Influence of Gemfibrozil on Sulfate Transport in Human Erythrocytes during the Oxygenation-Deoxygenation Cycle

E. TELLONE[1], S. FICARRA[1], R. SCATENA,[2] B. GIARDINA[2], A. KOTYK[3], A. RUSSO[1], D. COLUCCI[1], E. BELLOCCO[1], G. LAGANA'[1], A. GALTIERI[1]

[1]*Department of Organic and Biological Chemistry, University of Messina, 98166 Messina, Italy.*
[2]*C.N.R. Institute of Chemistry of Molecular Recognition and Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome, I-00168 Rome, Italy.*
[3]*Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.*

Summary

The effects of gemfibrozil (GFZ), an antihyperlipidemic agent, on the anionic transport of the human red blood cells (RBC) during the oxygenation-deoxygenation cycle were examined. Gemfibrozil clearly plays a role in the modulation of the anionic flux in erythrocytes; in fact it causes a strong increment of anions transport when the RBCs are in the high-oxygenation state (HOS). Such an effect is remarkably reduced in the low-oxygenation state (LOS). With the aim of identifying the dynamics of fibrate action, this effect has been investigated also in human ghost and chicken erythrocytes. These latter, in fact, are known to possess a B3 (anion transporter or Band 3) modified at the cytoplasmic domain (cdb3) which plays a significant role in the metabolic modulation of red blood cells. The results were analyzed taking into account the well known interactions between fibrates and both conformational states of haemoglobin i.e. the T state (deoxy-conformation) and the R state (oxy-conformation). The effect of gemfibrozil on anionic influx appears to be due to a wide interaction involving a "multimeric" Hb-GFZ-cdb3 macromolecular complex.

Introduction

Many molecules of pharmacological interest, besides having mainly a positive and important role in clinical therapy, can interfere with some cellular processes determining alterations of normal cell functions. Fibric acid derivatives (clofibrate, bezafibrate and gemfibrozil), particularly effective in reducing the incidence of coronary heart diseases (Frick *et al.* 1987; Manninen *et al.* 1988) and in lowering Tryglicerids (Brown and Goldstein 1990), are characterized by some important side effects such as an inhibitory activity of the mitochondrial respiratory chain, a significant production of oxygen free radicals (Chance and McIntosh 1995) and a strong allosteric interaction with hemoglobin.

In fact, these drugs permeating freely the erythrocyte membrane shift the oxygen equilibrium curve of human Hb to the right (Perutz *et al.* 1983; Abraham *et al.* 1983) lowering the affinity for oxygen more strongly than does the natural allosteric effector 2,3-bisphosphoglyerate (BPG) (Hyde *et al*. 1984; Marden *et al.* 1988). The binding sites of these antihyperlipidemic effectors with haemoglobin are known for both conformational states of the molecule (Perutz *et al.* 1983; Lalezari *et al*. 1990; Poyart *et al*. 1994; Safo *et al*. 2002; Yonetani *et al*. 2002; Tsuneshige *et al.* 2002; Imai *et al*. 2002; Shibayama *et al*. 2002; Tsuneshige *et al.* 2004; Laberge *et al*. 2005).

It has been demonstrated that gemfibrozil and clofibrate induce a shift of the O_2 dissociation curve (ODC) of erythrocytes toward the right (Scatena *et al.* 1995); moreover, the pO_2 seems to

represent a specific and selective signal that influences the anion transport function of band 3 through a molecular mechanism where the oxygen-linked T-R transition of Hb would play a key role (Galtieri *et al.* 2002; Russo *et al.* 2007). Thus the B3 cytoplasmic domain binds Hb through electrostatic interactions influenced by the oxygenation state of RBC (Salhany 1990; Chetrite *et al.* 1985); thus, the affinity of Hb for cdb3 is greater in the conformational T state than in the R state with K_{ass} approximately 10^4 M^{-1} and 10^2 M^{-1}, respectively (Walder *et al.* 1984). In addition, B3 undergoes numerous interactions with other cytoskeletal proteins, such as ankyrin (Bennett 1985), protein 4.2 and 4.1 (Korsgren *et al*. 1986; Han *et al.* 2000), with the hemichromes and several glycolytic enzymes (Chu *et al.* 2006), such as aldolase (Low 1986), phosphofructokinase (Murthy *et al.* 1981), glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase (Kliman *et al.* 1980), piruvate kinase (Campanella *et al.* 2005), glucose transporters (Jiang *et al.* 2006) protein tyrosine kinase $p72^{syk}$ (syk) (Harris *et al.* 1990; Harrison *et al.* 1994) and src $p56/53^{lyn}$ (lyn), and phosphatase protein PTP-1B (Zipser *et al*. 1996; Brunati *et al*. 2000).

These interactions are at the basis of molecular mechanisms which modulate cellular flexibility (Low *et al.* 1991; Goodall *et al.* 1994) and phosphorylation processes. In particular the phosphatase (PTP-1B), associated with the membrane, is responsible, through a redox system, for the low level of tyrosine phosphorylation normally present in these cells.

Moreover, the competition for cdb3 between Hb (in the T state) and glycolytic enzymes suggests a key role of O_2 in modulating erythrocyte's metabolism (Messana *et al*. 1996). In fact, at the level of tissues, the low O_2 saturation state of the red blood cell switch on the interaction of deoxy-Hb with cdb3 determining the release and therefore the activation of glycolytic enzymes that channel the glucose-6-phosphate (G6P) toward ATP and BPG production. In contrast, in HOS erythrocytes the lower affinity between oxy-Hb and cdb3 makes accessible the interaction sites of cdb3 to glycolytic enzymes the inhibition of which should reduce the glucose flux through the Embden-Meyerhof-Parnas pathway (EMP). As a consequence more G6P is available for the pentose phosphate pathway (PPP). In this way, RBC can satisfy the greater demand for NADPH, necessary at high oxygen saturation levels to counteract oxygen free radicals and ferrihemoglobin production (Giardina *et al*. 1995).

Based upon the fundamental role of Hb (connected with its oxygen-linked conformational transition T-R) in metabolic flux modulation and its known interaction with gemfibrozil, we tried to verify how much the presence of GFZ could influence the anionic transport of the human red blood cell, in the two extreme states of erythrocyte function, HOS and LOS. In addition, we tried to investigate the possible existence of metabolic changes caused by Hb-GFZ-cdb3 interactions.

Methods

Material

All reagents were from Sigma-Aldrich (St. Louis, MO, USA). Human erythrocyte samples were collected from informed healthy volunteers aged 30-50 years under the declaration that they had avoided any drug treatment at least one week before sample collection.

Preparation of red blood cells

Heparinized blood samples were washed three times with an iso-osmotic NaCl solution. During washings the white blood cells were discarded from the pellet. After washing the red blood cells were resuspended (hematocrit 3 %) in the incubation buffer (35 mM Na_2SO_4, 90 mM NaCl, 25 mM HEPES [*N*-(2-hydroxyethyl)-piperazine-N^1-2-ethanesulfonic acid], 1.5 mM $MgCl_2$), adjusted to pH 7.4 or 7.3 and 310 ± 20 mOsmol per kg, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco, Kyoto, Japan).

In experiments performed with deoxygenated erythrocytes, samples were submitted to cycles of in vacuo deoxygenation and nitrogen (ultrapure) saturation at a pressure of 100 kPa. This treatment allowed us to obtain different levels of deoxygenation (from 15 % up to 90 %), which were checked by determining hemoglobin saturation spectrophotometrically (Beckman DU 70 spectrophotometer) using the millimolar absorptivities reported by Zijlstra *et al*. (Zijlstra *et al*., 1991).

The buffer used to prepare deoxygenated erythrocytes was by 0.1 pH unit lower than that used for oxygenated erythrocytes, in order to compensate for the Haldane effect taking place during the deoxygenation step (Labotka *et al*. 1984). Thus, after the deoxygenation procedure, the oxygenated and deoxygenated samples had an external pH different by no more than 0.03 pH unit.

Preparation of RBC ghosts

Washed erythrocytes were lysed with iced hypotonic medium containing 5 mM Tris and 5 mM KCl. After hemolysis the hemoglobin and the intracellular contents were eliminated by centrifugation. The membranes were resealed by incubation at 37 °C for one hour with a closing buffer. The resealed ghosts were suspended in the same buffer used for sulfate transport in entire red blood cells.

Kinetic measurements

Cells were incubated in the above incubation buffer at 25 °C, under different experimental conditions. At several time intervals, 10 micromoles of the stopping medium SITS (4-acetamido-4´-isothiocyanostilbene-2,2´-disulfonic acid) were added to each test tube containing 5 mL of the red

blood cell suspension. Cells were then separated from the incubation medium by centrifugation (J2-HS Centrifuge, Beckman, Palo Alto, CA, USA) and washed three times at 4 °C with a sulfate-free medium to remove the sulfate trapped outside. After the last washing the packed cells were lysed with perchloric acid (4 %) and distilled water (2.8 mL final volume). Lysates were centrifuged for 10 min at 4000 × *g* (4 °C) and membranes were separated from the supernatant. Sulfate ions were precipitated from the supernatant by adding 1 mL of glycerol/distilled water mixture (1:1, V/V), 0.5 mL of 4 M NaCl and 1 M HCl, 0.5 ml of 1.23 M $BaCl_2 \cdot 2H_2O$ in order to obtain a homogeneous barium sulfate precipitate. The absorbance of this suspension was measured at 350–425 nm.

Using a calibrated standard curve, obtained by measuring the absorbance of suspensions obtained from solutions containing known sulfate amounts, the sulfate concentration was determined (Romano *et al.* 1998). Experimental data of sulphate concentration as a function of the time of incubation were analyzed by best fitting procedures according to the following equation:

$c(t) = c_\infty \left(1 - e^{-kt}\right)$ where $c(t)$ represents sulfate concentration at time t, c_∞ intracellular sulphate concentration at equilibrium, and k the rate constant of sulphate influx.

Determination of PTP activity

PTP activity was defined using *p*-nitrophenyl phosphate (*p*-NPP) as substrate. Briefly, membranes were suspended in 25 mM Hepes buffer of pH 7.3, containing 0.1 mM PMSF, 20 mM $MgCl_2$ and 15 mM *p*-NPP, and incubated at 37 °C for 30 min. After centrifugation, the release of *p*-nitrophenol was measured in the supernatant at 410 nm.

Statistical analysis

Differences were analyzed with a two-tailed Student's *t*-test for unpaired data. Upon occurrence the group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Dunnet test. The results are expressed as means ± S.D. $P < 0.05$ was considered significant.

Results

Here we study the gemfibrozil effects on the anionic transport of human erythrocyte in two extreme conditions represented by: HOS (about 90% of saturation) and LOS (about 15% of saturation).

As shown in Table 1 and Fig. 1 the mean of rate constant values determined for sulfate influx in HOS and LOS erythrocytes incubated with increasing concentrations of gemfibrozil reveal that the fibrate influences the anionic exchange kinetics under both conditions. More specifically the drug increases B3 activity proportionally to its concentration up to 3 mM; concentration levels higher than this value do not produce any further increase of B3 activity.

Fig. 2 shows the fibrate effect on the anionic transport kinetics in cells incubated with a gemfibrozil concentration of 3 mM. It may be seen that the drug modulation is higher in HOS erythrocytes (rate constant 0.052 $min^{-1}$and 0.012 min^{-1}, respectively, for treated and untreated RBCs) than in LOS (rate constant 0.008 min^{-1} and 0.005 min^{-1}, respectively, with and without GFZ). Since it is possible that O_2 affects the activity of other transport systems in red blood cells we carried out a set of experiments on integral cells and on ghosts pretreated with SITS, a specific inhibitor of B3 activity. Under these experimental conditions no sulfate transport was observed (data not shown); hence, we excluded the contribution of other transport systems in our observations.

Interestingly, we find that the fibrate effect on the anion influx is also present in particular structures like ghosts. These results are shown in Fig. 3 where a small increment of B3 activity in ghosts compared to the intact RBC can be noticed; this is due to the absence of several cytoplasmic factors that modulate the anion transport among which Hb is the most representative. However the persistence of gemfibrozil effect on the sulfate transport led us to investigate the nature of the drug action on the anionic channel.

A possible interaction path between the drug and B3 could involve a phosphorylative action toward Tyr 8, 21, 359 and 904; hence we tested the PTP-1B activity which is not affected by 3 mM gemfibrozil (for 1–3 h) (results not shown).

In order to go deeper into the GFZ molecular effect we performed a set of experiments on the anionic exchange in the presence of orthovanadate, a well-known phosphatase inhibitor which affects the phosphorylation/dephosphorylation state of B3. Kinetic measurements carried out on erythrocytes with 3 mM orthovanadate exhibit a modest increment of the B3 activity; these results were compared with those obtained with gemfibrozil. This comparison is reported in Table 2. The different modulation intensity of the anionic flux in response to orthovanadate and gemfibrozil and the absence of an effect of the latter on PTP-1B phosphatase activity allowed us to exclude a GFZ involvement on the phosphorylation state of the erythrocytic membrane. However, in order to better evaluate the results we carried out further kinetic experiments estimating the combined effects of orthovanadate and gemfibrozil.

The rate constant value measured under these conditions, the index of increment not being cumulative, indicated an effective role of fibrate, certainly connected with cdb3, the latter being the target of orthovanadate phosphorylation.

To confirm this hypothesis we used erythrocytes (chicken erythrocytes) characterized by B3 modified at the level of the cytoplasmic domain (Jay 1983). The chicken (*Gallus gallus*) red blood cells have been incubated in the presence and absence of 3 mM gemfibrozil; under the two experimental conditions the rate constant values were not significantly different (0.07 ± 0.019 min^{-1} and 0.068 ± 0.004 min^{-1}, respectively) indicating cdb3 as the fundamental target through which GFZ exercises its effect.

Discussion

In our previous studies (Galtieri *et al*. 2002; Russo *et al*. 2007) it was demonstrated that the anionic flux across the B3 is oxygen-dependent and is connected to the T-R conformational transition of Hb as also outlined by data reported in Fig.4. From these a role of gemfibrozil in the anionic flux modulation clearly emerges; the drug seems to have a "stimulating" action on the B3 activity that is much greater when the erythrocyte is at high oxygen saturation (HOS) conditions and less important in the LOS state. Experiments performed on the ghosts suggest that cdb3 is the main GFZ target; in fact, the observed effect can be attributed exclusively to the presence of gemfibrozil since these structures lack all the main cellular components and possible anionic transport mediators.

The results obtained may be interpreted on the basis of the strong interaction of GFZ with one of the main modulators of B3, hemoglobin. In this respect it should be recalled that X-ray crystallographic analysis demonstrated that two molecules of bezafibrate (BZF) bind to the central cavity of deoxy-Hb in a symmetrical fashion (Abraham *et al.* 1983) and that the BZF molecule which is structurally very similar to gemfibrozil, interacts with human Hb (HbA) not only in the T conformational state (Perutz *et al.* 1986; Noble *et al.* 1989; Bettati *et al*. 1997) but surprisingly also in the R state (Shibayama *et al.* 2002). Moreover recent modeling studies showed specific binding sites both in the T state and in the R state of HbA not only for BPG and inositol hexaphosphate (IHP) but also for clofibrate and BZF analogs (Laberge *et al*. 2005).

Due to the higher affinity of T-Hb for cdb3 compared to R-Hb a gradual increase of bound hemoglobin and, consequently, an increase of structural hindrance occur on going from HOS to LOS (Galtieri *et al.* 2002). Thus in the LOS of erythrocytes the Hb-GFZ complex, strongly

interacting with cdb3, may cause a structural hindrance of band 3 channel and, consequently, a decrease of anionic flux through the membrane.

In addition, BZF, a molecule similar to gemfibrotzil, is bound to Hb in the Z and R state not only at different sites but also at sites involving several numerical interactions (Perutz *et al.* 1986, Shibayama *et al.* 2002).

In this context the different modulation of the anionic flux observed in HOS and LOS states of the erythrocyte could be associated with the different binding free energy in the T-Hb-GFZ-cdb3 and R-Hb-GFZ-cdb3 complexes.

Deoxyhemoglobin binds the cdb3 by means of a series of electrostatic interactions (Jensen *et al*.1998) with the first 11 N-terminal residues of the anionic exchanger; such a binding is influenced by the oxygenation state of erythrocyte (K_{ass} approximately 4×10^{-4}M) (Chetrite *et al*. 1985). Hence, the remarkable difference in the anionic flux across B3, measured in red blood cell HOS and LOS states, could be ascribed to the various modes of binding of GFZ with Hb in the T and R conformational states (supported by the significant difference of K_{ass} between Hb and B3 on the oxygenation state).

The results could be interpreted in the light of the recent theory of the "global allostery model" which supposes that the allosteric effectors can bind to both the T and to R states of the Hb inducing direct tertiary conformational changes of the structure that can provoke remarkable variations of hemoglobin function (Yonetani *et al.* 2002; Shibayama *et al.* 2002; Laberge *et al.* 2005).

The anionic flux increase induced by the GFZ in the LOS state should be a positive physiological aspect because it may allow a more efficient CO_2 exchange (produced also "exogenously" through 6-phosphogluconate oxidation). However, a better analysis of RBC metabolism demonstrated a negative influence induced by the drug on the oxidative status of the cell.

In fact, the GFZ binding to Hb reduced remarkably the oxygen-binding affinity and delayed the separation from cdb3. This would cause a late binding of glycolytic enzymes to B3, extending the EMP pathway at the expense of NADPH production necessary to neutralize the oxygen radicals and to reduce the level of ferrihemoglobin. In other words, under these circumstances the GFZ would contribute significantly to the erythrocyte oxidative stress.

Hence, the interaction of GFZ with Hb may cause unexpected functional responses, such as those observed by Tsuneshige *et al.* (2002), Shibayama *et al.* (2002) and, significantly, by Coletta *et al*. (1999) where the addition of BZF to stripped Hb lowers the oxygen affinity of the R-state

hemoglobin by a factor of 8–10 at pH 7.0. The Hb-GFZ complex with new structural and functional characteristics, will be able to bind cdb3 even when haemoglobin is in its oxygenated state.

In this context the different modulation of the anionic flux observed here in HOS and LOS states of the erythrocyte could be tied to the different binding free energy in the complexes T-Hb-GFZ-cdb3 and R-Hb-GFZ-cdb3. In other words GFZ would interact with Hb and therefore with the whole complex with a binding free energy very different in the HOS and LOS states of the red blood cell. This consideration is supported by the different fibric effector stoichiometry in the T and R state of Hb (Laberge *et al.* 2005).

In summary, binding of GFZ to intraerythrocytic hemoglobin, beside decreasing the oxygen affinity of the cell, results in a substantial alteration of the oxygen-linked metabolic modulation since it makes the R state able to bind the cytoplasmic domain of band 3. This would greatly favour glycolysis with respect to the pentose shunt decreasing NADPH production and therefore the overall reducing power of the cell. On this basis we may say that erythrocytes treated with GFZ could be much more proned towards oxidative stress than normal untreated cells.

Last but not least, it is worthwhile to consider that the strong modification of anion transport at the level of band 3 by the gemfibrozil-hemoglobin complex could represent a useful model to further investigate other neglected aspects of peripheral oxygen delivery, suc as (a) the role of oxy-Hb in modulating isohydric transport and chloride shift at band 3 level, (b) the potential interplay between band 3 oxy-Hb and nitric oxide transport and release and (c) the reciprocal modulation between carbonate dehydratase II and oxy-Hb at the level of band 3 binding sites;
Above all, to better understand the complex and not yet totally clear function of erythrocyte-capillary functional unit.

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Reprint requests:

Dr. A. Kotyk, Institute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czechia; e-mail. kotyk@biomed.cas.cz

Table 1. Rates of sulfate transport measured in oxygenated and deoxygenated human red blood cells with and without gemfibrozil (p<0.05 was considered significant).

	Rate constant (min^{-1})	Rate constant (min^{-1})	p
Medium composition	Oxygenated	Deoxygenated	HOS vs. LOS
Control	0.012 ± 0.0026 *(n*=4*)*	0.005 ± 0.0006 *(n*=5*)*	0.0007
0.5 mM gemfibrozil	0.010 ± 0.0003 *(n*=3*)*	0.005 ± 0.0006 *(n*=5*)*	7.134×10^{-6}
1 mM gemfibrozil	0.020 ± 0.0075 *(n*=3*)*	0.005 ± 0.0018 *(n*=5*)*	0.0046
1.5 mM gemfibrozil	0.041 ± 0.0015 *(n*=4*)*	0.006 ± 0.0016 *(n*=3*)*	0.0041
3 mM gemfibrozil	0.052± 0.0014 *(n*=4*)*	0.008 ± 0.0014 *(n*=3*)*	0.0036
5 mM gemfibrozil	0.051 ± 0.0061 *(n*=3*)*	0.008 ± 0.0011 *(n*=3*)*	0.0002
10 mM gemfibrozil	0.052 ± 0.0080 *(n*=3*)*	0.008 ± 0.0021 *(n*=3*)*	0.0028

Table 2 Effect of concentration of orthovanadate and gemfibrozil on the rates of sulfate transport of oxygenated human adult red blood cells. (p<0.05 was considered significant).

Conditions	Rate constant (min^{-1})
Control	0.012 ± 0.0026 (n=4)
3mM orthovanadate	0.018 ± 0.0037 (n=4)
3mM gemfibrozil	0.052 ± 0.0014 (n=4)
3mM orthovanadate and 3mM gemfibrozil	0.032 ± 0.0051 (n=5)

Fig 1. Dose-dependent gemfibrozil induced modifications of the rate of sulfate transport measured in oxygenated (HOS) and deoxygenated (LOS) human red blood cells.(** = $p<0.01$ versus vehicle, by ANOVA).

Fig 2. Sulfate concentration (mg/l) determined in red blood cell lysates at different incubation times. Results of a typical experiment performed by incubating the erythrocytes with (closed symbols) and without (open symbols) 3 mmol/l of gemfibrozil under high (triangles) and low (circles) oxygen pressures. Curves were obtained by fitting experimental data with the equation $c(t) = c_\infty (1 - e^{-kt})$. See Methods for further experimental details.

Fig. 3. Sulfate concentration (mg/L) determined in ghost lysates at different incubation times. Results of a typical experiment performed by incubating the erythrocytes with (closed symbols) and without (opf symbols) 3 mmol/l of gemfibrozil. Curves were obtained by fitting experimental data with the equation: $c(t)= c_\infty (1 - e^{-kt})$. See Methods for further experimental details.

Fig 4. Rates of sulfate influx determined in human erythrocytes under different oxygen pressure. For experimental conditions see Methods. Rates are reported in min^{-1} and pO_2 values in mmHg

Fig. 1.

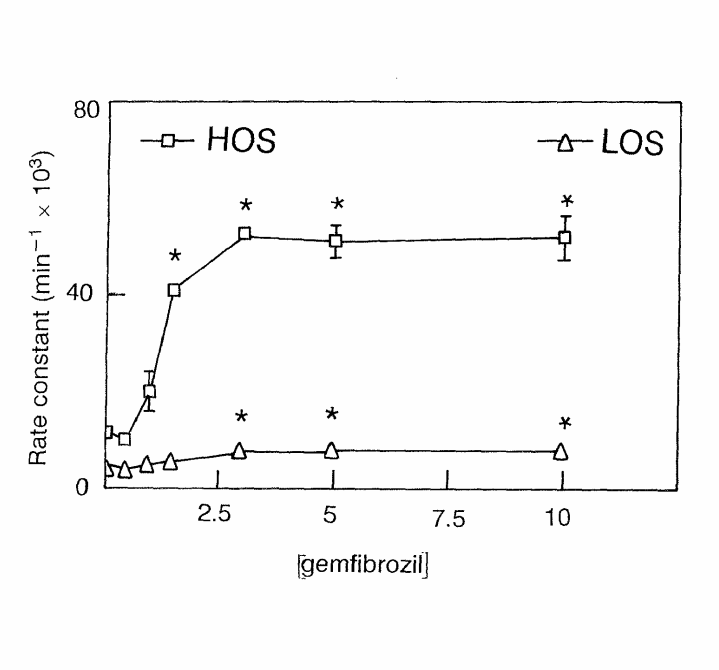

80
40
0
HOS
LOS
Rate constant (min^{-1} × 10^3)
2.5
5
7.5
10
[gemfibrozil]

Fig. 2.

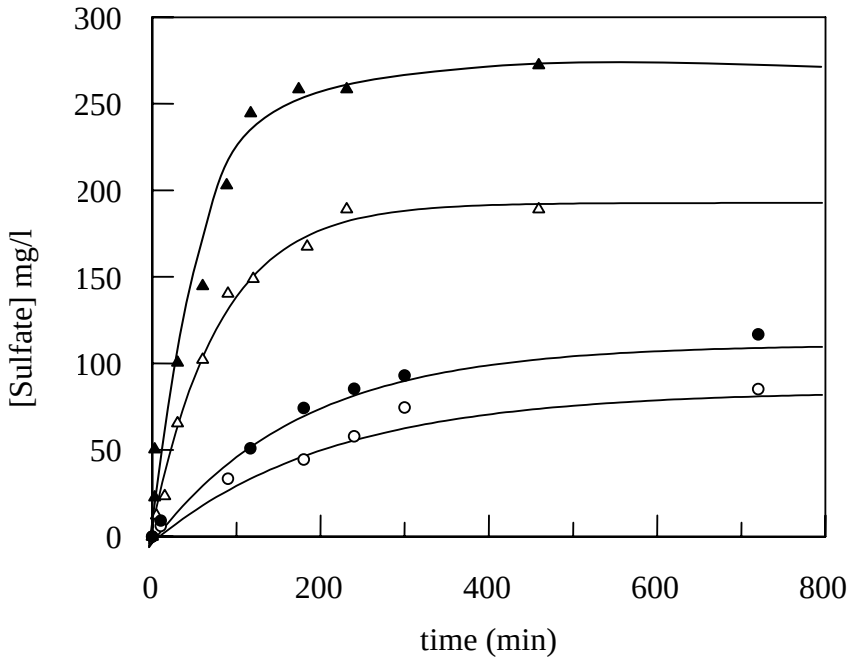

300
250
200
150
100
50
0
[Sulfate] mg/l
0
200
400
600
800
time (min)

Fig. 3.

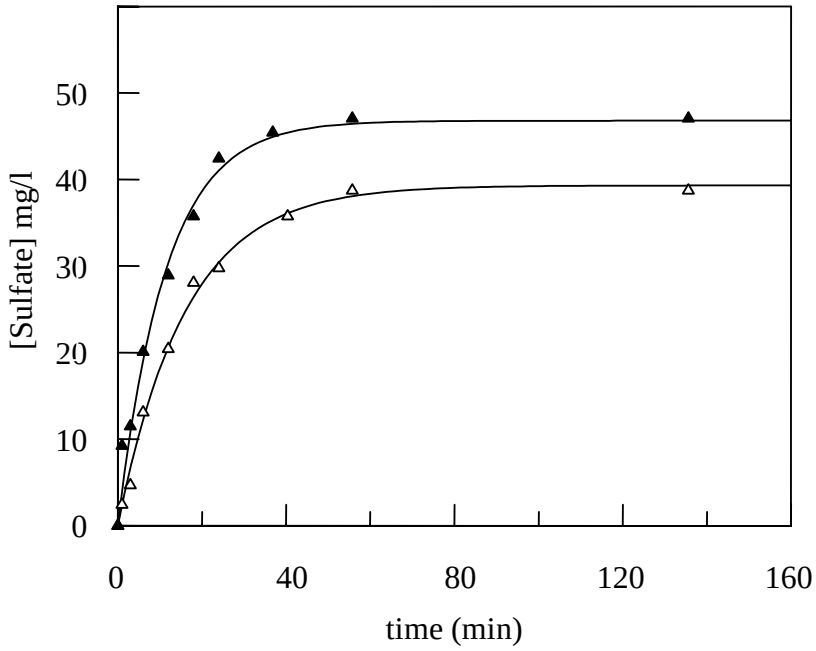
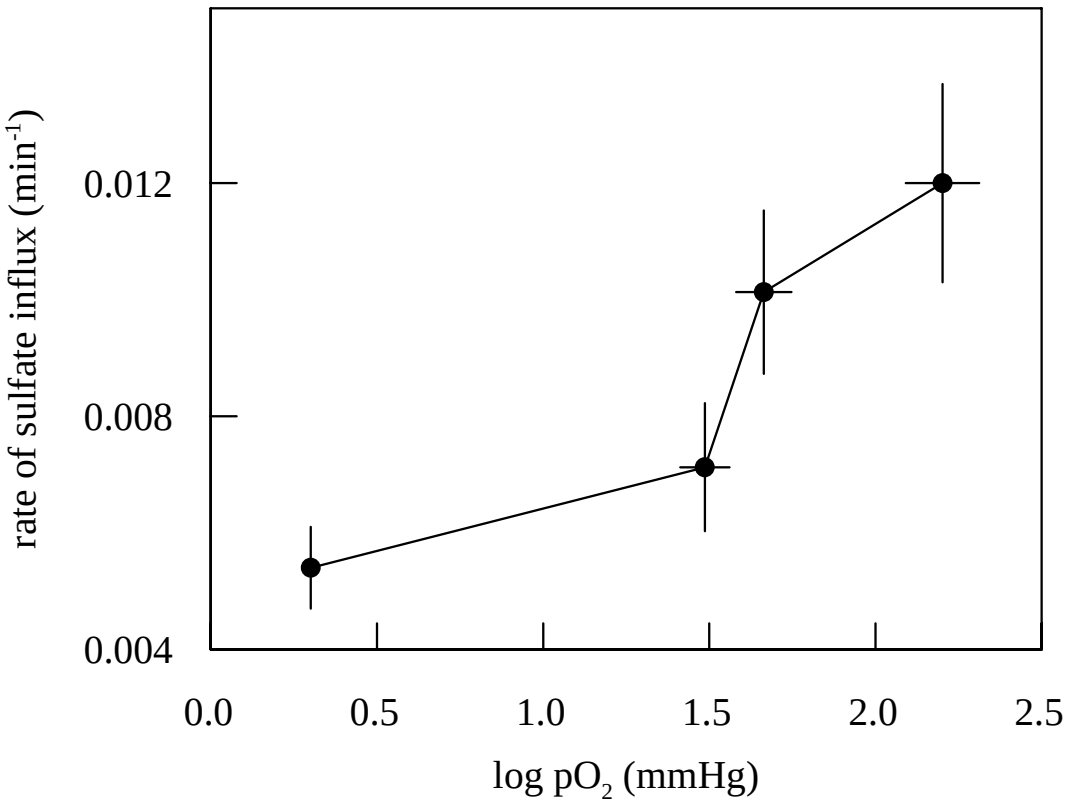


Fig 4.


rate of sulfate influx (min-1)
0.012
0.008
0.004
0.0
0.5
1.0
1.5
2.0
2.5
log pO2 (mmHg)