The antioxidant potential of pyruvate in the amitochondriate diplomonads *Giardia intestinalis* and *Hexamita inflata*

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***Giardia intestinalis* and *Hexamita inflata* are microaerophilic protozoa which rely on fermentative metabolism for energy generation. These organisms have developed a number of antioxidant defence strategies to cope with elevated O₂ tensions which are inimical to survival. In this study, the ability of pyruvate, a central component of their energy metabolism, to act as a physiological antioxidant was investigated. The intracellular pools of 2-oxo acids in *G. intestinalis* were determined by HPLC. With the aid of a dichlorodihydrofluorescein diacetate-based assay, intracellular reactive oxygen species generation by *G. intestinalis* and *H. inflata* suspensions was monitored on-line. Addition of physiologically relevant concentrations of pyruvate to *G. intestinalis* and *H. inflata* cell suspensions was shown to attenuate the rate of H₂O₂- and menadione-induced generation of reactive oxygen species. In addition, pyruvate was also shown to decrease the generation of low-level chemiluminescence arising from the oxygenation of anaerobic suspensions of *H. inflata*. In contrast, addition of pyruvate to suspensions of respiring *Saccharomyces cerevisiae* was shown to increase the generation of reactive oxygen species. These data suggest that (i) in *G. intestinalis* and *H. inflata*, pyruvate exerts antioxidant activity at physiological levels, and (ii) it is the absence of a respiratory chain in the diplomonads which facilitates the observed antioxidant activity.**

Keywords: parasite, oxidative stress, reactive oxygen species, yeast, dichlorodihydrofluorescein diacetate

INTRODUCTION

The diplomonads *Giardia intestinalis* and *Hexamita inflata* are flagellated protozoa which inhabit O₂-limited environments. *G. intestinalis* is a parasite which colonizes the mucosa of the gastrointestinal tract, causing one of the most common water-borne diseases in humans – giardiasis (Adam, 1991). *H. inflata* is free-living and can be found in the anoxic regions of marine and freshwater environments (Dando *et al.*, 1993; Fenchel *et al.*, 1995). In addition, parasitic species of *Hexamita* (which may have free-living stages) have been

observed in a variety of vertebrate and invertebrate animals (e.g. Kulda & Nohynková, 1978; Buchmann *et al.*, 1995). The energy demand in these organisms is met by carbohydrate and amino acid fermentation (Brown *et al.*, 1998; Biagini *et al.*, 1998). The principal products of glucose metabolism include ethanol, alanine, acetate (also lactate for *H. inflata*) and CO₂ (Brown *et al.*, 1998; Biagini *et al.*, 1998). Pyruvate is central to this metabolism, and the relative rates of generation of the end-products are influenced by the ambient values for O₂ tension (Biagini *et al.*, 1998; Paget *et al.*, 1993a). Both of these organisms lack mitochondria or detectable cytochromes (Brugerolle, 1974; Paget *et al.*, 1993b; Biagini *et al.*, 1997) (and hence oxidative phosphorylation), but do, however, have high affinities for O₂, comparable to those of aerobic protozoa (Paget *et al.*, 1993b; Biagini *et al.*, 1997). The consumption of O₂ has

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Abbreviation: SOD, superoxide dismutase.

been shown to rise linearly with the surrounding O_2 tension, up to a threshold level (30–100 μM O_2 , depending on the species), above which consumption is arrested due to the formation of reactive oxygen species (Paget *et al.*, 1993b; Biagini *et al.*, 1997; Lloyd *et al.*, 2000). The oxygen consumption has been attributed to the activity of NAD(P)H oxidase, and it has been postulated that the subsequent change in the redox state of the NAD(P)H pools affects the relative rates of production of end-products (Brown *et al.*, 1998; Paget *et al.*, 1993a; Biagini *et al.*, 1997).

Much of the energy metabolism in diplomonads more closely resembles that of bacteria than that of eukaryotic cells. Examples include the presence of pyrophosphate-dependent glycolytic enzymes (Mertens, 1990; Phillips *et al.*, 1997), the eubacterial-like pyruvate:ferredoxin oxidoreductase (Townson *et al.*, 1996), and the presence of the arginine dihydrolase pathway (Schofield *et al.*, 1990; Biagini *et al.*, 1998; Dimopoulos *et al.*, 2000). The antioxidant defence system in diplomonads is also unlike that found in most eukaryotes. Cysteine replaces glutathione as the major intracellular thiol (Brown *et al.*, 1993; G. A. Biagini, D. M. Brown & M. R. Edwards, unpublished observation in *H. inflata*), and catalase and non-specific peroxidase activities are undetectable (Biagini *et al.*, 1997; Brown *et al.*, 1995). An Fe-type superoxide dismutase (SOD) has been described for *H. inflata* (Biagini *et al.*, 1997) and, although SOD activity was initially reported in *Giardia* (Thompson *et al.*, 1993), in a more recent study SOD activity was undetectable even after induction with 1,10-phenanthroline (Brown *et al.*, 1995). In addition, *G. intestinalis* contains a thioredoxin-reductase-like disulphide reductase which has the ability to reduce cystine (Brown *et al.*, 1996), whereas *H. inflata* contains an as yet uncharacterized thiol reductase which may be involved in cystine reduction (Biagini *et al.*, 1997).

The ability of pyruvate to react with H_2O_2 (and $\cdot O_2^-$), forming acetate and CO_2 non-enzymically, has long been known (Holleman, 1904). However, whether pyruvate plays a physiological role as an H_2O_2 scavenger in cells is not clear. In this study, we report on the ability of pyruvate, a central component of diplomonad metabolism, to act as an intracellular scavenger of reactive oxygen species in both *G. intestinalis* and *H. inflata*. The contrasting physiological roles of pyruvate in oxidative and fermentative metabolism are discussed.

METHODS

Organisms and solutions. The *G. intestinalis* Portland 1 strain and *H. inflata* were grown as described previously (Knodler *et al.*, 1994; Biagini *et al.*, 1997). For experimentation, cells were grown to late-exponential phase, harvested by centrifugation at 650 g for 5 min and resuspended in PBS buffer (pH 7.2) containing 150 mM NaCl, 5 mM K_2HPO_4 and 1.8 mM KH_2PO_4 . *Saccharomyces cerevisiae* (a laboratory wild-type strain) was grown in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) bacteriological peptone and 2% (w/v) D-glucose, in a shaking incubator at 30 °C. For experimentation,

cells were grown to mid-exponential phase, harvested by centrifugation at 1000 g for 5 min and resuspended in PBS. Pyruvate (the Na salt), menadione and H_2O_2 solutions were prepared freshly on the day of experimentation.

Determination of intracellular 2-oxo acid pools in *G. intestinalis*. Pre-purification, derivatization and subsequent separation of 2-oxo acids in giardial extracts were based on the methods described by Hayashi *et al.* (1982) and Liao *et al.* (1977). Dry polyacrylamide beads (15 g Bio-Gel P-60) were allowed to swell overnight in distilled water (200 ml). The gel suspension was then added to a 98% hydrazine hydrate solution (120 ml) and mixed for 6 h at 50 °C. At the end of the reaction period, the gel was washed free of hydrazine with 0.1 M NaCl and suspended in a storage solution containing 0.2 M NaCl, 0.02 M Na_2EDTA , 0.1 M H_3BO_3 , 5 mM NaOH and 5 μM pentachlorophenol at 4 °C. Cell suspensions in PBS were centrifuged at 10000 g through oil (a mixture of dibutyl phthalate and diiso-octyl phthalate, 4:1, v/v; 1.03 g ml⁻¹) into 0.1 ml 1 M perchloric acid. The perchloric acid extract was placed on ice for 1 h and then centrifuged at 10000 g for 1 min. To 0.1 ml of the supernatant from the perchloric acid extract, 0.2 ml of an internal standard solution of 2-oxo-octanoate (80 μM) together with 1 ml 0.1 M acetic acid and 3 ml 0.1 M NaCl were added. The mixture was then loaded onto a glass column containing the 0.3 ml of hydrazide gel. After elution was complete, the gel was washed five times with 0.1 M NaCl and then transferred to a test tube; *o*-phenylenediamine solution (2 ml) was added to the gel and this was then incubated at 80 °C for 2 h, after which 0.5 g Na_2SO_4 was added. The derivatives of the 2-oxo acids were extracted with ethyl acetate, evaporated to dryness under a stream of N_2 , and the residue was dissolved in methanol. HPLC was carried out with a 250 × 4 mm Li-Chrosorb RP-8 (Capital HPLC) (5 μm particle size) column. The mobile phase consisted of a 6:4 methanol:water mix operating at a flow rate of 1 ml min⁻¹ at 35 °C. The overall recovery of 2-oxo acids measured using radiolabelled pyruvate and 2-oxoglutarate was 72 ± 7%.

Determination of intracellular amino acid pools in *G. intestinalis*. Intracellular amino acid analysis was performed as described previously (Knodler *et al.*, 1994), with 3-aminopropionate as the internal standard, on a Beckman 6300 amino acid analyser.

Flow cytometry. Cellular fluorescence (green emission, 530–540 nm) was monitored by flow cytometry using a MoFlo cytometer (Cytomation PTY) with excitation at 488 nm from a water-cooled 200 mW argon-ion laser. In addition, forward light scatter and right-angle side scatter were measured and used for gating data collection. Typically, signals from ≥ 50000 cells were acquired and analysed using Cyclops software (Cytomation PTY) for each sample. The flow-cytometric histogram shown is representative of at least three independent experiments performed with both *G. intestinalis* and *H. inflata*.

Monitoring of intracellular reactive oxygen species production. Dichlorodihydrofluorescein diacetate (H_2DCFDA ; Molecular Probes) was used to detect the intracellular generation of reactive oxygen intermediates (predominantly H_2O_2). H_2DCFDA was added (final concentration 2 μM) to suspensions of *G. intestinalis* and *H. inflata* in PBS (approx. 5×10^6 cells ml⁻¹) and fluorescence was measured by flow cytometry or on-line using a Perkin Elmer LS 50B luminescence spectrophotometer (excitation 504 nm, emission 527 nm). H_2DCFDA is non-fluorescent and is able to permeate biological membranes. Once in the cytosol, esterase activity

renders the indicator non-permeant by forming the fluorescent product dichlorofluorescein, and the fluorescence intensity of the dye is proportional to the rate of oxidation by reactive oxygen species (predominantly H_2O_2). The fluorescence signals detected from *H. inflata* H_2 DCFDA-loaded cells were larger than those from *G. intestinalis* and *S. cerevisiae* cells. These differences are believed to reflect dye-loading efficiencies and do not compromise the assays. The fluorescence traces shown are representative of at least three independent experiments performed with both *G. intestinalis* and *H. inflata*.

Measurement of photo-emissive O_2 -reduction products in *H. inflata*. *H. inflata* cells in PBS (approx. 6.5×10^6 cells ml^{-1}) were exposed to low levels of dissolved O_2 in a continuously stirred reactor (200 r.p.m.) fitted with an observation window for photon counting (Lloyd *et al.*, 1979, 1985). The reactor was open for gas exchange from a mobile gas phase, the composition of which was controlled using a gas mixer; dissolved O_2 was monitored using a Radiometer electrode. A Peltier-cooled, red-sensitive, photon-counting photo-multiplier device (EMI 9817) provided continuous monitoring of weak chemiluminescence emission (> 900 nm) without spectral discrimination. The O_2 concentration of air-saturated water at 25 °C was taken to be 253 μM O_2 (Wilhelm *et al.*, 1977). Data are representative of three experiments.

RESULTS

Initially, the study was concerned with measuring the physiological level of pyruvate and other 2-oxo acids in *G. intestinalis*. As described in Methods, 2-oxo acids were pre-purified from the cell extracts using hydrazide gel, and were derivatized with *o*-phenylenediamine, producing the 2-quinoxalinol derivatives. The pre-purification of 2-oxo acids was essential, since without this step, no discrete separation was observed. The intracellular 2-oxo acid pool concentrations in *G. intestinalis* together with those of their amino acid counterparts (for comparison) are given in Table 1. The number of intracellular 2-oxo acids was smaller, and their concentrations lower, than those of their amino acid counterparts, only seven 2-oxo acids versus 25 amino acids being detected (not all shown). The major 2-oxo acid detected was pyruvate (0.5 mM), which is consistent with its amino form, alanine, being present at the highest intracellular concentration. As *H. inflata* has a similar intracellular concentration of amino acids to

that of *G. intestinalis* (Biagini *et al.*, 2000), it was therefore assumed that their pyruvate levels would also be comparable. Experiments were then conducted to test whether pyruvate, added at concentrations close to physiological levels, would confer protection against reactive oxygen species.

We have previously observed that incubation of diplomonads in air-saturated solutions results in the generation of reactive oxygen species (e.g. H_2O_2) (Biagini *et al.*, 1997; Lloyd *et al.*, 2000). An H_2 DCFDA-based assay was designed (see Methods) to monitor the intracellular generation of reactive oxygen species on-line.

Flow cytometry was used to confirm that fluorescence of H_2 DCFDA-loaded *G. intestinalis* and *H. inflata* cells was arising intracellularly. Analysis of unstained *H. inflata* cells in PBS revealed a small degree of auto-fluorescence, whereas H_2 DCFDA-loaded cells (15 min incubation) were observed to have a significantly increased intracellular fluorescence intensity (Fig. 1). Addition of H_2O_2 (200 μM) to H_2 DCFDA-loaded cells further increased intracellular fluorescence, indicating free diffusion of H_2O_2 into the cell cytosol. Prior incubation of H_2 DCFDA-loaded cells with pyruvate (1 mM, 5 min) attenuated the H_2O_2 -induced fluorescence (Fig. 1). Similar results were also observed with *G. intestinalis* trophozoites (not shown).

The scavenging activity of pyruvate was monitored in real time with a luminescence spectrophotometer. Fluorescence from H_2 DCFDA-loaded *H. inflata* cells was monitored after the addition of 200 μM H_2O_2 (Fig. 2). Pyruvate was shown to reduce the rate of reactive oxygen species generation (as indicated by the rate of increase in fluorescence intensity), the degree of attenuation being proportional to the pyruvate concentration (Fig. 2). Heat-fixed (60 °C, 15 min) H_2 DCFDA-loaded cells were used as a negative control. A smaller but significant decrease in the rate of H_2O_2 -induced reactive oxygen species generation by pyruvate was also observed in *G. intestinalis* (Table 2). Addition of pyruvate metabolism end-products such as ethanol, alanine and acetate (up to 3 mM) was shown not to decrease the rate of H_2O_2 -induced reactive oxygen species generation in *H. inflata* or *G. intestinalis*.

Table 1. Intracellular 2-oxo acids and their amino forms in *G. intestinalis*

Values are means $\pm$ SD ($n = 10$).

2-Oxo acid	Concn (mM)	2-Amino form	Concn (mM)
2-Oxoglutarate	0.14 ± 0.03	Glutamate	17.8 ± 1.2
Glyoxylate	0.31 ± 0.05	Glycine	7.9 ± 0.8
Pyruvate	0.49 ± 0.08	Alanine	54.3 ± 1.9
2-Oxo-n-butyrate	0.31 ± 0.04	2-Amino-n-butyrate	0.5 ± 0.2
2-Oxoisovalerate	0.08 ± 0.02	Valine	5.3 ± 0.8
2-Oxoisocaproate	0.28 ± 0.05	Leucine	4.2 ± 0.5
2-Oxocaproate	0.16 ± 0.03	Isoleucine	2.2 ± 0.2

Fig. 1. Flow cytometric histograms of intracellular fluorescence intensity (488 nm excitation, 530–540 nm emission) in *H. inflata* trophozoites. The various cell populations reflect different treatments: (a) intrinsic autofluorescence, (b) fluorescence arising from H_2DCFDA -loaded cells, (c) H_2DCFDA -loaded cells in the presence of $200 \mu M$ H_2O_2 and (d) H_2DCFDA -loaded cells, previously incubated with pyruvate ($1 mM$), in the presence of $200 \mu M$ H_2O_2 .

Fig. 2. Fluorimetric traces of *H. inflata* H_2DCFDA -loaded cells. Increases in fluorescence are representative of increases in the rate of oxidative species generated (see Methods). Fluorescence was monitored from suspensions of live and heat-fixed cells ($60^\circ C$, 15 min) after the addition of H_2O_2 ($200 \mu M$) in the absence and presence of various concentrations of pyruvate.

Fig. 3. Fluorimetric traces of *G. intestinalis* H_2DCFDA -loaded cells. Fluorescence intensity was monitored from suspensions of live and heat-fixed cells ($60^\circ C$, 15 min) after the addition of menadione ($300 \mu M$) in the absence and presence of pyruvate ($1 mM$).

Menadione was also used to induce the generation of intracellular reactive oxygen species (predominantly $\cdot O_2^-$). Fluorescence arising from H_2DCFDA -loaded *G.*

intestinalis cells was shown to increase upon the addition of $300 \mu M$ menadione (Fig. 3). The increase in the rate of fluorescence intensity (and thus the increase in the rate

Table 2. Effect of pyruvate on H_2O_2 - and menadione ($\cdot O_2^-$)-induced generation of oxygen species in *G. intestinalis* and *H. inflata*, as measured by intracellular H_2DCFDA fluorescence

Organism	Major reactive oxygen species	Pyruvate concn (mM)	Inhibition (%)*	P†
<i>Giardia</i>	$\cdot O_2^-$	1	27.4 ± 0.8	< 0.001
<i>Giardia</i>	H_2O_2	0.6	2.3 ± 0.9	0.025
<i>Giardia</i>	H_2O_2	3	5.9 ± 0.6	< 0.001
<i>Giardia</i>	H_2O_2	6	8.7 ± 1.5	0.001
<i>Hexamita</i>	$\cdot O_2^-$	1	6.7 ± 1.2	0.001
<i>Hexamita</i>	H_2O_2	0.6	13.3 ± 0.5	< 0.001
<i>Hexamita</i>	H_2O_2	3	25.2 ± 0.4	< 0.001
<i>Hexamita</i>	H_2O_2	6	40.8 ± 0.3	< 0.001

* Values are means $\pm$ SD ($n = 3$) of independent experiments.

† P values (two-tailed *t*-test) for differences between fluorescence values in the presence and absence of pyruvate after 6 min incubation with either menadione ($300 \mu M$) or H_2O_2 ($200 \mu M$).

Fig. 4. Chemiluminescence of a whole-cell suspension of *H. inflata*. Low-level chemiluminescence was monitored in a continuously stirred reactor, open for gas exchange. Traces indicate the response of an anaerobic suspension of *H. inflata* in PBS to oxygenation. (a) $[O_2]$, (b) chemiluminescence.

of reactive oxygen species generation) with menadione was shown to be reduced by the addition of 1 mM pyruvate (Fig. 3). Heat-fixed (60°C , 15 min) H_2 DCFDA-loaded cells were used as a negative control. Duplication of the experiment using *H. inflata* cells also resulted in pyruvate (1 mM) reducing the rate of menadione-induced reactive oxygen species generation, but to a lesser extent than that shown for *G. intestinalis* (Table 2).

Low-level chemiluminescence arises from cells predominantly as a result of singlet O_2 generation (e.g. during lipid peroxidation). As such, measurement of low-level chemiluminescence is a useful assay for the monitoring of oxidative stress. Low-level chemiluminescence was observed upon oxygenation of an anaerobic suspension of *H. inflata* cells (Fig. 4). Prior anaerobic incubation of trophozoites with pyruvate (1 mM) dramatically lowered the burst of chemiluminescence occurring upon oxygenation of the suspending solution (Fig. 5).

One of the hypotheses leading to this study was that the observed reactive oxygen species scavenging activity of pyruvate favoured anaerobic energy metabolism over oxidative metabolism. To test this hypothesis, *S. cerevisiae* cells (which had been grown aerobically) in PBS were loaded with H_2 DCFDA and the subsequent fluorescence was monitored continuously with the luminescence spectrophotometer. The fluorescence arising from the H_2 DCFDA-loaded yeast cells was shown to increase proportionally with pyruvate concentration (Fig. 6a). In addition, the rate of increase in fluorescence intensity in H_2 DCFDA-loaded yeast cells, oxidatively

Fig. 5. Chemiluminescence of a whole-cell suspension of *H. inflata*. Traces indicate the response of an anaerobic suspension of *H. inflata* in PBS to oxygenation in the presence of pyruvate (1 mM). (a) $[O_2]$, (b) chemiluminescence.

Fig. 6. Fluorimetric traces of *S. cerevisiae* H_2 DCFDA-loaded cells. (a) Fluorescence intensity was monitored from a cell suspension of *S. cerevisiae* in PBS during the addition of various concentrations of pyruvate. (b) Fluorescence intensity was monitored from a cell suspension of *S. cerevisiae* in PBS after the addition of H_2O_2 (200 μM) and pyruvate (3 mM).

stressed by the addition of H_2O_2 (200 μM), was not attenuated by the addition of up to 3 mM pyruvate (Fig. 6b). These data indicate that, under our *in vitro* conditions, pyruvate does not have a net antioxidant

activity in aerobically grown yeast, but rather is acting as a generator of reactive oxygen species.

DISCUSSION

In this study we have measured the intracellular levels of 2-oxo acids, including pyruvate, in *G. intestinalis*. The ability of pyruvate, added at concentrations close to physiological levels, to act as an oxidative species 'scavenger' was then investigated in both *G. intestinalis* and *H. inflata*. The effect of pyruvate on the generation of reactive oxygen species in the fermentative diplomonads was then compared with that in respiring *S. cerevisiae*.

As shown in Table 1, the 2-oxo acid pools in *G. intestinalis* were shown to be smaller in number and at lower concentrations than their amino forms. Values for the intracellular amino acids were very similar to those reported previously (Knodler *et al.*, 1994). The ratio of the pyruvate pool to the alanine pool was almost identical to that of the 2-oxoglutarate pool to the glutamate pool, suggesting that the reaction catalysed by alanine aminotransferase is approximately at equilibrium in this parasite. In addition to alanine, the other neutral amino acids glycine, valine, leucine and isoleucine that contribute significantly to the total amino acid pool were represented by the respective oxo-acids glyoxylate, 2-oxoisovalerate, 2-oxoisocaproate and 2-oxocaproate in the oxo acid pool. However, no oxaloacetate pool was detected, although *G. intestinalis* contains aspartate aminotransferase activity (Edwards *et al.*, 1994). The lack of detectable oxaloacetate may be due to its instability; alternatively, it may simply be present at undetectable levels because of the high activity of malate dehydrogenase, favouring oxaloacetate reduction. In addition, the 2-oxo acid forms of the aromatic acids phenylalanine and tyrosine were undetectable. This was also unexpected in view of the aromatic aminotransferase activities that have been previously observed in *G. intestinalis* (Edwards *et al.*, 1994).

Biological membranes are readily permeable to pyruvate and it can therefore be assumed that the extracellular addition of pyruvate would raise, at least transiently, the levels of intracellular pyruvate. With the aid of the H₂DCFDA-based assays, intracellular generation of reactive oxygen species by *G. intestinalis* and *H. inflata* was monitored in real time. As expected, the addition of H₂O₂ (which is uncharged and therefore freely penetrates the plasma membrane) was shown to increase the rate of intracellular generation of reactive oxygen species (as indicated by the increase in fluorescence intensity; Fig. 2, Table 2). Similarly, the addition of the synthetic quinone menadione increased the rate of intracellular generation of reactive oxygen species (Fig. 3, Table 2). Both H₂O₂- and menadione-induced generation of reactive oxygen species in the diplomonads were reduced by the addition of pyruvate. Pyruvate was shown to be more effective at reducing menadione-induced generation of reactive oxygen species (pre-

dominantly $\cdot\text{O}_2^-$) in *G. intestinalis* than in *H. inflata* (Table 2). Conversely, pyruvate was shown to be more effective at reducing H₂O₂-induced generation of reactive oxygen species in *H. inflata* than in *G. intestinalis* (Table 2). This disparity could be explained by the different antioxidant systems present in these organisms, e.g. *H. inflata* contains an Fe-type SOD (Biagini *et al.*, 1997), whereas SOD is undetectable in *G. intestinalis* (Brown *et al.*, 1995).

Pyruvate was also shown to reduce the generation of weak chemiluminescence arising from the oxygenation of the suspending solution (Fig. 4). These data signify that pyruvate, *in vivo*, may play a significant role as a low-molecular-mass antioxidant in *G. intestinalis* and *H. inflata*, protecting the cells from both the generation and propagation (as indicated by the reduction of singlet O₂ production) of reactive oxygen species. It is also possible that the other 2-oxo acids detected, such as 2-oxoglutarate (which has known H₂O₂-scavenging activity; Halliwell & Gutteridge, 1999), may also act as physiological antioxidants.

In contrast to the effect of pyruvate in diplomonads, the addition of pyruvate to yeast had no observable antioxidant effect, but rather caused an increase in the rate of generation of reactive oxygen species (Fig. 6). The oxidative respiratory chain is a major source of H₂O₂ and other oxygen radicals (e.g. $\cdot\text{O}_2^-$) due to 'leaky' redox reactions (e.g. Halliwell & Gutteridge, 1999). It is therefore probable that pyruvate in respiring yeast cells acts to increase the generation of reactive oxygen species by increasing the flux of electrons down the respiratory chain.

In a recent elegant study performed by Brand & Hermfisse (1997), the phenomenon of aerobic glycolysis was investigated in mitogen-activated rat thymocytes. Resting thymocytes were observed to meet their ATP demand largely by oxidative glucose catabolism, whereas the energy demand of proliferating thymocytes was satisfied by glycolysis. Decreased reactive oxygen species generation, due to the shut-down of respiration as well as an observed increase in the pyruvate pool during glycolytic metabolism, were suggested as metabolic strategies employed by the cell to minimize oxidative stress during cell division. It appears, therefore, that pyruvate can act to induce or to scavenge reactive oxygen species, depending on the metabolic mode (e.g. oxidative or glycolytic) of the cell. In diplomonads, where components of the respiratory chain are undetectable (e.g. cytochromes), the fermentative energy metabolism results in pyruvate acting as an efficient scavenger of reactive oxygen species. Whether these two diplomonads have the ability to regulate pyruvate levels in response to oxidative stress remains to be determined. It is conceivable, however, that previously described changes in metabolic end-product formation (Paget *et al.*, 1993a; Biagini *et al.*, 1998) may indirectly promote a rise in the intracellular pyruvate availability in response to moderate increases in O₂ tension.

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