $\text{N}_2$  (deoxygenated blood) and the other with 0.4%  $\text{CO}_2/30\%$   $\text{O}_2/69.6\%$   $\text{N}_2$  (oxygenated blood), both at 15°C. Gas mixtures were delivered from cascaded Wösthoff (Bochum, FRG) gas-mixing pumps. Following 30 min of tonometry, equal portions of deoxygenated and oxygenated blood were mixed anaerobically, and  $P_{50}$  was measured by injecting the 50%  $\text{O}_2$ -saturated blood into the BMS 3  $P_{\text{O}_2}$  electrode.

Red cell pH (pHi) was measured with a BMS pH electrode after separation of red cells from 500  $\mu\text{l}$  of blood and freeze-thawing the cells twice in liquid nitrogen. The distribution ratio of hydrogen ions across the RBC membrane ( $r_{\text{H}^+} = [\text{H}^+]_e / [\text{H}^+]_i$ ) was calculated as  $10^{(\text{pHi} - \text{pHe})}$ . The concentration of nucleoside triphosphates (NTP) in the blood was determined enzymatically (Sigma Bulletin no. 366-UV) on supernatants from 1:1 deproteinizations of blood in 12% trichloroacetic acid (TCA), and the red cell concentrations were calculated from the corresponding Hct values. The red cell concentration of potassium (in  $\text{mmol l}^{-1}$  RBC) was determined from measurements of the blood concentration of  $\text{K}^+$  (using the TCA supernatant) and the plasma concentration of  $\text{K}^+$  (see above), according to the equation:

$$[\text{K}^+]_{\text{RBC}} = \{[\text{K}^+]_{\text{blood}} - (1 - F_{\text{Hct}})[\text{K}^+]_{\text{plasma}}\} / F_{\text{Hct}}, \quad (2)$$

where  $F_{\text{Hct}}$  is the fractional Hct. The mean cellular potassium content (MCK, in  $\text{mol K}^+ \text{RBC}^{-1}$ ) was determined from corresponding red cell counts as:

$$\text{MCK} = ([\text{K}^+]_{\text{RBC}} F_{\text{Hct}}) / N_{\text{RBC}}. \quad (3)$$

### In vitro experiments

#### Series I

To study the kinetics and oxygenation-dependence of the influx of nitrite from plasma into red cells, and of methHb formation, blood from individual normoxic carp was divided into two 3 ml subsamples and equilibrated at 15°C in the Eschweiler tonometer system. One sample was equilibrated with a gas mixture of 0.4%  $\text{CO}_2/99.6\%$   $\text{N}_2$  (deoxygenated blood) and the other with 0.4%  $\text{CO}_2/30\%$   $\text{O}_2/69.6\%$   $\text{N}_2$  (oxygenated blood). After 40 min of tonometry, control blood samples were withdrawn for measurements, and nitrite was added to each tonometer to produce a nominal nitrite concentration in the plasma of approximately  $3 \text{mmol l}^{-1}$ . The nitrite was added as microlitre samples from a  $140 \text{mmol l}^{-1}$   $\text{NaNO}_2$  stock solution, which has an osmolality similar to that of carp

plasma. Further blood samples were removed at specified times during the incubation, and measurements of plasma  $[\text{NO}_2^-]$ , MetHb content, blood [Hb] and Hct were performed (as above).

### *Series II*

To study possible routes of nitrite entry to the red cells, as well as the *in vitro* effects of nitrite on acid–base status, a set-up and procedure similar to that in series I was used, but the tonometers with oxygenated and deoxygenated blood were duplicated. Following withdrawal of nitrite-free control samples, DIDS (4,4'-diisothiocyanate-2,2'-stilbenedisulphonic acid, Aldrich Chemical Co.) was added to a concentration of  $5 \times 10^{-4} \text{ mol l}^{-1}$  to one of the duplicated tonometers for both the oxygenated and the deoxygenated blood. Nitrite was then added, and blood samples were removed at specified times for measurements of plasma  $[\text{NO}_2^-]$  and blood pH.

### *Series III*

This series studied the effects of nitrite on potassium permeation of red cells. Using incubation conditions as above, an extended sampling for measurement of plasma  $\text{K}^+$  was performed. Oxygenated and deoxygenated blood were examined for  $\text{K}^+$  changes both in the absence and presence of nitrite and in the absence and presence of DIDS.

### *Series IV*

This series investigated the mode, rate and concentration-dependence of nitrite-induced MetHb formation in carp haemoglobin solutions. Carp red cells were washed twice in a physiological saline and haemolysed in a weak phosphate buffer at pH 7.3. Subsamples of the haemolysate were transferred to a cuvette to which nitrite was added from a  $\text{NaNO}_2$  stock solution to produce variable concentrations. The time-dependence of MetHb formation was recorded from measurements of the absorbance at 560, 576 and 630 nm (Benesch *et al.* 1973).

## **Results**

### *In vivo changes in the respiratory properties of the blood*

During the 48 h period of nitrite exposure, carp accumulated nitrite in the plasma to concentrations above the environmental concentration, with an accompanying conversion of 71 % of the haemoglobin to methaemoglobin (Table 1). Blood haematocrit decreased by 34.1 % compared to the control level, whereas the total Hb concentration decreased by 24.8 % (Table 1). This resulted from a non-significant decrease in the number of red cells ( $N_{\text{RBC}}$ ) and a significant 15.3 % decrease in the mean cellular volume (MCV, Table 1). The mean cellular haemoglobin content (MCH) was unchanged, so that the cellular Hb concentration increased by 14.4 % in the nitrite-exposed carp (Table 1).

Table 1. *Plasma nitrite concentration, haematological parameters, blood oxygen affinity and the arterial oxygen tension in carp before (control) and after 48 h exposure to nitrite*

	Control	Nitrite-exposed	<i>P</i>
[NO <sub>2</sub> <sup>-</sup> ] (mmol l <sup>-1</sup> plasma)	0.01±0.02	3.00±1.41	0.0036
Methaemoglobin (%)	5.2±0.4	71.2±9.9	<0.0001
Haematocrit (%)	23.49±4.37	15.48±3.27	0.0049
Hb (mmol l <sup>-1</sup> blood)	1.09±0.21	0.82±0.16	0.03
Hb (mmol l <sup>-1</sup> RBC)	4.64±0.21	5.31±0.35	0.0023
<i>N</i> <sub>RBC</sub> (μl <sup>-1</sup> , ×10 <sup>-6</sup> )	1.324±0.291	1.030±0.249	NS
MCV (fl)	178.8±15.8	151.4±9.4	0.0044
MCH (fmol)	0.832±0.100	0.806±0.091	NS
<i>P</i> <sub>50</sub> (kPa)	1.2±0.25	4.7±1.3	0.005
<i>P</i> <sub>aO<sub>2</sub></sub> (kPa)	3.5±1.3	5.1±2.5	NS

Mean values±s.d. (*N*=6).

The statistical significance of the difference between two means (two-tailed Student's *t*-test); NS signifies a non-significant (*P*>0.05) change.

Hb, haemoglobin; *N*<sub>RBC</sub>, red blood cell count; MCV, mean cellular volume; MCH, mean cellular haemoglobin content.

The oxygen affinity of the blood decreased strongly during nitrite exposure. Thus, *P*<sub>50</sub> measured at 0.4 kPa *P*<sub>CO<sub>2</sub></sub> was elevated from a value of 1.2 kPa in control animals to 4.7 kPa in nitrite-exposed animals (Table 1). The arterial oxygen tension also increased in nitrite-exposed carp, but this change was not statistically significant (Table 1). From the changes in *P*<sub>50</sub> and *P*<sub>aO<sub>2</sub></sub> it could be predicted that the arterial oxygen saturation of the functional Hb was about 80 % in the control situation and about 50 % in the nitrite-exposed situation.

#### *In vivo changes in extracellular acid–base and electrolyte status*

The arterial blood pH was significantly elevated after 48 h of exposure to nitrite as a result of a decreased arterial *P*<sub>CO<sub>2</sub></sub> (Table 2). The plasma bicarbonate concentration was marginally decreased and the plasma lactate concentration marginally increased. The plasma chloride and sodium concentrations tended to decrease, whereas calcium and magnesium were unaffected by nitrite exposure (Table 2). The plasma potassium concentration, in contrast, showed a large significant increase (of 56 %) in nitrite-exposed carp (Table 2). A thorough analysis and discussion of extracellular electrolyte and acid–base changes during nitrite exposure in carp has been given earlier (Jensen *et al.* 1987). The present electrolyte and acid–base changes conform to this analysis, but the changes were relatively small owing to the moderate state of nitrite loading examined here.

#### *Red cell acid–base, nucleoside triphosphate and potassium ion changes*

The red cell pH was unchanged in nitrite-exposed carp when compared with the control situation (Table 3). However, as shown in Fig. 1, it was lower than the

Table 2. Extracellular acid-base status and electrolyte concentrations in carp blood before (control) and after 48 h of nitrite exposure

	Control	Nitrite-exposed	P
pHe	8.021±0.020	8.095±0.028	0.0004
$P_{\text{CO}_2}$ (kPa)	0.42±0.07	0.33±0.05	0.025
[HCO <sub>3</sub> <sup>-</sup> ] (mmol l <sup>-1</sup> plasma)	11.99±2.38	11.00±1.39	NS
[Lactate] (mmol l <sup>-1</sup> plasma)	0.83±0.32	2.87±2.57	NS
[Cl <sup>-</sup> ] (mmol l <sup>-1</sup> plasma)	117.5±4.9	111.9±7.9	NS
[Na <sup>+</sup> ] (mmol l <sup>-1</sup> plasma)	128.5±2.7	125.7±3.4	NS
[Ca <sup>2+</sup> ] (mmol l <sup>-1</sup> plasma)	2.30±0.24	2.33±0.19	NS
[Mg <sup>2+</sup> ] (mmol l <sup>-1</sup> plasma)	0.94±0.12	0.87±0.12	NS
[K <sup>+</sup> ] (mmol l <sup>-1</sup> plasma)	2.92±0.53	4.56±0.82	0.0022

Mean values±s.d. (N=6).

Statistics as in Table 1.

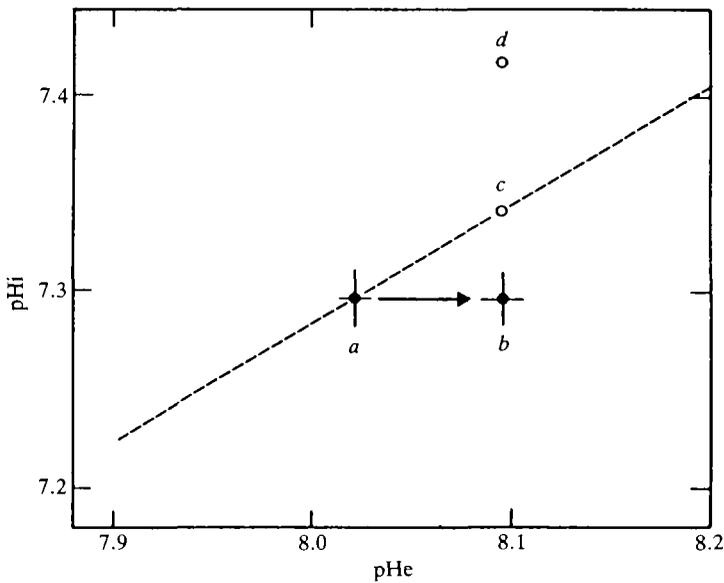


Fig. 1. Red cell pH (pHi) vs plasma pH (pHe) diagram, depicting the *in vivo* change in carp between the control (a) and the nitrite-exposed (b) situation. The predicted values in nitrite-free blood subjected to a rise in pHe (c) and decrease in O<sub>2</sub> saturation (d), as observed during nitrite exposure, are shown for comparison.

values expected for nitrite-free blood at similar pHe and O<sub>2</sub> saturation. In oxygenated nitrite-free blood the  $\Delta p\text{Hi}/\Delta p\text{He}$  value is about 0.6 (Albers and Goetz, 1985; F. B. Jensen, unpublished results), which would predict a pHi value at point c in Fig. 1 because of the rise observed in pHe (Table 2). An additional deoxygenation of the haemoglobin to about 50% would bring pHi to the value at

Table 3. *Red cell pH, nucleoside triphosphate and potassium ion concentration in carp before (control) and after 48 h of nitrite exposure*

	Control	Nitrite-exposed	P
pHi	7.296±0.038	7.295±0.034	NS
$r_{H^+}$	0.189±0.011	0.160±0.018	0.0071
[NTP] (mmol l <sup>-1</sup> RBC)	10.06±1.55	12.44±1.45	0.02
NTP/Hb	2.18±0.39	2.35±0.36	NS
[K <sup>+</sup> ] (mmol l <sup>-1</sup> RBC)	103.6±5.2	101.2±7.3	NS
MCK (fmol)	18.55±2.15	15.33±1.48	0.0128

Mean values±s.d. (N=6).

NTP, nucleoside triphosphate; Hb, haemoglobin;  $r_{H^+}$ , distribution ratio of H<sup>+</sup> across the red blood cell membrane; MCK, mean cellular potassium content.

Statistics as in Table 1.

point *d* (if all Hb remained functional). The actual *in vivo* pHi (point *b*) in nitrite-exposed carp was clearly lower than these predictions (Fig. 1). As a consequence, the Donnan distribution ratio of H<sup>+</sup> across the red cell membrane was significantly decreased during nitrite exposure (Table 3).

The red cell nucleoside triphosphate (NTP) concentration increased significantly following nitrite exposure (Table 3), whereas the red cell NTP content (as reflected by the NTP/Hb ratio) was only marginally elevated. Thus, the rise in NTP concentration appeared to be primarily a consequence of the reduced red cell volume (Table 1).

The arterial red cell potassium concentration was 103.6 mmol l<sup>-1</sup> RBC. With a RBC water content of 64.6% (Albers and Goetz, 1985) this corresponds to a value of 160.4 mmol kg<sup>-1</sup> cell water and 292.7 mmol kg<sup>-1</sup> dry mass, comparing well with previously reported values at similar pH (Salama and Nikinmaa, 1988). The red cell potassium concentration was unaffected by nitrite exposure (Table 3). The mean potassium content of the individual cells (MCK) was, however, significantly reduced in nitrite-exposed carp (Table 3), suggesting loss of K<sup>+</sup> from the cells during red cell shrinkage with maintenance of a constant concentration.

#### In vitro experiments

##### *Nitrite entry, methaemoglobin formation and potassium ion changes*

When red cells were brought into contact with nitrite at a plasma concentration of 3 mmol l<sup>-1</sup>, the subsequent disappearance of nitrite from the plasma was found to be strongly dependent on the oxygenation state of the blood. In oxygenated blood, the plasma [NO<sub>2</sub><sup>-</sup>] decrease was small, whereas in deoxygenated blood the plasma nitrite concentration was halved within 120 min (Fig. 2). Thus the nitrite influx into the red cells was much larger in deoxygenated cells than in oxygenated cells. From the initial  $\Delta[\text{NO}_2^-]/\Delta t$  and Hct, the initial rate of NO<sub>2</sub><sup>-</sup> entry to the deoxygenated red cells was calculated to be about 0.16 mmol l<sup>-1</sup> RBC min<sup>-1</sup>. This

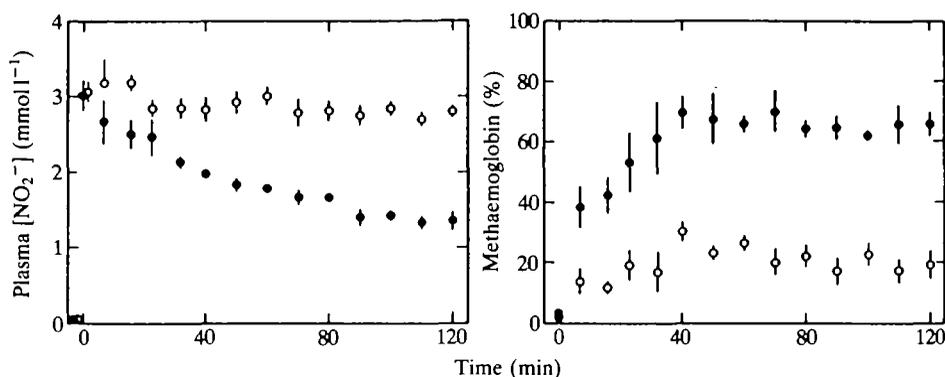


Fig. 2. Time-dependent changes in plasma nitrite concentration and methaemoglobin content in oxygenated (○) and deoxygenated (●) carp blood brought into contact with nitrite at time zero. Means  $\pm$  s.e.m. are shown for three individual carp. Haematocrit (time zero):  $21.1 \pm 1.4$  in oxygenated and  $23.0 \pm 2.4$  in deoxygenated blood.

rate decreased with time as a result of an exponential decrease in the plasma nitrite concentration with time (Fig. 2).

The nitrite-induced methaemoglobin formation exhibited a strong oxygenation-dependence that corresponded with the oxygenation-dependence of nitrite entry to the red cells. In oxygenated blood the MetHb content was elevated to approximately 20% MetHb, whereas in deoxygenated blood the MetHb content rose to about 70% (Fig. 2). In both oxygenated and deoxygenated blood an apparently steady level was reached within 40 min, with only small subsequent fluctuations in the MetHb content up to 120 min of incubation (Fig. 2).

When the blood was treated with DIDS (at  $5 \times 10^{-4} \text{ mol l}^{-1}$ ), no change in the kinetics of the plasma [NO<sub>2</sub><sup>-</sup>] was observed (Fig. 3). Thus, the entry of nitrite to the red cells appeared to be unaffected by inhibition of the red cell anion exchanger. Also, during the 120 min treatment with nitrite there were no significant changes in the extracellular pH, either in the absence or in the presence of DIDS (Fig. 3).

In nitrite-treated oxygenated blood the plasma potassium concentration increased during the incubation. However, a similar increase in plasma [K<sup>+</sup>] was observed in oxygenated blood in the absence of nitrite (Fig. 4, upper left panel), and the effect must therefore be ascribed to a K<sup>+</sup> efflux from the red cells related to their incubation in the oxygenated state *per se*. In deoxygenated blood, in contrast, a clear effect of nitrite on the net flux of potassium between red cells and plasma was evident. Thus, whereas in the absence of nitrite the plasma [K<sup>+</sup>] fell, reflecting a net K<sup>+</sup> uptake of the deoxygenated cells, there was a large increase in the plasma potassium concentration when nitrite was present (Fig. 4, upper right panel). This nitrite-induced K<sup>+</sup> loss from deoxygenated cells appeared after some 50 min of nitrite treatment (Fig. 4), which coincided with the time of reaching the high quasi-steady MetHb level (Fig. 2). Despite this delay in the onset of the

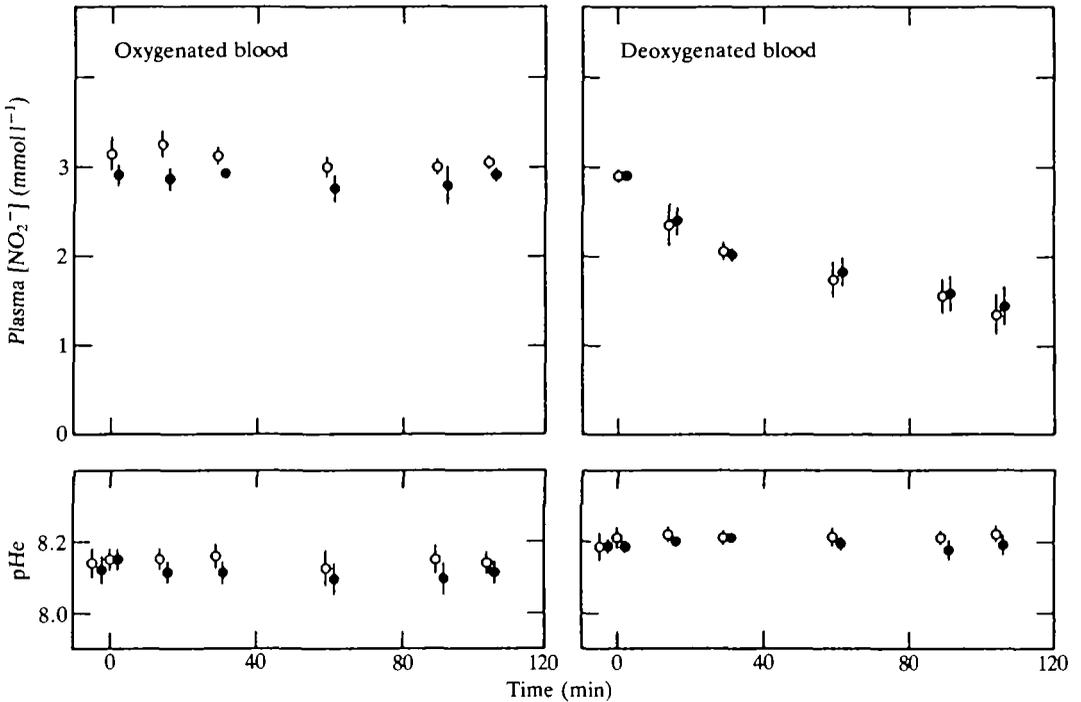


Fig. 3. Time-dependent changes in plasma [NO<sub>2</sub><sup>-</sup>] and blood pH in oxygenated (left panels) and deoxygenated (right panels) blood treated with nitrite in the absence (○) and presence (●) of DIDS. Means ± S.E.M. for three carp are shown.

plasma K<sup>+</sup> rise, similar plasma concentrations to those of oxygenated blood were reached at 3 h of incubation (Fig. 4). Treatment of the red cells with DIDS abolished both the plasma [K<sup>+</sup>] increase induced by nitrite in deoxygenated blood and that induced in oxygenated blood (Fig. 4, lower panels).

#### *Methaemoglobin formation in haemoglobin solutions*

The formation of MetHb after addition of nitrite to oxygenated carp haemoglobin solutions was characterized by an initial lag phase followed by a rapid increase in the rate of MetHb formation and a levelling off in reaction rate when the MetHb level approached 100% (Fig. 5). The lag phase, and thus the velocity of MetHb formation, depended on the nitrite concentration (Fig. 5). Using the time taken for oxidation of half the haem groups,  $t_{1/2}$ , as a measure of the rapidity of the reaction, the data of Fig. 5 were best described by the equation:  $t_{1/2} = 15.3[\text{NO}_2^-]^{-2}$  ( $r = 0.999$ ,  $N = 3$ ). Thus the rate of MetHb formation appeared to be inversely proportional to the square of the nitrite concentration, as may also be the case in mammalian haemoglobins (Kiese, 1974).

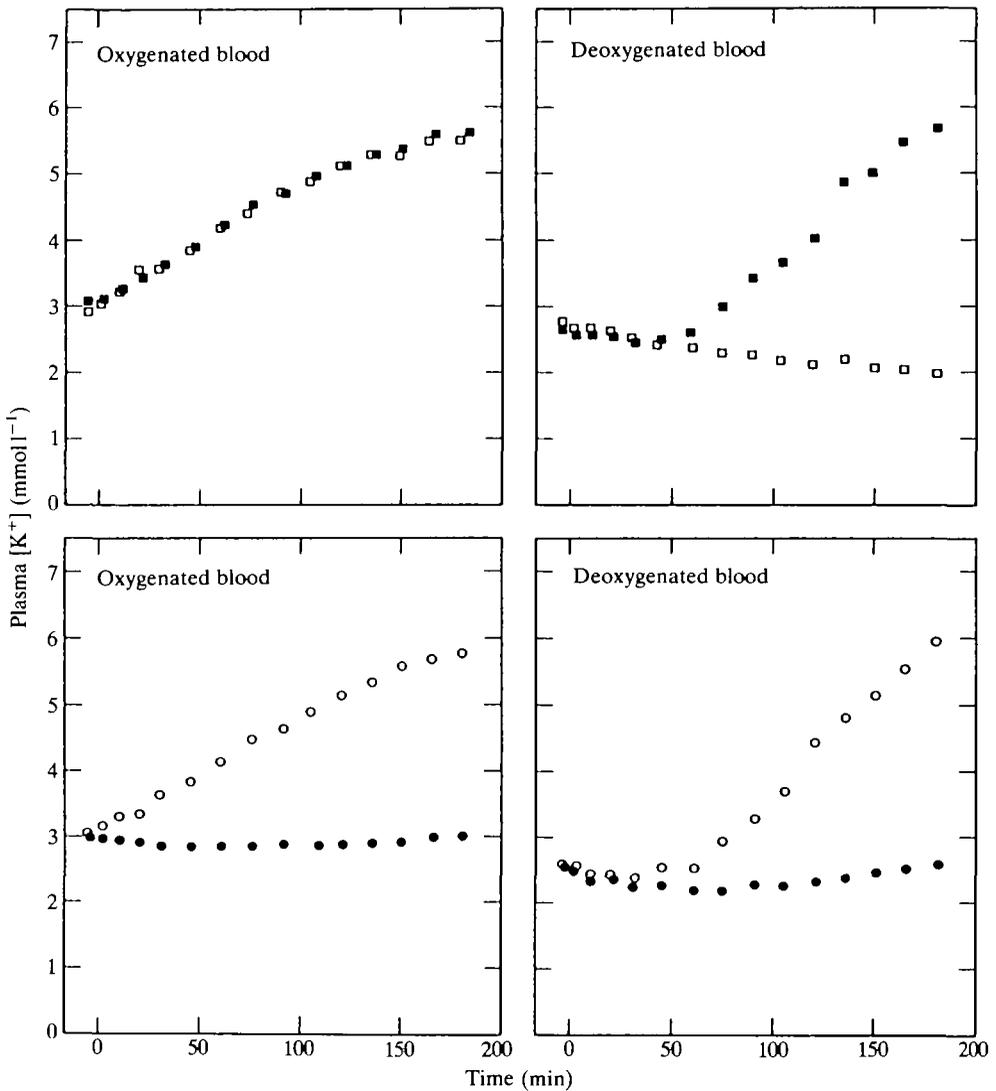


Fig. 4. Upper panels: time-dependent changes in plasma  $K^+$  concentration of oxygenated (left) and deoxygenated (right) carp whole blood in the absence ( $\square$ ) and presence ( $\blacksquare$ ) of nitrite (at a plasma concentration of  $3 \text{ mmol l}^{-1}$  at time zero). Lower panels: changes in plasma  $[K^+]$  in nitrite-treated oxygenated (left) and deoxygenated (right) blood in the absence ( $\circ$ ) and presence ( $\bullet$ ) of DIDS. Mean values are shown for three carp in the upper panels and for three further carp in the lower panels (s.d.  $< 0.22 \text{ mmol l}^{-1}$ ).

## Discussion

### *Nitrite uptake in red cells*

*In vivo* accumulation of nitrite in carp and other fish species causes a severe methaemoglobinaemia (Table 1; Eddy and Williams, 1987). This implies that part

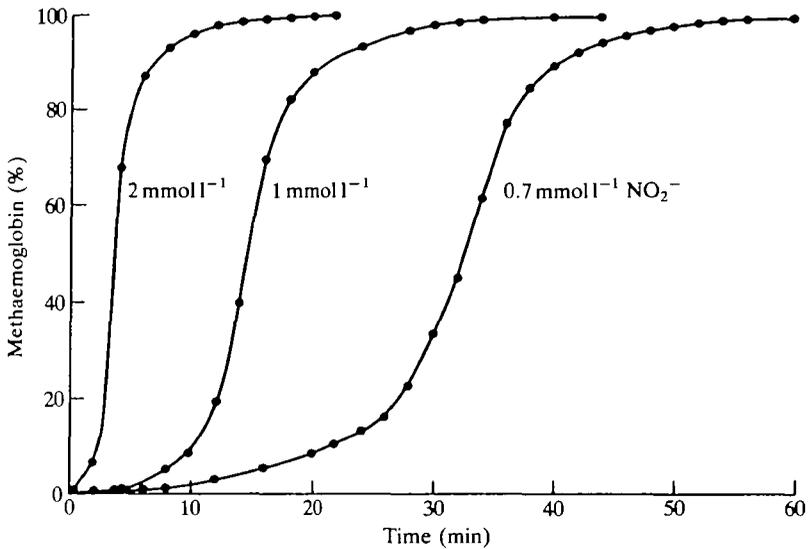


Fig. 5. Methaemoglobin formation in a carp haemolysate (haem concentration  $0.14 \text{ mmol l}^{-1}$ ) at different nitrite concentrations.

of the nitrite that enters the blood plasma at the blood/water interface in the gills must cross the red cell membrane, but the factors that govern this entry of nitrite to the red cells have not hitherto been investigated. In carp the entry of nitrite to the red cells was found to be strongly dependent on the oxygenation state of the blood, with nitrite entering deoxygenated red cells much more rapidly than oxygenated red cells (Fig. 2). Thus, deoxygenation increases the nitrite permeability. This finding is of interest in the light of the recent findings of an oxygenation-dependence of other red cell membrane transport processes, including the  $\text{K}^+$  permeation observed here (Fig. 4) and beta-adrenergically stimulated  $\text{Na}^+/\text{H}^+$  exchange (Motais *et al.* 1987; Salama and Nikinmaa, 1988). The oxygenation state of haemoglobin accordingly appears to be an important control factor for many red cell membrane transport functions.

*A priori*, a likely candidate to mediate the transport of nitrite into the red cell would be the red cell anion exchanger (Band 3), which has a relatively low anion specificity (Lowe and Lambert, 1983), suggesting that nitrite could compete with chloride and bicarbonate for the outward-facing site of the carrier. Inhibition of the anion exchanger with DIDS, however, did not change the kinetics of nitrite entry to carp red cells (Fig. 3). Assuming a similar potency and specificity of DIDS in carp red cells and in human red cells, this result suggests that nitrite enters *via* some route not involving the anion exchanger. This could be by some other transport protein, by conductive transport or by diffusion of  $\text{HNO}_2$ . Regarding the latter possibility, the high plasma pH means that only a small fraction of the nitrite exists in the acid  $\text{HNO}_2$  form (the  $\text{pK}$  of nitrous acid is about 3.3). Additionally, the red cell pH is lower than plasma pH, so that the fraction of  $\text{HNO}_2$  will be larger

in the red cells. However, as nitrite is constantly removed inside the red cell by the process of methaemoglobin formation, a diffusion gradient of  $\text{HNO}_2$  from plasma to red cells may exist.  $\text{HNO}_2$  diffusion thus cannot be entirely ruled out. However, the constancy of plasma pH during nitrite entry (and in the presence of DIDS, where rapid pH re-equilibration across the RBC membrane *via* the anion exchanger is inhibited, Fig. 3), does not agree with the increase in pH expected from  $\text{HNO}_2$  diffusion. The present data, therefore, may be most compatible with the idea that nitrite enters the red cells by conductive transport. The oxygenation-dependence of the transport could then be explained by a change in the membrane potential as the Donnan equilibrium changes with the degree of oxygenation. A firm conclusion regarding the transport mechanism must, however, await further study.

#### *Nitrite-induced methaemoglobin formation*

Oxidation of oxygenated carp haemoglobin by nitrite occurred after an initial lag followed by a rapid increase in the rate of MetHb formation (Fig. 5). Thus, there was an 'autocatalytic' increase in the reaction rate, as in mammalian Hbs (Kiese, 1974; Kosaka and Tyuma, 1987), suggesting that the mechanism of oxidation is the same (Kosaka and Tyuma, 1987).

It is known that the reaction of nitrite with haemoglobin may be different in the absence and in the presence of oxygen, with a slower formation of ferriHb in the absence of oxygen (Kiese, 1974). In whole blood, however, MetHb formation was more pronounced in deoxygenated than in oxygenated red cells with the same plasma nitrite load (Fig. 2). This difference can be attributed to changes at the red cell membrane that cause the much faster entry of nitrite to deoxygenated than to oxygenated red cells (Fig. 2).

In both deoxygenated and oxygenated blood a quasi-steady MetHb level was reached at about 70 % and 20 % MetHb, respectively. Since fish red cells possess methaemoglobin reductase activity (Freeman *et al.* 1983), it appears that a balance is reached between nitrite-induced oxidation and reduction *via* the methaemoglobin reductase. During the  $\text{O}_2$  affinity measurements on blood drawn from nitrite-exposed carp, it was observed that the MetHb content was not significantly increased during the 30 min of incubation, pointing to a similar balance in the blood *in vivo* (not illustrated). The oxygenation-dependence of nitrite permeation and MetHb formation suggests that *in vivo* these processes will primarily occur in venous blood, so that the deoxygenation of the blood in the tissues determines the MetHb content reached at a given nitrite load.

#### *Red cell volume*

The red cells shrink during nitrite exposure, judging from the large increase in the red cell Hb concentration and the concomitant increase in the red cell NTP concentration at an unaltered NTP/Hb ratio (Tables 1 and 3; Jensen *et al.* 1987), and also from direct measurements of a reduced mean red cell volume (Table 1).

Liberation of immature red cells could contribute to reduce RBC volume, but this explanation appears unlikely as immature red cells have reduced Hb and NTP contents (Schindler and DeVries, 1986; Lane, 1984). Shrinkage of the red cells explains part of the Hct decrease (Table 1), but the number of red cells and the blood Hb concentration also decrease, and by more than expected from blood sampling. Assuming a blood volume of 4–5%, the average fish of the present study should possess about 50 ml of blood, and sampling of 2 ml should only decrease blood Hb from 1.09 to about 1.04 mmol l<sup>-1</sup> and not to 0.82 mmol l<sup>-1</sup> as was observed (Table 1). A decrease in the total Hb concentration of the blood has been observed in other fish exposed to nitrite (e.g. Brown and McLeay, 1975; Eddy *et al.* 1983) and may reflect an increased haemolytic breakdown of red cells or, alternatively, an increased blood volume.

#### *Blood oxygen-affinity*

Methaemoglobin formation is traditionally believed to increase the oxygen affinity of haem groups that remain functional (e.g. Kiese, 1974). The basis for this change is that oxidation of haem groups increases the tendency of the Hb molecule to assume an R- or 'oxy'-like quaternary structure, which has a higher O<sub>2</sub> affinity than the T or 'deoxy' quaternary structure. Nitrite exposure in carp, however, was associated with a large decrease in oxygen affinity of the haemoglobin that had not become oxidized to methaemoglobin (Table 1). This result corroborates the conclusion drawn previously from measurements of arterial O<sub>2</sub> saturation and O<sub>2</sub> tension in nitrite-exposed carp (Jensen *et al.* 1987), but it is the opposite of the increase in O<sub>2</sub> affinity reported in nitrite-treated mammalian blood (Darling and Roughton, 1942).

In carp, the reduction in blood O<sub>2</sub>-affinity may be a main consequence of the red cell shrinkage. By increasing the intracellular concentrations of Hb and NTP, red cell shrinkage tends to increase NTP–Hb complexing, thereby lowering O<sub>2</sub> affinity (Jensen *et al.* 1987). This interpretation is in line with the large increase in *P*<sub>50</sub> in carp Hb solutions when the Hb concentration is increased towards natural red cell concentrations at constant NTP/Hb (Lykkeboe and Weber, 1978). How does this mechanism connect to the O<sub>2</sub> affinity increase expected from MetHb formation *per se*? A large fraction of the Hb molecules in the blood, which contained about 70% MetHb (Table 1), may have an R-like structure (indeed, this is supported by the K<sup>+</sup> data, see below), and not have NTP bound to it. Together with the concentrating mechanism *via* shrinkage, this would increase the amount of NTP potentially available for interaction with other Hb molecules. Thus, some fraction of the Hb molecules, probably those with the lesser number of haems oxidized (and therefore with the largest influence on O<sub>2</sub>-binding properties), may become fixed in the T structure and have a greatly lowered O<sub>2</sub> affinity because of saturating binding of NTP. This would explain the overall decrease in blood O<sub>2</sub>-affinity. Other mechanisms may, however, also contribute.

By using multiple regression analysis on the *in vivo* data (including both control

and nitrite-exposed values) in order to relate O<sub>2</sub> affinity to other variables, the most significant combination of variables turned out to be:

$$\log P_{50} = 0.0427[\text{NO}_2^-]_e + 0.2684[\text{Hb}]_i + 0.08[\text{NTP}]_i - 0.0029\text{MCV} - 1.154\text{pHi} + 7.846 \quad (r = 0.997; F = 220.3; P < 0.0001). \quad (4)$$

Although highly significant, such a statistical correlation should be interpreted with caution when inferring causal interrelationships. Nevertheless, the correlation seems to support the idea, stated above, that red cell shrinkage is a key factor underlying the decrease in O<sub>2</sub> affinity. Furthermore, it suggests a contribution from red cell pH (see below) and nitrite itself. Nitrite may indeed decrease O<sub>2</sub> affinity, as do other anions such as NTPs, Cl<sup>-</sup> etc. Nitrite, however, is probably converted to nitrate in the red cell by the process of Hb oxidation (Kosaka and Tyuma, 1987), perhaps making nitrate a more likely candidate to contribute to the change in O<sub>2</sub> affinity than nitrite. Aggregation of Hb tetramers could be a further mechanism for lowering O<sub>2</sub> affinity. However, no significant increase in Hill's constant, *n*, was observed in those cases where full O<sub>2</sub> equilibrium curves were recorded on nitrite-treated blood.

#### *Red cell acid-base status*

The red cell pH was unchanged in nitrite-exposed carp when compared to control values, but it was lower than the value expected for nitrite-free blood subjected to a similar rise in extracellular pH and decrease in O<sub>2</sub> saturation (Fig. 1). Thus, when referred to constant pHe and O<sub>2</sub> saturation, the red cell pH was decreased during nitrite exposure, which may contribute to lower O<sub>2</sub> affinity (see above). This finding probably represents the net result of several changes that affect red cell pH. Partial oxidation of the Hb may reduce the large oxygenation-linked change in pHi associated with the Bohr-Haldane effect (Jensen, 1986), so that the change in pHi resulting from the change in O<sub>2</sub> saturation will be less than that indicated in Fig. 1. The oxidation process itself, however, consumes hydrogen ions (Kiese, 1974; Kosaka and Tyuma, 1987) and MetHb formation may decrease the non-permeable negative charge on the Hb, tending to increase the Donnan distribution ratio of hydrogen ions across the RBC membrane, thus elevating pHi. This may, however, be counteracted by the rise in the red cell NTP concentration, which tends to lower pHi. An additional influence on pHi may arise from the reduced RBC cation content (Table 3, Hladky and Rink, 1977).

#### *Extracellular potassium ion changes*

One characteristic finding during nitrite exposure in carp is a large elevation of the extracellular potassium concentration (Table 2; Jensen *et al.* 1987). The present *in vivo* data provide some insight into the origin of this extracellular hyperkalaemia. Thus, the decrease in the mean cellular K<sup>+</sup> content (Table 3), suggests that the red cells lose K<sup>+</sup> during their shrinkage, which may contribute significantly to the rise in plasma K<sup>+</sup>. From Table 3 the loss amounts to  $3.22 \times 10^{-15}$  mol RBC<sup>-1</sup> during the 48 h of nitrite exposure. Taking the number of

red cells and the plasma volume,  $(100 - \text{Hct})\%$ , into account (Table 1), this RBC  $\text{K}^+$  release, if distributed only in plasma, can elevate the plasma  $\text{K}^+$  concentration by about  $4 \text{ mmol l}^{-1}$ . The  $\text{K}^+$  will, however, be distributed not only in blood plasma but also in the interstitial fluid. Against this background the red cell  $\text{K}^+$  release can be expected to elevate the extracellular  $[\text{K}^+]$  by about  $0.8 - 1.0 \text{ mmol l}^{-1}$ . Therefore, the  $\text{K}^+$  lost from the red cells can indeed explain a large fraction of the measured increase in plasma  $\text{K}^+$  ( $1.64 \text{ mmol l}^{-1}$ , Table 2). However, it appears that other intracellular compartments may also contribute to elevate the plasma  $\text{K}^+$  concentration.

#### *Factors affecting $\text{K}^+$ permeation of the red cell membrane*

The conclusion drawn from the *in vivo* experiments, that nitrite induces a net red cell  $\text{K}^+$  efflux, was confirmed by the *in vitro* experiments. The nitrite-induced red cell  $\text{K}^+$  release was oxygenation-dependent, but in a manner similar to that demonstrated for nitrite entry to the red cells and for MetHb formation. Only in deoxygenated blood did nitrite cause a large increase in the plasma  $\text{K}^+$  concentration compared to control incubations of blood in the absence of nitrite (Fig. 4). This  $\text{K}^+$  efflux from the red cells could be abolished by DIDS (Fig. 4). The  $\text{K}^+$  loss and accompanying red cell shrinkage (see above), accordingly resembles the volume-reducing KCl extrusion reported in fish red cells swollen in a hypotonic medium, a volume regulatory mechanism that is also sensitive to DIDS (Lauf, 1982).

An interesting observation during the present experiments was that incubation of the blood under different oxygenation conditions influenced the potassium balance *per se*. In oxygenated blood the plasma  $[\text{K}^+]$  rose as a function of incubation time, reflecting a net red cell  $\text{K}^+$  efflux. In deoxygenated blood the plasma  $[\text{K}^+]$  decreased, reflecting a net red cell  $\text{K}^+$  uptake (Fig. 4). A release of  $\text{K}^+$  from oxygenated erythrocytes has also been observed in rainbow trout blood incubated in an oxygen atmosphere (Railo *et al.* 1985). These  $\text{K}^+$  changes are of considerable magnitude during long-term *in vitro* experiments but, when related to the relatively fast circulation time *in vivo*, the plasma  $\text{K}^+$  fluctuations that may occur from  $\text{K}^+$  release in arteries and subsequent uptake in veins will be small.

As was the case with the nitrite-induced  $\text{K}^+$  loss in deoxygenated cells, the  $\text{K}^+$  release from oxygenated cells could be abolished by DIDS (Fig. 4). These findings suggest that, in both cases, the  $\text{K}^+$  permeation of the red cell membrane was mediated *via* Band 3 protein. At first sight this appears surprising, given the normal anion exchange function of Band 3. Similar results have been reported, however, in human erythrocytes. Thus, the  $\text{K}^+$  efflux from human red cells in low-chloride media is also inhibited by DIDS and appears to occur *via* Band 3 (Jones and Knauf, 1985). The present differences between oxygenated and deoxygenated blood suggest that the DIDS-sensitive  $\text{K}^+$  permeation depends on the oxygenation-dependent interaction of haemoglobin with Band 3, with a red cell  $\text{K}^+$  loss being induced when the Hb assumes the oxy or R quaternary structure. OxyHb binds less strongly to the cytoplasmic fragment of Band 3 than does deoxyHb

(Chetrite and Cassoly, 1985), and this oxygenation-dependent interaction may be associated with some structural modification of Band 3 that allows the potassium permeation (either *via* Band 3 itself or *via* some other pathway that depends on the Band 3 structural change). That a significant fraction of the haemoglobin should assume an R-like quaternary structure in order for the DIDS-sensitive net  $K^+$  efflux to occur also agrees with the red cell  $K^+$  loss induced by nitrite in deoxygenated blood. DeoxyHb attains the T quaternary structure, but as more and more haem groups are oxidized, the fraction of Hb molecule assuming an R-like structure is likely to increase, since MetHb has an R-like structure (Perutz, 1978). Accordingly, the  $K^+$  loss should occur from nitrite-treated deoxygenated cells only at later stages of the incubation when a high MetHb content has been developed. This was indeed the case (Fig. 4).

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