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 ($\Delta pH$) across the cytoplasmic membrane is one component of the proton-electrochemical potential ($\Delta \psi$); the other component is the membrane potential ($\Delta \psi$). These forces are related thus: $\Delta \psi = \Delta \psi - 59 \Delta pH$ (Mitchell, 1966). $\Delta \psi$ 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 $\Delta \psi$ reflects the energy status of the cell and is thus variable. However, of the two components which make up the $\Delta \psi$, the $\Delta pH$ 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 $\Delta pH$, i.e. when the cell is 'de-energized', $\Delta \psi$ 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 abiotically 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 (Ingledew, 1982). The distribution of weak acids across a membrane separating phases of different pH is a well-documented physical process, the

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

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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 \( \text{pH}_{\text{in}} \), \( \text{pH}_{\text{out}} \), and \( \text{pK}_a \) 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 \( A_{\text{in}} \) and \( A_{\text{out}} \) are the concentrations of the anionic form inside and outside the cell (Addanki et al., 1968). For a dibasic acid, if \( \text{pK}_a \) and \( \text{pK}_b \) are the same the accumulation ratio is squared when compared to a monobasic acid with the same \( \text{pK}_a \) (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, \( \text{pK}_a \) values, the absence of positively charged groups (which may affect distribution and membrane permeability) and the \( \text{pH} \) 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 \( \text{SO}_4^{2-} \) at supporting Fe(II) oxidation or cell growth. This has led to the suggestion that \( \text{SO}_4^{2-} \) is directly involved in the mechanism of Fe(II) oxidation and models of Fe(II) oxidation incorporating a direct role for \( \text{SO}_4^{2-} \) have been proposed (Dugan & Lundgren, 1965; Lazaroff, 1983). \( \text{SO}_4^{2-} \) 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 \( \Delta \psi = (59/n) \log_{10}(A_{\text{in}}/A_{\text{out}}) \)]. In the case of an acidophile the \( \Delta \psi \) is often positive inside, because, with the need to maintain the cytosol at neutrality, the \( \Delta \text{pH} \) 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) \( a \) (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 601 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\(_2\)SO\(_4\), 0.2 mM-K\(_2\)HPO\(_4\), 1 mM-(NH\(_4\))\(_2\)SO\(_4\), plus 0.2 ml\(^{-1}\) 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\(_2\)SO\(_4\), pH 1.8, and twice in 50 mM-Na\(_2\)SO\(_4\). Cells (3–5 g wet wt) were then resuspended in appropriate buffer for experimentation and used fresh.

**Measurement of \( \Delta \text{pH} \) 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 $[^3$H]acetic acid and $[^{14}$C]chloroacetic acid (ΔpH), and $[^{14}$C]thiocyanate (Δψ), was determined. Initially, $[^3$H]acetic was used to determine the ΔpH; however, it became apparent that pHma was, in some cases, declining to values close to the pK of acetic acid (4.75) thus giving accumulation ratios that were too low for accurate determination of pHma. Chloroacetic acid (pKₐ = 2.86) was therefore also used to determine the ΔpH. $[^3$H]O₂ was used as a reference isotope with the $[^{14}$C]-labelled probes; $[^3$H]sucrose was used with the $[^3$H]-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 μCi ml⁻¹ (2.775 kBq ml⁻¹) $[^{14}$C]chloroacetate (normally 1 μM) and 0.5 μCi ml⁻¹ (18.5 kBq ml⁻¹) $[^3$H]O₂ were used. For measurement of Δψ $[^{14}$C]thiocyanate [0.2 μCi ml⁻¹ (7.4kBq ml⁻¹); 7 μM] and $[^3$H]O₂ [0.5 μCi 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 13 800 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 °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 NO₃⁻ distribution.** This was done by using a similar procedure to that used for the determination of Δψ by isotope distribution, except that the NO₃⁻ 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 NO₃⁻ 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 $[^3$H]O₂ 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 (H₂O₂), to the other substrate (5 mM-FeSO₄) 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 °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 Δp 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 Δψ. These two mechanisms can be distinguished because of the acidophilic nature of the bacterium which allows the magnitude of the Δψ 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-Na₂SO₄ and 25 μM-DNP in 4 ml buffer. Substrate (2 mM-FeSO₄) 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-FeSO₄ (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-FeSO₄ 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-FeSO₄ 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

<table>
<thead>
<tr>
<th>Anion</th>
<th>Q</th>
<th>pH 0.94 Addition</th>
<th>pH 0.94 DNP</th>
<th>pH 3.0 Addition</th>
<th>pH 3.0 DNP</th>
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<tr>
<td>SO₄²⁻</td>
<td>3-1</td>
<td>None</td>
<td>10</td>
<td>0.5</td>
<td>150</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>2-4</td>
<td>10</td>
<td>0.5</td>
<td>120</td>
<td>7</td>
</tr>
<tr>
<td>Br⁻</td>
<td>2-0</td>
<td>10</td>
<td>0.5</td>
<td>120</td>
<td>7</td>
</tr>
<tr>
<td>I⁻</td>
<td>1-6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>1-6</td>
<td>1</td>
<td>0.5</td>
<td>25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

pH 3.0 and pH 0.94. NO₃⁻, Cl⁻, Br⁻ and I⁻ all have a single charge whereas SO₄²⁻, 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⁻ this will tend to form larger (and more permeant) I₂ species. Traces of Fe(III) present will oxidize some of the I⁻ to I₂, 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 pHᵢₒ. 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 Δψ will be approximately −40 mV inside (tends to electrophoretically exclude anions), whereas in the presence of uncoupler the Δψ will approach +180 mV inside, tending to
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 $+70 \text{ mV}$ inside, and is projected to be approximately $+250 \text{ mV}$ 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 $\text{Br}^-$: trace (a) is the control ($\text{SO}_4^{2-}$) and trace (b) (note slower time base) shows the effect of preincubating cells with buffer and uncoupler, in the presence of $\text{Br}^-$, before substrate addition. This is the most deleterious condition, presumably because the anion has had time to

Fig. 2. Effect of inorganic anions on pH$_i$ and $\Delta \psi$ in non-respiring *T. ferrooxidans* cells. (a, b) Effect of anions on $\Delta \psi$: (a) coupled; (b) uncoupled (+25 $\mu\text{M}$-DNP). (c, d) Effect of anions on pH$_{in}$: (c) coupled; (d) uncoupled (+25 $\mu\text{M}$-DNP). The concentration of the anions was 100 mM made up in 20 mM-$\beta$-alanine sulphate, pH 3.0. pH$_{in}$ and $\Delta \psi$ were calculated from the distribution of radioisotopes as described in Methods. $\bigcirc$, Na$_2$SO$_4$; $\blacktriangle$, NaCl; $\blacklozenge$, NaBr; $\bullet$, NaNO$_3$; $\triangle$, NaI; $\blacksquare$, no salt added.
Table 2. Effects of organic acids on $\Delta p$H$_{in}$ 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-$\beta$-alanine sulphate, pH 3.0. The measurements of $\Delta p$H and $\Delta \psi$ were calculated from the distribution of radioisotopes as described in Methods.

<table>
<thead>
<tr>
<th>Species</th>
<th>$pK_a$</th>
<th>$\Delta p$H$_{in}$</th>
<th>$\Delta \psi$</th>
<th>$\Delta p$H$_{in}$</th>
<th>$\Delta \psi$</th>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td>5.9 ± 0.12</td>
<td>131 ± 2</td>
<td>5.6 ± 0.1</td>
<td>136 ± 3</td>
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<tr>
<td>Propionate</td>
<td>4.75</td>
<td>5.0 ± 0.06</td>
<td>76 ± 3</td>
<td>5.0 ± 0.03</td>
<td>106 ± 2</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.9</td>
<td>5.0 ± 0.06</td>
<td>82 ± 2</td>
<td>5.0 ± 0.04</td>
<td>117 ± 2</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.1</td>
<td>4.8 ± 0.03</td>
<td>72 ± 2</td>
<td>4.9 ± 0.03</td>
<td>106 ± 2</td>
</tr>
<tr>
<td>Chloroacetate</td>
<td>2.86</td>
<td>4.2 ± 0.04</td>
<td>65 ± 2</td>
<td>4.3 ± 0.07</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>2.5</td>
<td>4.3 ± 0.02</td>
<td>72 ± 2</td>
<td>4.3 ± 0.03</td>
<td>86 ± 2</td>
</tr>
</tbody>
</table>

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 $\Delta p$ 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 $\Delta \psi$ 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 pH$_{in}$. 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 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 $\Delta p$H are in balance; a drop in $\Delta \psi$...
Effects of organic acids

We studied the effects of a selected range of acids on \( \Delta \text{pH} \) and \( \Delta \psi \) and on respiratory activity. In addition we studied the converse — the effects of \( \Delta \text{pH} \) and \( \Delta \psi \) on the distribution of the acids — and distinguished between the role of the \( \Delta \text{pH} \) 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 \( \text{pH}_{\text{in}} \) and on the \( \Delta \psi \) across the bacterial cytoplasmic membrane is also shown. The control value for \( \text{pH}_{\text{in}} \) obtained with chloroacetic acid as a probe is lower than when acetate was used as the probe (\( \text{pH}_{\text{in}} \) 6.5 and 6.2 in the absence and presence of DNP respectively), probably because chloroacetic acid affects \( \text{pH}_{\text{in}} \) 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 \( \Delta \text{pH} \) 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 \( \text{pH}_{\text{in}} \) appears to occur within the first 10 min of incubation. The effects of organic acids on \( \text{pH}_{\text{in}} \) and \( \Delta \psi \) when DNP is present are also shown in Table 2. The protonophore did not potentiate the collapse of \( \Delta \text{pH} \) 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 \( \Delta \text{pH} \) and \( \Delta \psi \). The effects of oxalate (two carboxyls), glutamate (two carboxyls, one amino group) and \( \beta \)-alanine (one carboxyl and one amino group) on the \( \Delta \text{pH} \) are shown in Fig. 3. The \( \Delta \text{pH} \) in the control (10 mM-H\(_2\)SO\(_4\)) and in the presence of oxalate were determined at pH 1.8, whereas the effects of \( \beta \)-alanine sulphate and glutamate were determined at pH 3.0. The \( \text{pH}_{\text{in}} \) 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 μM. Each value is the mean of five or more determinations. The respiration rates [μg-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].

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<tr>
<th>Species</th>
<th>Ascorbate/TMPD</th>
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<tbody>
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</tbody>
</table>

slowly than in the case of the monobasic acids (where collapse of pHᵢₙ 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 pHᵢₙ, 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 Δψ 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 pHᵢₙ, thus lowering the maximum accumulation ratio (e.g. if the pHᵢₙ falls from 6.5 to 6 then the maximum theoretical accumulation ratio of pyruvate falls from 2.4 × 10³ to 7.6 × 10²). If the anions could be accumulated electrophoretically then the large Δψ, 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 a₁ 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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