Hydrogen peroxide production in uncoupled mitochondria of the parasitic nematode worm *Nippostrongylus brasiliensis*

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1. Mitochondria from the parasitic nematode worm *Nippostrongylus brasiliensis* produce H_2O_2 in the energized state; higher rates of H_2O_2 production were observed in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazine. 2. Antimycin A inhibits respiration and H_2O_2 production by 70 and 65% respectively; the residual activities can be attributed to alternative electron-transport pathway(s). 3. *o*-Hydroxydiphenyl and 1,3,5-trihydroxybenzene, inhibitors of alternative electron transport, inhibit respiration by 37% and H_2O_2 production by 26%. 4. Another inhibitor of alternative electron transport, salicylhydroxamic acid, shows a complex mode of action; low concentrations (< 0.5 mM) stimulate respiration and H_2O_2 production, whereas 2 mM-salicylhydroxamic acid inhibited respiration by 35% and stopped H_2O_2 production completely. 5. O_2 thresholds were observed for the inhibition of respiration at O_2 concentrations $> 57.7 \mu M$ and inhibition of H_2O_2 production ($> 20.5 \mu M-O_2$); apparent K_m values for oxygen were $5.5 \mu M$ and $3.0 \mu M$ respectively. 6. In the presence of antimycin A the O_2 -inhibition thresholds and apparent K_m values for O_2 of respiration and H_2O_2 production matched closely, suggesting that the alternative oxidase is a likely site of H_2O_2 production. 7. These results are discussed in relation to O_2 toxicity to *N. brasiliensis*.

INTRODUCTION

The small gut-dwelling nematode *Nippostrongylus brasiliensis* (length 3–6 mm, diameter 0.1–0.2 mm) lives in an environment close to the mucosal epithelium where oxygen fluctuates over the range 0–60 μM (Atkinson, 1980). Paget *et al.* (1987) have shown that respiration is inhibited, both in whole worms and in mitochondria-enriched fractions, by oxygen above a threshold of about 60 μM . Respiration in these mitochondria is predominantly via a mammalian-like phosphorylating electron-transport chain inhibited by antimycin A and terminating in cytochrome aa_3 , as well as an alternative salicylhydroxamic acid-sensitive pathway (Fry *et al.*, 1983); some two-thirds of the respiration may occur through the aa_3 oxidase, although this proportion depends ultimately on the O_2 concentration prevailing (Paget *et al.*, 1987). The components of the alternative electron-transport pathway have not been characterized.

Respiration of *N. brasiliensis* mitochondria in the presence of an uncoupler of energy conservation results in the production of H_2O_2 (Paget *et al.*, 1987), and the rate of H_2O_2 production, like that of respiration, can be stimulated by low concentrations of salicylhydroxamic acid (< 0.5 mM). Increased respiration under these conditions requires the operation of the main chain, since the extra O_2 consumption observed in the presence of salicylhydroxamic acid is antimycin A-sensitive (Paget *et al.*, 1987).

The present paper describes simultaneous measurement of the effects of inhibitors on respiration and H_2O_2 formation at different O_2 concentrations. We demonstrate that electron transport along the main and alternative electron-transport chains can separately

generate H_2O_2 ; a strong correlation between the O_2 -dependence of respiration and H_2O_2 production in the presence of antimycin A suggests that the major site of H_2O_2 formation is at the alternative oxidase when the main chain is blocked.

MATERIALS AND METHODS

Maintenance and growth of *Nippostrongylus brasiliensis*

The Wellcome strain of *N. brasiliensis* was maintained by weekly passage in male, category 2, Sprague–Dawley rats. The method for production of adult worms has been described in detail by Fry *et al.* (1983). Fourth-stage adult *N. brasiliensis* were obtained from 8–10-week-old rats, each initially infected with 5000 larvae. Maintenance of adult *N. brasiliensis* was carried out in a complete medium (Jenkins & Carrington, 1982) with the modifications described by Fry & Jenkins (1983). The adult worms were placed in fresh medium daily and kept in 9-cm-diameter Petri dishes at 37 °C; all washings were carried out in 100 mM-potassium phosphate buffer, pH 7.2, supplemented with the following antibiotics: sodium benzoylpenicillin, 2000 i.u./ml; streptomycin sulphate, 2 mg/ml; 5-fluorocytosine, 0.01 mg/ml; nystatin, 5 units/ml.

Isolation of mitochondria

Isolation of mitochondria from whole *N. brasiliensis* and the details of assay and isolation media were as described previously (Paget *et al.*, 1987). Mitochondria with ADP/O quotients and respiratory-control ratios of 1.5 ± 0.2 and 3.6 ± 0.9 respectively were used. Assays were made at 37 °C by using 10 mM-sodium succinate in

a closed oxygen-electrode reaction vessel (Lloyd & Brookman, 1967).

Combined respiration and spectrophotometric measurements

Respiration was measured simultaneously with spectrophotometric measurements in an open oxygen-electrode system positioned in a modified scanning dual-wavelength spectrophotometer (Hitachi/Perkin-Elmer, model 557) (Degn *et al.*, 1980; Lloyd *et al.*, 1983; Lloyd, 1985). The open reaction vessel used was of hexagonal cross-section with quartz windows, giving a light path of 1.6 cm and incorporating an oxygen electrode (Radiometer, Copenhagen, Denmark). All spectrophotometric measurements were carried out with monochromator slit widths equivalent to 1 nm. Gases used were N_2 and air (Air Products, Cardiff, Wales, U.K.). Gas mixtures were obtained using a digital gas mixer giving defined ratios of the two gases in 5% steps (Lundsgaard & Degn, 1973). To calibrate gas mixtures in the reaction medium, an anaerobic baseline was determined under N_2 ; air was then introduced into the gas phase in 5% steps. Air-saturated buffer contained $210 \mu M-O_2$ at $37^\circ C$ (Wilhelm *et al.*, 1977). Calibrations were performed before each experiment.

Enzyme assays

Superoxide dismutase (EC 1.15.1.1) was assayed spectrophotometrically at 550 nm by using the ferricytochrome *c* reduction assay for crude homogenate as described by Salin & McCord (1974), but $10 \mu M-NaN_3$ was added instead of $2 \mu M-KCN$ to block interference due to peroxidase activity (Rigo *et al.*, 1975). Catalase (EC 1.11.1.6) was assayed spectrophotometrically at 240 nm as described by Cohen *et al.* (1970). Results were expressed as the mean of five determinations.

Analytical methods

H_2O_2 production was measured concomitantly with dissolved O_2 by using $3 \mu M$ -microperoxidase (MP-11) at 419–407 nm ($\epsilon = 78 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) (Paget *et al.*, 1987). Controls indicated that inhibitors, at the concentration used in these experiments, had no effect on the ability of MP-11 to trap H_2O_2 . Ethanol at a final concentration $> 0.5\%$ (v/v) produced a marked reduction in the effectiveness of MP-11; however, at concentrations $< 0.5\%$ (v/v), no inhibition could be observed.

The effect of various catalase concentrations on the MP-11/ H_2O_2 trap was monitored at various H_2O_2 production rates. H_2O_2 was produced by the action of glucose oxidase (EC 1.1.3.4) on 10 mM-D-glucose. All rates of H_2O_2 production shown in the Results section are those values uncorrected for the catalase content of mitochondrial preparations.

Protein was measured by using a Bio-Rad protein assay kit employing bovine γ -globulin (Bio-Rad) as a protein standard. Samples were diluted to give an absorbance between 0.2 and 0.4 at 595 nm after 15 min at $20^\circ C$.

Chemicals

All antibiotics, enzymes and chemicals, except for myxathiazol and salicylhydroxamic acid (Boehringer Mannheim) were from Sigma. Newborn-calf serum (heat-inactivated) was from Gibco. Carbonyl cyanide *m*-chlorophenylhydrazine, antimycin A, salicylhydroxa-

mic acid and myxathiazol were dissolved in Analar-grade ethanol.

RESULTS

Effect of inhibitors on uncoupled succinate oxidase activity in mitochondria

As previously observed by Paget *et al.* (1987), H_2O_2 production could not be detected in succinate-respiring mitochondrial preparations from *N. brasiliensis* in which ADP was limiting; Fig. 1 shows levels of dissolved O_2 monitored simultaneously with H_2O_2 production. Addition of $50 \mu M$ -ADP in the presence of P_i gave increased respiration (i.e. decreased O_2 concentration) in State 3 (Chance & Williams, 1955) and increased H_2O_2 generation. Complete phosphorylation of exogenous ADP led eventually to a decrease in respiration rate and a cessation of H_2O_2 production (State 4). Addition of uncoupler gave an acceleration of respiration, which was accompanied by production of H_2O_2 . Table 1 shows the effects of various inhibitors on succinate oxidase and H_2O_2 production in *N. brasiliensis* mitochondria after uncoupling with $1 \mu M$ -carbonyl cyanide *m*-chlorophenylhydrazine. The addition of antimycin A at $75 \text{ nmol} \cdot \text{mg}$ of protein $^{-1}$ decreased H_2O_2 production and respiration by 76 and 65% respectively, and further additions showed that this represents the maximum inhibition attainable. Respiration in the presence of 0.2 mM-salicylhydroxamic acid was stimulated by 55%, and H_2O_2 production increased 10-fold; further additions of salicylhydroxamic acid to a final concentration of 4.5 mM inhibited respiration by 35% and reduced H_2O_2 production to zero. *o*-Hydroxydiphenyl and 1,2,3-trihydroxybenzene at concentrations of 1.5 mM and 1 mM respectively decreased respiration by 36% and H_2O_2 production by 26%. Expression of H_2O_2 production as a percentage of O_2 utilized showed that salicylhydroxamic acid-stimulated respiration gave the highest value, 1.8%; in the presence of *o*-hydroxydiphenyl and 1,2,3-trihydroxybenzene the values were 0.4%, which was also higher than that observed in the absence of inhibitors or in the presence of antimycin A. The response of H_2O_2 production and O_2 uptake to addition of salicylhydroxamic acid (Fig. 2) were clearly quite similar; both showed maximum stimulation at 0.25 mM-salicylhydroxamic acid and maximum inhibition at 2.5 mM-salicylhydroxamic acid.

Effects of inhibitors on salicylhydroxamic acid-stimulated respiration and H_2O_2 production

Table 2 shows the effect of some inhibitors on salicylhydroxamic acid-stimulated H_2O_2 production and respiration. Addition of antimycin A ($85 \text{ nmol} \cdot \text{mg}$ of protein $^{-1}$), myxathiazol ($100 \text{ nmol} \cdot \text{mg}$ of protein $^{-1}$) or 2,3-dimercaptopropanol (1.3 mM) gave 78% inhibition of respiration and 98% inhibition of H_2O_2 production respectively. Azide (1.5 mM) inhibited respiration by 77%, but only reduced H_2O_2 production by 4%.

O_2 affinities of H_2O_2 production by uncoupled mitochondria in the presence or absence of inhibitors

Oxygen-dependencies of respiration and H_2O_2 production over the range 2–210 $\mu M-O_2$ were determined for *N. brasiliensis* mitochondria in the presence or absence of inhibitors with $3 \mu M$ -MP-11 as peroxide trap and

10 mM-sodium succinate as substrate. Fig. 3 shows the O₂-dependence of respiration and H₂O₂ production in the absence of inhibitors. Observed maximum respiration and H₂O₂ production rates were 9.3 $\mu\text{M-O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ and 27.8 $\text{nM-H}_2\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ respectively, the value of H₂O₂ production expressed as a percentage of respiration was 0.33%. O₂ inhibition thresholds were at 20.5 $\mu\text{M-O}_2$ for H₂O₂ production and

at 57.7 $\mu\text{M-O}_2$ for respiration. At 210 $\mu\text{M-O}_2$, H₂O₂ production was 40% of its maximum value and O₂ uptake was 52% of its maximum value. The apparent K_m values for O₂ of H₂O₂ production and O₂ uptake were 3.0 μM and 5.5 μM respectively.

In the presence of antimycin A (100 nmol·mg of protein⁻¹) the O₂-dependencies of H₂O₂ production and O₂ uptake followed similar patterns (Fig. 4). From

Fig. 1. Effects of ADP and uncoupler on respiration and H₂O₂ production in suspensions of *N. brasiliensis* mitochondria

Steady-state levels of O₂ (a) were measured polarographically in an open oxygen electrode of 7 ml volume, and H₂O₂ production (b) was monitored simultaneously with 3 $\mu\text{M-MP-11}$ at 419–407 μM and slit widths of 1 nm. Experiments were performed in mitochondrial assay medium under a gas phase of 5 kPa of O₂ at 37 °C. Mitochondrial protein concentration was 0.6 mg/ml. Parentheses indicate, for trace (a), respiration rates in $\mu\text{M-O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹; for trace (b), rate of H₂O₂ production in $\text{nM-H}_2\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹.

Table 1. Effects of inhibitors on respiration and H₂O₂ production in mitochondria from *N. brasiliensis*

Respiration was measured in an open system under a mobile gas phase consisting of 5 kPa of O₂ in N₂; H₂O₂ was monitored simultaneously by using MP-11 at 419–407 μM . The mitochondrial suspension (protein concentration 0.55 $\text{mg} \cdot \text{ml}^{-1}$) was incubated with 10 mM-sodium succinate and 1 μM -carbonyl cyanide *m*-chlorophenylhydrazone at 37 °C.

Inhibitor and concn.	Rates of:		(B)/(A) (%)
	Respiration ($\mu\text{M-O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein ⁻¹)	H ₂ O ₂ production ($\mu\text{M-H}_2\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein ⁻¹)	
	(A)	(B)	
None	6.3	0.0189	0.3
Antimycin A			
75 nmol·mg of protein ⁻¹	2.21	0.0045	0.2
100 nmol·mg of protein ⁻¹	2.21	0.0045	0.2
Salicylhydroxamic acid			
0.2 mM	9.78	0.185	1.8
4.5 mM	4.02	0	0
1.5 mM- <i>o</i> -Hydroxydiphenyl	3.96	0.0138	0.4
1.0 mM-1,2,3-Trihydroxybenzene	3.98	0.0140	0.4

Table 2. Effects of inhibitors on salicylhydroxamic acid-stimulated respiration and H_2O_2 production in mitochondria from *N. brasiliensis*

Conditions used were as described for Table 1, except that 0.2 mM-salicylhydroxamic acid was present.

	Rates of:	
	Respiration ($\mu\text{M}\cdot\text{O}_2\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$)	H_2O_2 production ($\text{nM}\cdot\text{H}_2\text{O}_2\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$)
Control	9.8	181
NaN_3 (1.5 mM)	2.2	168
Antimycin A (85 nmol·mg of protein $^{-1}$)	2.2	6
Myxathiazol (100 nmol·mg of protein $^{-1}$)	2.2	3.6
2,3-Dimercaptopropanol (1.3 mM)	2.2	3.7

Fig. 2. Effects of salicylhydroxamic acid on H_2O_2 production (●) and respiration (▲) in *N. brasiliensis* mitochondria

Respiration and H_2O_2 production were measured as described in Fig. 1. All conditions were identical, except that the reaction mixture was supplemented with 10 mM-sodium succinate and $5\mu\text{M}$ -carbonyl cyanide *m*-chlorophenylhydrazone and protein concentrations of 0.85 mg/ml were used. Unstimulated rates were $6\mu\text{M}\cdot\text{O}_2\cdot\text{min}^{-1}$ for respiration and $17.5\text{nM}\cdot\text{H}_2\text{O}_2\cdot\text{min}^{-1}$ for H_2O_2 production respectively.

double-reciprocal plots, maximum O_2 uptake and H_2O_2 production rates of $3.9\mu\text{M}\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$ and $7.9\text{nM}\cdot\text{H}_2\text{O}_2\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$ were calculated, and H_2O_2 production expressed as a percentage of respiration was 0.20%. The O_2 inhibition threshold was $142\mu\text{M}\cdot\text{O}_2$ for both H_2O_2 production and O_2 uptake. Apparent K_m values for O_2 uptake ($5.4\mu\text{M}\cdot\text{O}_2$) and H_2O_2 production ($6\mu\text{M}\cdot\text{O}_2$) were similar.

In the presence of 0.2 mM-salicylhydroxamic acid, the O_2 dependencies of H_2O_2 production and O_2 uptake were markedly different (Fig. 5). O_2 uptake followed the

pattern observed in Figs. 3 and 4, with an O_2 inhibition threshold at $30\mu\text{M}\cdot\text{O}_2$ giving a maximum respiratory rate of $16\mu\text{M}\cdot\text{O}_2\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$ and an apparent K_m value (for O_2) of $7.2\mu\text{M}\cdot\text{O}_2$. At $210\mu\text{M}\cdot\text{O}_2$, respiration was only 21% of the maximum value. H_2O_2 production was observed to decrease with increasing O_2 , and at $210\mu\text{M}\cdot\text{O}_2$ the rate of H_2O_2 production ($1.1\text{nM}\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$) was less than 1% of the observed maximum value of $240\text{nM}\cdot\text{H}_2\text{O}_2\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$. H_2O_2 production, expressed as a percentage of respiration, varied dramatically from 32 to 0.1% as O_2 concentrations increased from $4\mu\text{M}\cdot\text{O}_2$ to $210\mu\text{M}\cdot\text{O}_2$ (air saturation).

Catalase and SOD activity in mitochondrial preparations

Mitochondrial fractions contained catalase and SOD at 29.2 ± 4 units·mg of protein $^{-1}$ and 54 ± 5.6 units·mg of protein $^{-1}$ respectively.

In order to correct observed H_2O_2 production rates for the endogenous catalase activities of mitochondria-enriched fractions, a system was set up to test the efficiency of H_2O_2 trapped by MP-11 in the presence of catalase (Fig. 6). With a glucose/glucose oxidase H_2O_2 -generating system, a linear relationship was observed between the formation and the detection of H_2O_2 for a range of catalase activities. Appropriate correction factors, taking into account the catalase content of the mitochondrial preparations, can be estimated from these data. Consequently, observed H_2O_2 production in mitochondria was estimated to be only 5–15% of its real value.

DISCUSSION

The production of H_2O_2 by mammalian mitochondria has been well documented. Boveris *et al.* (1977) have shown the major sites for this process to be located at points in the electron-transport chain, e.g. at certain flavoproteins or in the vicinity of the ubiquinone-cytochrome *b* region, as indicated by antimycin A stimulation of H_2O_2 generation at these sites.

In mitochondria from *N. brasiliensis* the number of potential sites of H_2O_2 generation is greater than in mammalian mitochondria, because of the possibilities for electron transport via route(s) alternative to the main phosphorylating electron-transport chain (Fry *et al.*, 1983; Paget *et al.*, 1987). As has been shown for

Fig. 3. Reciprocal plots showing O₂-dependencies of respiration (▲) and H₂O₂ production (●) in uncoupled *N. brasiliensis* mitochondria

Respiration ($\mu\text{M}^{-1} \cdot \text{min} \cdot \text{mg of protein}^{-1}$) and H₂O₂ production ($\text{nm}^{-1} \cdot \text{min} \cdot \text{mg of protein}^{-1}$), were measured over an increasing range of O₂ concentrations from 2 to 210 μM -O₂. Methods and conditions used were as described in Fig. 1. Maximum values for respiration and H₂O₂ production were those observed at oxygen inhibition thresholds. Error bars indicate S.E.M. values calculated from data obtained in five experiments.

Fig. 4. Reciprocal plots showing O₂-dependencies of respiration (▲) and H₂O₂ production (●) in antimycin A-inhibited uncoupled *N. brasiliensis* mitochondria

Respiration ($\mu\text{M}^{-1} \cdot \text{min} \cdot \text{mg of protein}^{-1}$), and H₂O₂ production ($\text{nm}^{-1} \cdot \text{min} \cdot \text{mg of protein}^{-1}$) were measured under the conditions described in Fig. 3, except that 100 nmol of antimycin A/mg of protein was added to the incubation medium. Error bars indicate S.E.M. values calculated from data obtained in five experiments.

Fig. 5. Reciprocal plots showing the O_2 -dependencies of respiration (Δ) and H_2O_2 production ($\bullet$) in salicylhydroxamic acid-stimulated uncoupled *N. brasiliensis* mitochondria

Respiration ($\mu M^{-1} \cdot \text{min} \cdot \text{mg of protein}^{-1}$) and H_2O_2 production ($\text{nmol} \cdot \text{min} \cdot \text{mg of protein}^{-1}$) were measured under the conditions described in Fig. 3, except that the experiment was carried out in the presence of 0.2 mM-salicylhydroxamic acid. Error bars indicate S.E.M. values calculated from the data obtained in five experiments.

mammalian mitochondria (Boveris *et al.*, 1977), uncoupling of energy conservation also favours H_2O_2 production in nematode mitochondria.

In *N. brasiliensis* mitochondria, comparable decreases in O_2 consumption and H_2O_2 generation, in the presence of antimycin A, indicate a site of H_2O_2 production on the main respiratory chain. The residual rate of H_2O_2 production in the presence of antimycin A can be accounted for by the effect of alternative-pathway inhibitors (*o*-hydroxydiphenyl and 1,2,3-trihydroxybenzene) on H_2O_2 production (Table 1). Thus the rate of H_2O_2 production in the absence of inhibitors is equivalent to the residual rate in the presence of antimycin A plus the residual rate in the presence of *o*-hydroxydiphenyl or 1,2,3-trihydroxybenzene. This accountability would suggest only two major sites of H_2O_2 production, one presumably associated with the main respiratory pathway and the other with the alternative pathway.

In the presence of salicylhydroxamic acid (0.25 mM), the stimulated rate of H_2O_2 production is sensitive to inhibition by antimycin A. The comparable effects of other *b-c*₁-complex inhibitors to that shown by antimycin A, would indicate this site to be placed between energy conservation Site II and O_2 . The O_2 -dependencies of salicylhydroxamic acid-stimulated respiration and H_2O_2 production are markedly different; this would tend to discount the possibility of H_2O_2 generation at cytochrome *c* oxidase.

The O_2 -dependencies of respiration and H_2O_2 production, in the presence of antimycin A, are very similar with respect to inhibition threshold, O_2 concentrations and apparent K_m values for O_2 and may indicate that an

Fig. 6. Effects of catalase on H_2O_2 trapping by MP-11

The effects of catalase (Δ) 1 unit $\cdot \text{ml}^{-1}$, ($\blacksquare$) 6 units $\cdot \text{ml}^{-1}$, ($\bullet$) 13 units $\cdot \text{ml}^{-1}$ and (∇) 26 units $\cdot \text{ml}^{-1}$ on various rates of H_2O_2 production were determined in the presence of 3 μM -MP-11. Experiments were performed by using the closed oxygen electrode. All experiments were performed in mitochondrial assay medium under a gas phase consisting of 5 kPa of O_2 in N_2 at 37 °C. H_2O_2 was generated by using 10 mM-D-glucose and glucose oxidase.

alternative oxidase, with an apparent K_m for O_2 of 6 μM , is responsible for production of H_2O_2 .

The present studies have identified some major differences regarding H_2O_2 production in nematode mitochondria compared with mammalian mitochondria. Unlike in mammalian mitochondria, the addition of antimycin A does not stimulate H_2O_2 production in mitochondria of *N. brasiliensis*. The most likely explanation for this is that the site of H_2O_2 generation is on the O_2 side of the antimycin A block. However, other explanations are possible; given that the main respiratory pathway may branch to an alternative oxidase in the area of the *b-c*₁ complex, then the function of the so-called 'Q-cycle' in this region will indeed be complicated and the effects of antimycin A less predictable.

Low concentrations (< 0.5 mM) of salicylhydroxamic acid have been shown to stimulate respiration in *Acanthamoeba castellanii* (Edwards & Lloyd, 1977); this was explained by the diverting of electron flux from the alternative (inhibited by salicylhydroxamic acid) to the main respiratory pathway. A similar mechanism may operate in mitochondria of *N. brasiliensis*, where respiratory stimulation and increased H_2O_2 production in the presence of salicylhydroxamic acid are inhibited by antimycin A, but other inhibitors of the alternative pathway, i.e. *o*-hydroxydiphenyl and 1,2,3-trihydroxybenzene, gave no stimulation of respiration or H_2O_2 production at any concentration. As the components of alternative electron transport have, as yet, not been elucidated, and the action of salicylhydroxamic acid is not fully understood (Siedow & Bickett, 1981), the discrepancy between the effects of salicylhydroxamic acid and other alternative-pathway inhibitors requires further investigation. The disproportionate stimulation in H_2O_2 production compared with respiration caused by salicylhydroxamic acid can be explained as follows. The site

of H₂O₂ production can be oxidized via two routes (1) by direct interaction with O₂ to produce H₂O₂ or (2) by transfer of electrons to the next component in the main respiratory chain so as ultimately to produce H₂O in the cytochrome *c* oxidase reaction. In the presence of stimulatory concentrations of salicylhydroxamic acid, the terminal oxidase is saturated and the site of H₂O₂ production will 'leak' excess electrons to produce H₂O₂, thus producing a marked increase in the low, physiological, level of H₂O₂. In the presence of azide the components of the main respiratory chain will be fully reduced, and the insensitivity of salicylhydroxamic acid-stimulated H₂O₂ production to azide therefore suggests that the site of H₂O₂ production is already in a highly reduced state.

The residual rates of H₂O₂ production and respiration, in the presence of antimycin A, are presumably due to the activity of the alternative pathway.

High concentrations of salicylhydroxamic (> 2.5 mM) inhibit the alternative pathway, but also lead to complete inhibition of H₂O₂ production. This would indicate that higher concentrations of salicylhydroxamic acid may interact with the main respiratory pathway as well as with the alternative pathway. Possible mechanisms to explain the inhibition observed by salicylhydroxamic acid have been discussed by Siedow & Bickett (1981); chelation of Fe³⁺ may interfere with the formation of H₂O₂ via superoxide radical (O₂⁻) and hydroxy radical (OH[•]) as intermediates (Bors *et al.*, 1980).

Mechanisms of O₂ toxicity have been investigated in the nematodes *Turbatrix aceti* and *Caenorhabditis elegans* (Blum & Fridovich, 1983). In these nematodes evidence for the formation of oxygen radical species (O₂⁻; OH[•]) was obtained at hyperbaric (more-than-atmospheric) O₂ concentrations. Whether formation of H₂O₂ is accompanied by other reduced oxygen products (Foote, 1976; Sawyer & Valentine, 1981) in *N. brasiliensis* is at present not known. Exploitation of the capacity of the mitochondrial electron-transport systems of nematodes to produce potentially toxic oxygen species under conditions favouring oxidative stress may provide some chemotherapeutic potential in the treatment of helminth infections.

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