The Relationship between Chemiosmotic Parameters and Sensitivity to Anions and Organic Acids in the Acidophile *Thiobacillus ferrooxidans*

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We investigated the relationships between the trans-cytoplasmic-membrane chemiosmotic parameters, viz. the membrane potential and the pH difference, and the toxicity of anions and organic acids in the acidophile *Thiobacillus ferrooxidans*. Organic acids accumulated in the cytoplasm in response to the transmembrane pH difference and inorganic anions could be caused to accumulate in response to the membrane potential. The distribution of the organic acids was unaffected by the membrane potential and that of the anions was not influenced by the pH difference. These accumulations may be toxic because of a direct effect of a high concentration of the anion in the cytoplasm or by acidification of the cytoplasm. The point of inhibition of respiration was at the level of the respiratory chain cytochrome oxidase when Fe(II) was the respiratory substrate.

INTRODUCTION

Thiobacillus ferrooxidans is capable of growth by oxidation of Fe(II) iron or inorganic sulphur species, using oxygen as respiratory oxidant. This growth occurs in an acidic environment and is itself acidogenic when the substrate is native pyrite. During these processes the internal pH (pH_{in}) of the cell is maintained at approximately 6.5 while the pH of the suspending medium (pH_{out}) may be as low as 1.0 (Cox *et al.*, 1979). The pH difference (ΔpH) across the cytoplasmic membrane is one component of the proton-electrochemical potential (Δp); the other component is the membrane potential ($\Delta\psi$). These forces are related thus: $\Delta p = \Delta\psi - 59\Delta\text{pH}$ (Mitchell, 1966). Δp is the initially conserved form of the energy available from the redox potential difference donor and acceptor substrates in the functioning respiratory chain. The magnitude of the Δp reflects the energy status of the cell and is thus variable. However, of the two components which make up the Δp , the ΔpH component is not (and cannot be for physiological reasons) very variable under normal circumstances. $\Delta\psi$ varies, in response to the energy state of the cell, from close to zero to a value which is large and approximately equal and opposite to the ΔpH , i.e. when the cell is 'de-energized', Δp must be zero (Cox *et al.*, 1979).

Organic acids, in general, are deleterious to Fe(II) oxidation and cell viability. It has been suggested that one major factor contributing to the effectiveness of an organic acid in inhibiting Fe(II) oxidation is the relative electronegativity of the species, and that they act (i) via a direct effect on the Fe(II) oxidizing enzyme system; (ii) by reacting abiologically with Fe(II) outside the cell; and (iii) by non-selectively disrupting the cell envelope or membrane (Tuttle & Dugan, 1976; Tuttle *et al.*, 1977). We proposed that the toxicity of 'weak' acids can, in general, be ascribed to their accumulation in the cell matrix in response to the pH difference between the cytoplasm and the supporting medium (Ingledeew, 1982). The distribution of weak acids across a membrane separating phases of different pH is a well-documented physical process, the

Abbreviations: ΔpH , transmembrane pH difference; $\Delta\psi$, membrane potential; Δp , proton-electrochemical potential.

condition for the following treatment being that only the fully protonated form is significantly membrane-permeable. The concentrations of the anionic form in the two phases are related by a consideration of the Henderson-Hasselbalch equation in each phase and equating the internal and external concentrations of the undissociated form. For a monobasic acid the relationship of accumulation to pH_{in} , pH_{out} and pK_a is given by

$$\text{pH}_{\text{in}} = \text{pK}_a + \log_{10}[A_{\text{in}}/A_{\text{out}}(1 + 10^{\text{pH}_{\text{out}} - \text{pK}_a}) - 1]$$

where A_{in} and A_{out} are the concentrations of the anionic form inside and outside the cell (Addanki *et al.*, 1968). For a dibasic acid, if pK_{a1} and pK_{a2} are the same the accumulation ratio is squared when compared to a monobasic acid with the same pK_a (*et seq.* for polybasic acids). Accumulation of the anion of the weak acid could be toxic in three ways: a simple concentration effect (which can be very large), an acidification of the cell matrix by the protons which accompany the accumulation, or osmotic damage to the cytoplasmic membrane. Thus, all else being equal, the toxicity of organic acids to *T. ferrooxidans* can be predicted to be related to their basicity, pK_a values, the absence of positively charged groups (which may affect distribution and membrane permeability) and the pH of the suspending medium. In this paper we present evidence in support of this proposal.

As *T. ferrooxidans* is used in the leaching of minerals from different ore deposits there have been many studies on the effects of inorganic ions on cell growth and Fe(II) oxidation (for references see Ingledew, 1982). In general, *T. ferrooxidans* is unusually resistant to cations but relatively sensitive to anions. All inorganic anions are less effective than SO_4^{2-} at supporting Fe(II) oxidation or cell growth. This has led to the suggestion that SO_4^{2-} is directly involved in the mechanism of Fe(II) oxidation and models of Fe(II) oxidation incorporating a direct role for SO_4^{2-} have been proposed (Dugan & Lundgren, 1965; Lazaroff, 1983). SO_4^{2-} does, as any dominant anion will, have a direct physicochemical effect on the mid-point potential of the Fe(II)/Fe(III) couple; these effects are, however, due to complexation and do not necessarily imply a role in the reaction mechanism. Noting that the oxidation of sulphur and its reducing oxy-anions by *T. ferrooxidans* is also sensitive to the anionic composition of the medium (Razzell & Trussell, 1963; Lazaroff, 1977), we have sought a general physiological explanation for the toxicity of anions. On the basis of our findings we propose that one of the ways in which inorganic anions are toxic is via the accumulation of the anion into the cell cytoplasm as a result of the combination of a finite permeability of the cytoplasmic membrane to the charged anion and a membrane potential ($\Delta\psi$) positive inside, i.e. the anions tend to respond to the membrane potential as lipophilic ions [the $\Delta\psi$ and distribution are related by $\Delta\psi = (59/n) \log_{10}(A_{\text{in}}^{n-}/A_{\text{out}}^{n-})$]. In the case of an acidophile the $\Delta\psi$ is often positive inside, because, with the need to maintain the cytosol at neutrality, the ΔpH is very large (Cox *et al.*, 1979; Matin *et al.*, 1982).

The respiratory chain of *T. ferrooxidans* is short and comprises a blue copper protein, cytochromes *c*, and the oxidase cytochrome(s) a_1 (Ingledew, 1982). Organic acids, and under appropriate conditions anions, inhibit the respiratory chain, and we suggest this is by acidification of the cytoplasm. We have determined, herein, the site of inhibition to be the terminal O_2 -reducing reaction of the respiratory chain. This finding is consistent with earlier suggestions that most of the respiratory chain components involved in Fe(II) oxidation are located periplasmically or on the outer surface of the cytoplasmic membrane, and that only the oxidase is in contact with the cytoplasm (Ingledew *et al.*, 1977).

METHODS

Growth and harvesting. *T. ferrooxidans* (strain NCIB 8455) cells were grown at room temperature in a 60 l chemostat, sparged with air, and at a dilution rate of approximately 0.02 h^{-1} . The medium contained 180 mM- FeSO_4 , 37.5 mM- H_2SO_4 , 0.2 mM- KH_2PO_4 , 1 mM- $(\text{NH}_4)_2\text{SO}_4$ plus 0.2 ml l^{-1} of a salts solution (Cobley & Haddock, 1975). The cells were harvested by passage through an MSE continuous-flow rotor operating in an MSE 18 centrifuge at 17000 r.p.m. at a flow rate of approximately 200 ml min^{-1} . The cells were separated from a ferric hydroxysulphate-unhydrated ferric oxide layer (limonite) and washed twice in 10 mM- H_2SO_4 , pH 1.8, and twice in 50 mM- Na_2SO_4 . Cells (3–5 g wet wt) were then resuspended in appropriate buffer for experimentation and used fresh.

Measurement of ΔpH and $\Delta\psi$. This was done essentially as described by Nicholls (1974) but modified by

separating the cells by centrifugation through silicone oil (Rottenberg, 1979). The oil used was BDH silicone fluid 550 (GLC grade). The distribution across the cytoplasmic membrane of [^3H]acetic acid and [^{14}C]chloroacetic acid (ΔpH), and [^{14}C]thiocyanate ($\Delta\psi$), was determined. Initially, [^3H]acetate was used to determine the ΔpH ; however, it became apparent that pH_{in} was, in some cases, declining to values close to the pK_a of acetic acid (4.75) thus giving accumulation ratios that were too low for accurate determination of pH_{in} . Chloroacetic acid ($\text{pK}_a = 2.86$) was therefore also used to determine the ΔpH . $^3\text{H}_2\text{O}$ was used as a reference isotope with the ^{14}C -labelled probes; [^{14}C]sucrose was used with the ^3H -labelled probes. The suspending buffer normally used was 20 mM- β -alanine sulphate (pH 3.0), together with the specified organic acids and cells (approximately 1 mg protein ml^{-1}). For ΔpH measurement 0.075 $\mu\text{Ci ml}^{-1}$ (2.775 kBq ml^{-1}) [^{14}C]chloroacetate (normally 1 μM) and 0.5 $\mu\text{Ci ml}^{-1}$ (18.5 kBq ml^{-1}) $^3\text{H}_2\text{O}$ were used. For measurement of $\Delta\psi$ [^{14}C]thiocyanate [0.2 $\mu\text{Ci ml}^{-1}$ (7.4 kBq ml^{-1}); 7 μM] and $^3\text{H}_2\text{O}$ [0.5 $\mu\text{Ci ml}^{-1}$ (18.5 kBq ml^{-1})] were used. A suspension (250 μl) containing isotopes and approximately 0.25 mg (protein) of cells was used for each determination and placed in an Eppendorf tube. The Eppendorf tube contained, in the bottom, 50 μl of a dinonyl phthalate/silicone oil (45:55, v/v) mixture. The tubes were spun in an Eppendorf bench centrifuge at 13800 g for 1 min. The cells separated from the supernatant, pelleting through the oil. The supernatant was aspirated off, and 1 ml of distilled water was added to wash the pellet and tube. This was then aspirated off with the oil, leaving the pellet. SDS (50 μl ; 5 mM) was then added and the tubes were incubated at 60 $^{\circ}\text{C}$ for 2 h to solubilize the pellets. Scintillation fluid (1.2 ml; toluene/Triton X-100, 2:1, v/v) was added to each tube and the tubes placed in glass scintillation vials for scintillation counting. Samples from the original incubation and samples from incubations using cells denatured by heat treatment were also counted. In all incubations each determination was made in triplicate.

Measurement of NO_3^- distribution. This was done by using a similar procedure to that used for the determination of $\Delta\psi$ by isotope distribution, except that the NO_3^- concentrations in the pellet and supernatant were assayed chemically (colorimetrically) using the Szechrome NAS method (Polysciences Inc., Paul Valley Industrial Park, Warrington, PA 18976, USA). The values for the internal concentration of NO_3^- were calculated both from the concentration of the anion in the matrix and from the loss of the anion from the supernatant; the values were in good agreement.

Matrix volume. This was determined from the difference between the [^{14}C]sucrose permeable space and the $^3\text{H}_2\text{O}$ permeable space (Rottenberg *et al.*, 1972). The matrix volume was found to be 2.3 μl (mg protein) $^{-1}$. This result is in good agreement with that of Cox *et al.* (1979), who recorded a matrix volume of 2.4 μl (mg protein) $^{-1}$ for *T. ferrooxidans*.

Liquid nitrogen temperature spectrophotometry. Liquid nitrogen temperature optical difference spectra of cells in various respiratory states were obtained using a split-beam instrument constructed in the workshops of this department. The optical path length was 0.2 cm. The steady-state level of cytochrome reduction in control and experimental incubations was studied by comparison of the steady state *minus* oxidized difference spectra after freeze-clamping of the steady-state condition, and by comparison with fully reduced *minus* oxidized spectra. The cuvette holder consisted of an aluminium spade with two compartments of approximately 1 ml volume. Appropriate media containing cells (4 mg protein ml^{-1}) were placed in each cuvette. One cuvette was oxidized (H_2O_2); to the other substrate (5 mM- FeSO_4) was added. The samples were frozen by immersion after mixing (steady-state) or after a period to allow anaerobiosis to occur (fully reduced).

Assays. Respiratory activities were assayed in a Clark oxygen electrode at 30 $^{\circ}\text{C}$. Protein was measured by the method of Lowry with the inclusion of 1% SDS; bovine serum albumin was used as standard.

Bovine serum albumin, 2,4-dinitrophenol and β -alanine sulphate were from Sigma. All other chemicals were of AnalaR grade and were supplied by BDH. Radioisotopes and scintillation fluid (United Technologies Packard toluene scintillant) were from Amersham.

RESULTS AND DISCUSSION

The two forces of the $\Delta\mu$ can affect the distribution of an anion in two ways: (i) as a 'weak' acid when the uncharged form is permeable and is present at a finite concentration, and (ii) electrophoretically, when the charged form is permeable. In the former mechanism accumulation is driven by the ΔpH and in the latter by the $\Delta\psi$. These two mechanisms can be distinguished because of the acidophilic nature of the bacterium which allows the magnitude of the $\Delta\psi$ to be varied from close to 0 mV to greater than 150 mV, positive inside, under conditions which alter the ΔpH little (Cox *et al.*, 1979).

Effects of anions

The anions used in this study are listed in Table 1, with the approximate concentration of anion required to inhibit Fe(II) oxidation by 50% in the presence and absence of uncoupler at

Fig. 1. Clark oxygen electrode traces showing the effect of Br^- ions on the oxidation of Fe(II) by *T. ferrooxidans* under different conditions. (a) Control; 100 mM- Na_2SO_4 and 25 μM -DNP in 4 ml buffer. Substrate (2 mM- FeSO_4) was added where indicated (arrow) after 2 min preincubation of cells and buffer. (b) Substrate added last; 100 mM- NaBr and 25 μM -DNP were placed in the vessel. Cells and buffer were preincubated for 2 min before the addition of 2 mM- FeSO_4 (arrow). Note the chart speed is one-sixth that in the other traces. (c) Cells added last; 100 mM- NaBr , 25 μM -DNP and 2 mM- FeSO_4 were placed in the vessel. Respiration was initiated by the addition of cells (arrow). (d) No uncoupler; 100 mM- NaBr and cells were preincubated for 2 min before the addition of 2 mM- FeSO_4 where indicated (arrow). All incubations were at 30 °C in a 4 ml vessel. The basic buffer was 20 mM- β -alanine sulphate, pH 3.0, in all incubations, and the cells were added to 0.25 mg protein ml^{-1} .

Table 1. Anion inhibition of *Fe(II)* respiration in *T. ferrooxidans*

Q is the nominal charge per nm^2 of ion surface area. Values are calculated from data from *The Handbook of Chemistry & Physics* (Boca Raton: CRC Press). The I_{50} values for Fe(II) oxidation were determined with respect to the SO_4^{2-} controls of similar ionic strength; this did not necessarily correspond to the half-maximum rate as some ions were found to stimulate respiration at low concentrations. Rates of O_2 consumption were measured as described in Methods. DNP, where present, was at 25 μM . The sodium salts of the anions were used; the pH 3.0 buffer was 20 mM- β -alanine sulphate and the basic pH 0.94 buffer was 100 mM- H_2SO_4 . NA, Not assayed.

Anion	Q	Addition . . .	I_{50} (mM)			
			pH 0.94		pH 3.0	
			None	DNP	None	DNP
SO_4^{2-}	3.1		—	—	—	—
Cl^-	2.4		10	0.5	150	1
Br^-	2.0		10	0.5	120	7
I^-	1.6		NA	NA	NA	NA
NO_3^-	1.6		1	0.5	25	0.1

pH 3.0 and pH 0.94. NO_3^- , Cl^- , Br^- and I^- all have a single charge whereas SO_4^{2-} , which is the least inhibitory anion, and used as control, has a double charge. Of the halides I^- is the largest ion, and in the presence of I_2 this will tend to form larger (and more permeant) I_3^- species. Traces of Fe(III) present will oxidize some of the I^- to I_2 , which is why I^- was not tested for effects on Fe(II) respiration. The surface charge (Q) is a parameter that is calculated from the ionic surface area of the unhydrated ion and the sum of the modules of the individual charges. It provides a correlation with the toxicity of the anion and its ability to collapse pH_{in} . It can be seen from a comparison of the concentrations of anions required for 50% inhibition of oxidation that the presence of uncoupler (the protonophore DNP) greatly enhances the toxicity of the anions tested (Table 1). This is because at pH 3.0, when the bacteria are respiring in the absence of uncoupler, the $\Delta\psi$ will be approximately -40 mV inside (tends to electrophoretically exclude anions), whereas in the presence of uncoupler the $\Delta\psi$ will approach $+180$ mV inside, tending to

Fig. 2. Effect of inorganic anions on pH_{in} and $\Delta\psi$ in non-respiring *T. ferrooxidans* cells. (a, b) Effect of anions on $\Delta\psi$: (a) coupled; (b) uncoupled (+25 μM -DNP). (c, d) Effect of anions on pH_{in} : (c) coupled; (d) uncoupled (+25 μM -DNP). The concentration of the anions was 100 mM made up in 20 mM- β -alanine sulphate, pH 3.0. pH_{in} and $\Delta\psi$ were calculated from the distribution of radioisotopes as described in Methods. $\circ$, Na_2SO_4 ; $\blacktriangle$, NaCl ; $\blacklozenge$, NaBr ; $\bullet$, NaNO_3 ; $\triangle$, NaI ; $\blacksquare$, no salt added.

accumulate anions (Cox *et al.*, 1979). At pH 0.94, respiration both in the absence and presence of uncoupler will be more sensitive to anions, because the $\Delta\psi$ in the absence of uncoupler will be +70 mV inside, and is projected to be approximately +250 mV inside in the presence of uncoupler (in both cases tending to accumulate anions). Sensitivity to anions is thus a pH-dependent phenomenon: the lower the pH the greater the toxicity, as a result of the pH-dependence of the magnitude and polarity of $\Delta\psi$. The I_{50} values given in Table 1 were obtained from oxygen electrode traces of Fe(II) oxidation after the rates had stabilized, as described in the legend. Overall, the traces obtained for the effects of anions on Fe(II) oxidation are complex because order of addition, physiological state of the cells and length of preincubation time affect the oxidation rates, and these rates are often not linear. If cells are added to the medium containing the anion before substrate then the toxicity is greater, presumably because the cell has been exposed to the anion when unenergized. Uncoupler potentiates the inhibition. Some recovery of the oxidation rate may occur depending on the anion used, its concentration, the presence of uncoupler and extent of preincubation. These phenomena are illustrated in Fig. 1 for the anion Br^- : trace (a) is the control (SO_4^{2-}) and trace (b) (note slower time base) shows the effect of preincubating cells with buffer and uncoupler, in the presence of Br^- , before substrate addition. This is the most deleterious condition, presumably because the anion has had time to

Table 2. *Effects of organic acids on pH_{in} and $\Delta\psi$ in the absence and presence of DNP*

Each value is the mean of 18 determinations. The cells were non-respiring and the concentration of the organic acids was 5 mM; the buffer was 20 mM- β -alanine sulphate, pH 3.0. The measurements of ΔpH and $\Delta\psi$ were calculated from the distribution of radioisotopes as described in Methods.

Species	pK_a	-DNP		+DNP	
		pH_{in}	$\Delta\psi$	pH_{in}	$\Delta\psi$
None		5.9 ± 0.12	131 ± 2	5.6 ± 0.1	136 ± 3
Propionate	4.75	5.0 ± 0.06	76 ± 3	5.0 ± 0.03	106 ± 2
Acetate	4.9	5.0 ± 0.06	82 ± 2	5.0 ± 0.04	117 ± 2
Lactate	3.1	4.8 ± 0.03	72 ± 2	4.9 ± 0.03	106 ± 2
Chloroacetate	2.86	4.2 ± 0.04	65 ± 2	4.3 ± 0.07	85 ± 2
Pyruvate	2.5	4.3 ± 0.02	72 ± 2	4.3 ± 0.03	86 ± 2

accumulate inside before the onset of respiration. Trace (c) shows the effects of adding the cells last to the incubation mixture (in the presence of DNP): in this case the inhibition is progressive, presumably as the anion is accumulated. Trace (d) shows that in the absence of uncoupler Br^- is not very toxic; the respiration rate is not linear because the preincubation may have allowed some anion to accumulate in the cytoplasm, but not enough to render the process irreversible so that the respiratory process can re-establish the Δp and pH_{in} (hence the acceleration in rate).

The effect of the anions on the pH_{in} as a function of time is shown in Fig. 2. It can be seen that all species tested, except SO_4^{2-} , caused some acidification of the cell cytoplasm both in the absence and presence of DNP. In the absence of DNP only I^- was very effective in collapsing pH_{in} . The pH_{in} values obtained for SO_4^{2-} , Cl^- , Br^- and NO_3^- , in the absence of DNP did not fall further after 1 h (experiments were done for up to 6 h). However, in the presence of I^- , pH_{in} did continue to fall, reaching a low of 3.5 (pH_{out} 3.0) after 3 h. It must be remembered that endogenous substrate reserves may help to maintain the pH_{in} by providing energy for H^+ and ion transport in the absence of uncoupler, and may be able to cope for a while if the rate of entry is not too great. In the presence of DNP the pH_{in} falls further and more rapidly. There are two reasons for this: firstly, the possible loss of any ATP from endogenous metabolism, and secondly, because in the presence of uncoupler the $\Delta\psi$ will be more positive inside, pulling in the anions. The uncoupler will also facilitate the cotransport of protons with the anion movement. In the absence of substrate the effect of uncoupler will be less dramatic than in the presence of substrate because Δp is already much lower before uncoupler is added (cf. Table 1 and Fig. 2).

The effects of the anions on the $\Delta\psi$ in the presence and absence of DNP are also shown in Fig. 2. A comparison of the effects of the anions shows that the presence of DNP produces a larger $\Delta\psi$, positive inside, and potentiates the efficacy of the anions in collapsing the $\Delta\psi$. These results indicate that there is a predominating electrophoretic contribution to the distribution of the anions, in agreement with the findings in Table 1 and Fig. 1, and in contrast to the distribution of 'weak' acids which is unaffected by the presence of uncoupler (see below). The order of toxicity of the anions towards Fe(II) oxidation follows that of their efficacy in collapsing the pH_{in} suggesting that the inhibition arises from acidification of the cytoplasm. In addition, both the inhibition of substrate oxidation and the collapse of pH_{in} are potentiated by uncoupler.

To show, directly, that these anions distribute electrophoretically, we studied the accumulation in the cytoplasm of NO_3^- . At an initial external concentration of 0.1 mM NO_3^- accumulation reached equilibrium after approximately 15 min. Measurements were made in the presence of uncoupler, and under the conditions used (low concentration of permeant anion) the pH_{in} and the $\Delta\psi$ did not fall significantly, allowing an accumulation ratio for NO_3^- of approximately 140, representing a $\Delta\psi$ (calculated from the NO_3^- distribution) of approximately 130 mV. The internal NO_3^- concentration was calculated both directly from the amount of NO_3^- trapped in the matrix and indirectly from the decrease in concentration in the supernatant; the two values were in good agreement.

The acidification of the cytoplasm as a consequence of anion entry (at inhibitory concentrations of the anion) is due to the fact that the $\Delta\psi$ and ΔpH are in balance; a drop in $\Delta\psi$

Fig. 3. Effects of complex organic acids on pH_{in} . Cells were suspended in 10 mM- H_2SO_4 , pH 1.8 (●), 10 mM- H_2SO_4 + 1 mM-sodium oxalate, pH 1.8 (▲), 20 mM- β -alanine sulphate, pH 3.0 (○), or 20 mM- β -alanine sulphate + 10 mM-sodium glutamate, pH 3.0 (■). DNP (25 μM) was present in all incubations. pH_{in} was determined as described in Methods.

(inside positive) due to the movement of anions will mean that the ΔpH will tend to be greater than the opposing $\Delta\psi$, and thus protons will enter until the two forces are again balanced. In the absence of a protonophore the entry of protons may be slow, and when coupled respiration is occurring respiration can remove these protons. Thus, the collapsing of $\Delta\psi$ by anions leads to the acidification of the cytoplasm either in the presence of high concentrations of the anions or when the cells are 'de-energized'. Although *T. ferrooxidans* is sensitive to anions, it is unusually resistant to heavy metal cations. Specific mechanisms of resistance may exist but a general mechanism for resistance may be the opposite of the anion case, i.e. non-specifically permeable cations may be excluded by the $\Delta\psi$, whereas in neutrophiles they may be accumulated by a $\Delta\psi$ of normal polarity.

Effects of organic acids

We studied the effects of a selected range of acids on ΔpH and $\Delta\psi$ and on respiratory activity. In addition we studied the converse – the effects of ΔpH and $\Delta\psi$ on the distribution of the acids – and distinguished between the role of the ΔpH and the $\Delta\psi$ in these phenomena. The organic acids used are listed in Table 2. The effect of the monobasic acids (5 mM) on the pH_{in} and on the $\Delta\psi$ across the bacterial cytoplasmic membrane is also shown. The control value for pH_{in} obtained with chloroacetic acid as a probe is lower than when acetate was used as the probe (pH_{in} 6.5 and 6.2 in the absence and presence of DNP respectively), probably because chloroacetic acid affects pH_{in} through its larger accumulation ratio, but it was used because of its lower pK_a (as explained in Methods). However, taking into account the effect of the probes themselves on ΔpH and $\Delta\psi$, it can still be seen that all of the organic acids tested caused some acidification of the cell cytoplasm, propionic acid being the least effective of this group, followed by acetic, lactic, pyruvic and chloroacetic acids. This sequence approximately follows the increase in predicted accumulation ratios (and decreasing pK_a values). The time-course of the effects were followed between 2 min and 1 h. In the case of all those monobasic acids tested the collapse of pH_{in} appears to occur within the first 10 min of incubation. The effects of organic acids on pH_{in} and $\Delta\psi$ when DNP is present are also shown in Table 2. The protonophore did not potentiate the collapse of ΔpH and $\Delta\psi$ in the presence of organic acids, in contrast to its effect in the presence of permeant anions. The more complex glutamic and oxalic acids were slower in their effects on ΔpH and $\Delta\psi$. The effects of oxalate (two carboxyls), glutamate (two carboxyls, one amino group) and β -alanine (one carboxyl and one amino group) on the ΔpH are shown in Fig. 3. The ΔpH in the control (10 mM- H_2SO_4) and in the presence of oxalate were determined at pH 1.8, whereas the effects of β -alanine sulphate and glutamate were determined at pH 3.0. The pH_{in} falls significantly in the presence of low concentrations of oxalic acid (1 mM) but more

Table 3. *Inhibition of respiration by organic acids*

Buffer (20 mM- β -alanine sulphate, pH 3.0) plus cells were preincubated for 10 min before addition of substrate (5 mM-ascorbate/0.1 mM-TMPD, or 10 mM-FeSO₄), at 30 °C. The concentration of acids was 10 mM; that of DNP was 25 μ M. Each value is the mean of five or more determinations. The respiration rates [μ g-atoms O min⁻¹ (mg protein)⁻¹] equivalent to 100% were as follows: 0.72 (ascorbate/TMPD; -DNP); 0.92 (ascorbate/TMPD; +DNP); 0.81 [Fe(II); -DNP]; 0.82 [Fe(II); +DNP].

Species	Respiration rate (percentage of control)			
	Substrate . . . Ascorbate/TMPD		Fe(II)	
	-DNP	+DNP	-DNP	+DNP
None	100	100	100	100
Propionate	79	69	70	58
Acetate	63	54	68	78
Lactate	82	63	74	59
Chloroacetate	8	14	10	14
Pyruvate	16	36	34	40

slowly than in the case of the monobasic acids (where collapse of pH_{in} is complete after 10 min). Theory predicts massive accumulation of oxalic acid into the cell cytoplasm. This slow effect may be because of slow equilibration with a chelated form of the oxalate (oxalate readily complexes with a range of cations). The distribution of glutamic acid is complicated by the amino group which will be positively charged and is likely to reduce non-specific membrane permeability, and may introduce an electrophoretic element into the distribution (this acid is not deleterious at these concentrations).

A number of reports cite specific interactions between the Fe(II)/Fe(III) couple and anions (Huffman & Davidson, 1956; Tuttle & Dugan, 1976; Tuttle *et al.*, 1977). To determine the importance of these direct effects we also tested the action of the acids on the oxidation of the artificial substrate ascorbate with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). The results obtained both for Fe(II) and ascorbate/TMPD oxidation are complex because order of addition, physiological state of the cells and times of preincubation affect the oxidation rates, and these rates are generally not linear. However, the sensitivities to organic acids of oxidation of the two substrates by *T. ferrooxidans* parallel each other, indicating general rather than specific mechanisms. If the cells are added to the medium containing the organic acid before substrate then the toxicity is greater, presumably because the cell has been exposed to the acid when unenergized. Some recovery of the oxidation rate may occur depending on the organic acid used and extent of preincubation. The data shown in Table 3 are taken from oxygen electrode traces of substrate oxidation by *T. ferrooxidans* cells. The cells were preincubated with the organic acid for 10 min before addition of the substrate and the maximum oxidation rate was measured; this developed after a variable lag period. The most potent inhibitors both of Fe(II) and ascorbate/TMPD oxidation were chloroacetate and pyruvate. Propionate, acetate and lactate inhibited to approximately similar extents. This order of toxicity approximately follows that of the efficacy of the acids in collapsing the pH_{in} , suggesting that the inhibition arises from acidification of the cytoplasm.

Cox *et al.* (1979) have shown that *T. ferrooxidans* respiring with either Fe(II) or ascorbate/TMPD at pH 3.0 maintains a $\Delta\psi$ of +40 mV; on the addition of DNP this falls to approximately -180 mV. The effect of organic acids on respiration in the presence of DNP is shown in Table 3. The inhibition of respiration in the presence or absence of DNP was qualitatively similar. With DNP chloroacetate and pyruvate are less effective, probably because DNP does cause a small fall in pH_{in} , thus lowering the maximum accumulation ratio (e.g. if the pH_{in} falls from 6.5 to 6 then the maximum theoretical accumulation ratio of pyruvate falls from 2.4×10^3 to 7.6×10^2). If the anions could be accumulated electrophoretically then the large $\Delta\psi$, positive inside in the presence of DNP, would lead to greater accumulation and hence greater sensitivity. These results should be contrasted with the ability of DNP to potentiate the toxicity of inorganic anions, and indicate that there is no significant electrophoretic contribution to the

distribution of the organic acids. If the distribution of the organic acids were significantly influenced by electrophoretic processes then their ability to inhibit Fe(II) and ascorbate/TMPD oxidation, and to be accumulated inside the cell, would be greatly potentiated by the addition of DNP.

We attempted to determine the specific site in the respiratory chain at which these acids inhibited respiration. To do this we studied the extent of oxidation/reduction of respiratory chain components during steady-state respiration. If a component is on the substrate [Fe(II)] side of the block it will become reduced relative to the control; if on the O₂ side it will become oxidized. Comparisons of the optical difference spectra (at 77 K; not shown) indicates that during steady-state respiration in the absence of inhibitor the cytochromes *c* and *a*₁ are only partially reduced; however, during the residual respiration occurring in the presence of chloroacetic acid all of these cytochromes are extensively reduced. This indicates that the site of inhibition is on the O₂ side of the cytochromes, i.e. at or close to the terminal O₂ reduction reaction. This location of the first pH-sensitive cytosolic site in the respiratory chain at the terminal step in the chain is in accord with the model of the Fe(II)-oxidizing respiratory chain proposed by Ingledew *et al.* (1977). In this model most of the respiratory chain components are located periplasmically or on the external surface of the cytoplasmic membrane, except for the O₂ reduction site.

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