The O₂-dependence of respiration and H₂O₂ production in the parasitic nematode *Ascaridia galli*

Timothy A. PAGET,* Mitchell FRY† and David LLOYD*

*Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, Wales, U.K.,

and †Department of Biochemical Sciences, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

1. Respiration in the parasitic nematode worm *Ascaridia galli* was inhibited at O₂ concentrations in excess of 255 µM, and an apparent K_{m,O_2} of 174 µM was determined. 2. Mitochondria-enriched fractions isolated from the tissues of *A. galli* have much lower apparent K_{m,O_2} values (approx. 5 µM). They produce H₂O₂ in the energized state; higher rates of H₂O₂ production were observed in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone. 3. Antimycin A inhibited respiration in muscle tissue mitochondria by 10%, but had no effect on respiration in gut+reproductive tissue mitochondria; the major portion of respiration in both types of mitochondria could be attributed to an alternative electron-transport pathway. 4. *o*-Hydroxydiphenyl, an inhibitor of alternative electron-transport pathways, inhibits respiration by 98% and completely inhibits the production of H₂O₂ in gut-plus-reproductive-tissue mitochondria; respiration and H₂O₂ production in muscle tissue mitochondria were inhibited by 90 and 86% respectively. 5. Another inhibitor of alternative electron transport, salicylhydroxamic acid, had the same effect as *o*-hydroxydiphenyl on H₂O₂ production and respiration in gut-plus-reproductive-tissue mitochondria. However, its effect on muscle tissue mitochondria was complex; a low concentration (0.35 mM) stimulated H₂O₂ production, whereas 3 mM inhibited respiration by 87% and prevented H₂O₂ production completely. 6. The similarities between the apparent K_{m,O_2} values for H₂O₂ production and respiration in muscle mitochondria and in gut-plus-reproductive-tissue mitochondria suggests that the site of H₂O₂ production on the alternative electron-transport chain is cytochrome 'o'. 7. These results are discussed in relation to potential O₂ toxicity in *A. galli*.

INTRODUCTION

The role of O₂ in the metabolism of gut-dwelling nematodes is uncertain. As the gut is a microaerobic habitat (Atkinson, 1980), it might be expected that nematodes would show adaptation wherever possible to optimize the use of O₂ for maximum energy yields. Adaptation to low O₂ concentrations within nematodes has been correlated with variations in electron-transport and associated pathways (Fry & Jenkins, 1984; Paget *et al.*, 1987a).

The large nematode worm *Ascaridia galli* (length 50–116 mm, diameter 1–2 mm), lives in the gut lumen, where O₂ concentrations fluctuate over the range 0–20 µM (Atkinson, 1980). As nematodes have no circulatory system, their size will limit O₂ diffusion and intracellular O₂ concentrations (Rogers, 1962). However, even large nematodes, such as *A. galli*, may receive sufficient O₂ within their natural environment to supply at least peripheral tissues with O₂.

Previous work on the small gut-dwelling nematode *Nippostrongylus brasiliensis* (Paget *et al.*, 1987a,b) showed that respiration was inhibited by concentrations of O₂ greater than those found within their environments. It was also demonstrated that electron transport along the main phosphorylating chain and by way of alternative electron-transport pathway(s) can each separately generate H₂O₂.

Using *A. galli*, Fry *et al.* (1983) showed that mitochondria isolated from the muscle were morphologically very different from those prepared from gut plus reproductive tissue. Whereas muscle-tissue mitochondria

showed well-developed cristae, gut-plus-reproductive-tissue mitochondria contained only very poorly developed cristae. These two types of mitochondria also showed differences in their response to respiratory substrates and inhibitors. *A. galli* muscle mitochondria showed a preference for succinate as substrate, with only 10% of total respiratory activity being sensitive to antimycin A or azide. Gut-plus-reproductive-tissue mitochondria showed a substrate preference for L-α-glycerophosphate, their respiratory activity being completely insensitive to antimycin A, but sensitive to alternative-pathway inhibitors.

The present paper describes measurements of respiration and H₂O₂ production in *A. galli* at several O₂ concentrations and in the presence of various inhibitors. We demonstrate that peripheral tissues of *A. galli* can maintain an oxidative metabolism. On the other hand, cortex tissues are at best supplied with O₂ at a very low level. H₂O₂ generation appears to occur at two distinct sites, one of which is located on the classical chain and the other at an alternative oxidase. We also suggest that alternative electron transport occurs by way of more than one pathway, one of which is a cytochrome-‘o’ producing H₂O₂.

MATERIALS AND METHODS

Maintenance and growth of *Ascaridia galli*

The Wellcome strain of *A. galli* was maintained in immuno-depressed Wickham chickens. The method for production of adult worms was described previously by

Fry *et al.* (1983). After a period of 8 weeks, *A. galli* were fully matured. Chickens were killed by cervical dislocation, and adult *A. galli* were dissected from the proximal half of the lower intestine. To remove contaminating bacteria from *A. galli*, worms were washed three times in a buffer (antibiotic buffer) containing 50 mM-potassium phosphate, 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. After having been washed, worms were placed in the antibiotic buffer, which was changed every 8 h.

Isolation of mitochondria

Before homogenization, *A. galli* were separated into gut plus reproductive tissues and muscle plus chitin (after separation, tissues were thoroughly washed in antibiotic buffer). This was achieved by removing one end of the worm, and teasing out gut plus reproductive tissue through the cut end using a scalpel blade, drawn along the length of the worm. The remaining muscle tissue, associated with the outer chitin, was then cut into small pieces by using crossed scalpels and further comminuted by suspending the pieces of muscle tissue plus chitin in cold mitochondrial isolation medium and blending for 1 min in an MSE Waring blender. Isolation of mitochondria from tissues of *A. galli* and the details of assay and isolation media were as described previously for *N. brasiliensis* (Paget *et al.*, 1987a). Substrates used were sodium succinate for muscle mitochondria and L- α -glycerophosphate for gut-plus-reproductive-tissue mitochondria (both substrates at a final concentration of 10 mM).

Muscle-tissue mitochondria with ADP/O quotients and respiratory-control ratios of 0.85 ± 0.1 and 2.6 ± 0.5 respectively were used. Mitochondria isolated from gut plus reproductive tissues and with ADP/O quotients of 0.8 ± 0.1 and respiratory control ratios of 1.9 ± 0.4 were used. Assays were performed at 37 °C in a closed oxygen-electrode reaction vessel (Lloyd & Brookman, 1967).

Measurements of oxygen-uptake rates

Measurements of oxygen uptake, under defined O_2/N_2 ratios, were made at 37 °C by two methods: (1) in an oxygen-electrode system, open for gases (Degn *et al.*, 1980) comprising a 7 ml stirred reaction vessel; (2) using a membrane inlet mass spectrometer (Lloyd & Scott, 1983; Lloyd, 1985). The mass spectrometer probe consisted of a silica glass tube, of 1.5 mm internal diameter, sealed at one end with a 500 μ m-diameter inlet, covered by a silicone membrane. This probe continuously and directly measured oxygen dissolved in the liquid phase, in a vessel sparged with a defined gas mixture (1.5 ml working volume). t_1 values and respiratory rates were calculated as described by Paget *et al.* (1987a). t_1 values of 5.05 ± 0.02 and 0.72 ± 0.01 min were obtained for the oxygen-electrode and the membrane-inlet-mass-spectrometer systems respectively. Gases used were N_2 , O_2 and air (Air Products, Cardiff, Wales, U.K.). O_2 -saturated buffer contained 1015 μ M- O_2 at 37 °C (Wilhelm *et al.*, 1977).

Respiratory activity measurements were carried out either in washing buffer for whole worms, or in assay medium for isolated mitochondria, as described previously (Paget *et al.*, 1987a). Apparent oxygen affinities

are presented as means $\pm$ S.D. calculated from five determinations.

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.

Enzyme assays

Catalase (EC 1.11.1.6) was assayed spectrophotometrically at 240 nm as described by Cohen *et al.* (1970). Results were expressed as means of five determinations.

Analytical methods

H_2O_2 production was measured simultaneously with dissolved O_2 using 3 μ M-microperoxidase (MP-11) at 419–407 nm, which has a difference coefficient (ϵ) of 78 $mm^{-1} \cdot cm^{-1}$ (Paget *et al.*, 1987a). 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 decrease in the effectiveness of MP-11; however, at concentrations $< 0.5\%$ (v/v), no inhibition could be observed. 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 α -globulin as a protein standard (Bio-Rad Laboratories). Samples were diluted to give an absorbance between 0.2 and 0.4 at 595 nm after 15 min at 20 °C.

Chemicals

All antibiotics, enzymes and chemicals, except for myxathiazol and salicylhydroxamic acid (Boehringer-Mannheim), were from Sigma Chemical Co. (Dorset, U.K.). Newborn-calf serum (heat-inactivated) was from Gibco. Carbonyl cyanide *m*-chlorophenylhydrazon, antimycin A, salicylhydroxamic acid and myxathiazol were dissolved in Analar-grade ethanol.

RESULTS

Oxygen affinities of whole *A. galli* in the presence or absence of inhibitors

The oxygen-dependence of respiration over the range 5–400 μ M- O_2 was determined for whole *A. galli*, suspended in 50 mM-potassium phosphate buffer, pH 7.2, supplemented with antibiotics. Double-reciprocal plots were used to calculate apparent K_m values for oxygen of whole *A. galli*, utilizing endogenous substrates. For four batches of untreated *A. galli* (Fig. 1) respiration was diminished at oxygen concentrations of $225 \pm 13.2 \mu$ M. At 400 μ M- O_2 , respiration was only 18% of its maximum value. An apparent K_m value of 174 μ M- O_2 was calculated, and the observed maximum respiratory rate was $11 \pm 0.1 \text{ nm-O}_2 \cdot \text{min}^{-1} \cdot \text{mg wet wt.}^{-1}$.

Fig. 1. Reciprocal plot of respiration rate against oxygen concentration for whole *A. galli*

The oxygen-dependence of endogenous respiration ($\mu\text{M} \cdot \text{min}^{-1} \cdot \text{mg wet wt.}^{-1}$) in whole *A. galli* was measured using a membrane-inlet mass spectrometer at an m/z value of 32, in a system open for gas flow. All experiments were carried out at 37 °C in 50 mM-potassium phosphate buffer, pH 7.2, supplemented with antibiotics as described in the text. Approx. 300 mg wet weight of *A. galli* was used throughout. Error bars indicate S.E.M. values calculated from data obtained in five experiments.

In the presence of antimycin A the oxygen-dependence of respiration followed a similar pattern to that observed in Fig. 1; the results from the double-reciprocal plot are shown in Table 1. In the presence of antimycin A, the rate of respiration and the apparent K_m for O₂ were not significantly different from those observed in the absence of the inhibitors. However, respiration was less sensitive to inhibition at high O₂ concentration, with respiration decreasing at an O₂ concentration of $273 \pm 15 \mu\text{M}$; at $400 \mu\text{M-O}_2$, respiration was only 24% of its maximum value.

Oxygen-dependencies of respiration in mitochondria isolated from various tissues of *A. galli* in the presence or absence of inhibitors

Dependencies of respiration over the range 2–210 $\mu\text{M-O}_2$ were determined for mitochondria isolated from various tissues of *A. galli* in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazine. (Uncoupler was added to mitochondria to remove ADP-dependent respiratory control, allowing for a more clear-cut comparison of results between whole worms and isolated mitochondria, and for consistency in respiratory response during the course of the oxygen-dependency assays.)

Respiratory activity in the presence or absence of inhibitors followed a similar pattern to that observed in whole *A. galli* (Fig. 1); results obtained from the double-reciprocal plots are shown in Table 1. For mitochondria isolated from muscle tissues, it can be seen that the lowest affinity for oxygen was observed in the presence of

Table 1. Oxygen kinetics of respiration in *A. galli*

Maximal respiratory rates (V_{max}), apparent K_m values and oxygen-inhibition threshold values were obtained from double-reciprocal plots showing respiration as a function of oxygen concentrations. Conditions used were as described in Fig. 1, except that protein concentrations of 4.2 and 5.1 mg were used for muscle and gut-plus-reproductive-tract mitochondrial-respiration experiments respectively; 300 mg wet weight of whole *A. galli* was used for all whole-worm respiration experiments. Determinations of mitochondrial respiratory activity were carried out in the presence of 10 mM-sodium succinate (muscle mitochondria) or 10 mM-L- α -glycerophosphate (gut-plus-reproductive-tissue mitochondria) and 4 μM -carbonyl cyanide *m*-chlorophenylhydrazine. Abbreviation: ND, experiment not performed.

Inhibitor	Whole worms			Muscle			Gut + reproductive tract		
	V_{max} , (nmol·min ⁻¹ ·mg wet wt. ⁻¹)	Apparent K_m value for O ₂ (μM)	O ₂ ⁻ inhibition threshold (μM)	V_{max} , (μM·min ⁻¹ ·mg of protein ⁻¹)	Apparent K_m value for O ₂ (μM)	O ₂ ⁻ inhibition threshold (μM)	V_{max} , (μM·min ⁻¹ ·mg of protein ⁻¹)	Apparent K_m value for O ₂ (μM)	O ₂ ⁻ inhibition threshold (μM)
None	11 ± 0.7 (4)	174 ± 5.6 (4)	225 ± 13.2 (4)	6.6 ± 0.1 (5)	5 ± 0.4 (5)	115 ± 5.2 (5)	4.9 ± 0.2 (6)	6.4 ± 0.4 (6)	125 ± 6.1 (6)
Antimycin A	10.3 ± 0.7 (4)	183 ± 6.2 (4)	273 ± 15.8 (4)	5.8 ± 0.5 (4)	6.3 ± 0.4 (4)	153 ± 7.4 (4)	4.8 ± 0.3 (4)	6.5 ± 0.5 (4)	132 ± 5.3 (4)
[3.0 nmol·mg wet wt. ⁻¹]									
Salicylhydroxamic acid (4.5 mM)	ND	ND	ND	0.7 ± 0.05 (5)	4.7 ± 0.3 (5)	92 ± 8.6 (5)	ND	ND	ND

Table 2. Effects of inhibitors on respiration and H_2O_2 production on mitochondria from various tissues of *A. galli*

Respiration was measured under a mobile gas phase consisting of 5 kPa of O_2 in N_2 in a system open for gases; H_2O_2 was monitored simultaneously using MP-11 at 419–407 nm. Experiments were performed in the presence of $4 \mu M$ -carbonyl cyanide *m*-chlorophenylhydrazone. Mitochondrial respiratory substrates L- α -glycerophosphate (gut plus reproductive tract) and sodium succinate (muscle tissue) were used at a concentration of 10 mM. Mitochondrial protein concentrations of 0.6 and $0.7 \text{ mg} \cdot \text{ml}^{-1}$ for muscle- and gut-plus-reproductive-tract mitochondria respectively were used throughout.

Inhibitor	Rate (per mg of protein)					
	Muscle			Gut + reproductive tract		
	Respiration ($\mu M-O_2 \cdot \text{min}^{-1}$) (A)	H_2O_2 production ($\mu M-H_2O_2 \cdot \text{min}^{-1}$) (B)	(B)/(A) (%)	Respiration ($\mu M-O_2 \cdot \text{min}^{-1}$) (A)	H_2O_2 production ($\mu M-H_2O_2 \cdot \text{min}^{-1}$) (B)	(B)/(A) (%)
None	5.2 ± 0.6	0.25 ± 0.02	4.8	4.4 ± 0.8	0.18 ± 0.02	4.0
Antimycin A ($100 \text{ nmol} \cdot \text{mg of protein}^{-1}$)	4.7 ± 0.3	0.19 ± 0.02	4.0	4.3 ± 0.4	0.17 ± 0.03	4.0
Salicylhydroxamic acid						
0.1 mM	5.7 ± 0.8	0.37 ± 0.05	6.8	4.0 ± 0.4	0.16 ± 0.02	4.0
4.5 mM	0.6 ± 0.05	0	—	0.1 ± 0.02	0	—
<i>o</i> -Hydroxydiphenyl (1.5 mM)	0.6 ± 0.05	0.033 ± 0.005	5.7	0.1 ± 0.02	0	—

Fig. 2. Effects of salicylhydroxamic acid on H_2O_2 production (●) and respiration (▲) in mitochondria isolated from muscle tissue of *A. galli*

Respiration was measured polarographically in an open electrode vessel of 7 ml volume. H_2O_2 production was monitored simultaneously with $3 \mu M$ -MP-11 at 419–407 nm; slit widths of 1 nm were used. Experiments were performed in mitochondrial assay medium under a gas phase of 5 kPa of O_2 at $37^\circ C$. A mitochondrial protein concentration of $0.95 \text{ mg} \cdot \text{ml}^{-1}$ was used. Assay medium contained 10 mM-sodium succinate and $4 \mu M$ -carbonyl cyanide *m*-chlorophenylhydrazone. Unstimulated rates were $5.2 \pm 0.6 \mu M-O_2 \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ for respiration and $0.25 \pm 0.02 \mu M-H_2O_2 \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ for H_2O_2 production.

antimycin A ($6.3 \pm 0.4 \mu M-O_2$). Apparent K_m values for oxygen in the absence of inhibitors or in the presence of salicylhydroxamic acid showed no significant differences. The observed oxygen-inhibition threshold in the absence of inhibitors was increased in the presence of antimycin A but diminished in the presence of salicylhydroxamic acid. A similar variation was also observed when comparing the rates of respiration at air saturation. In mitochondria without inhibitors the rate of respiration at air saturation was only 48% of the observed maximum; in the presence of antimycin A or salicylhydroxamic acid these values were 65 and 40% respectively.

For mitochondria isolated from gut plus reproductive tissues there were no significant differences between the

Table 3. Effects of inhibitors on salicylhydroxamic acid-stimulated H₂O₂ production in *A. galli* muscle-tissue mitochondria

H₂O₂ production in *A. galli* muscle-tissue mitochondria were measured as described in Table 2. Experimental conditions used were identical, except that the assay medium was supplemented with 0.1 mM-salicylhydroxamic acid.

Inhibitor	Rate of H ₂ O ₂ production (μM-H ₂ O ₂ · min ⁻¹ · mg of protein ⁻¹)
None (control)	0.38 ± 0.05
(1.5 mM)	0.36 ± 0.06
Antimycin A (100 nmol · mg of protein ⁻¹)	0.18 ± 0.04
2,3-Dimercaptopropanol (1.3 mM)	0.17 ± 0.03

apparent oxygen affinities of respiration and the oxygen-inhibition thresholds observed for mitochondria in the presence or absence of antimycin A.

H₂O₂ production in mitochondria isolated from various tissues of *A. galli*

H₂O₂ production could not be detected in respiring mitochondrial preparations of *A. galli*, from either muscle or gut plus reproductive tissues when ADP was limiting. However, in the presence of exogenous ADP or uncoupler, H₂O₂ production was detectable. Table 2 shows the effects of various inhibitions on H₂O₂ production and on succinate oxidase activity in muscle-tissue mitochondria, and L-α-glycerophosphate oxidase activity in gut-plus-reproductive-tissue mitochondria, after first uncoupling with 2 μM-carbonyl cyanide *m*-chlorophenylhydrazone.

The addition of antimycin A at 100 nmol · mg of protein⁻¹ to *A. galli* muscle mitochondria decreases respiration by 9% and H₂O₂ production by 24%. Addition of 0.1 mM-salicylhydroxamic acid did not significantly affect respiration, but stimulated H₂O₂ production by 48%. Further additions of salicylhydroxamic acid to a final concentration of 4.5 mM inhibited respiration by 90% and decreased H₂O₂ production to zero. *o*-Hydroxydiphenyl at a concentration of 1.5 mM decreased respiration by 90% and H₂O₂ production by 99%. Expression of H₂O₂ production as a percentage of oxygen utilized showed that salicylhydroxamic acid-stimulated H₂O₂ production gave the highest value; in the presence of *o*-hydroxydiphenyl the value was lower, but was still higher than that observed in the absence of inhibitors or in the presence of antimycin A. The response of H₂O₂ production to the addition of increasing concentrations of salicylhydroxamic acid (Fig. 2) showed a maximum stimulation at 0.15 mM and maximum inhibition at 4 mM.

The addition of antimycin A at 100 nmol · mg of protein⁻¹ to *A. galli* gut-plus-reproductive-tissue mitochondria (Table 2) did not significantly alter the rate of respiration of H₂O₂ production. In the presence of salicylhydroxamic acid (4.5 mM), respiration was inhibited by 98% and H₂O₂ production was decreased to zero. *o*-Hydroxydiphenyl at a concentration of 1.5 mM

Fig. 3. Reciprocal plots showing the oxygen-dependencies of respiration (▲) and H₂O₂ production (●) in uncoupled mitochondria isolated from muscle tissues of *A. galli* in the absence of inhibitors

The oxygen-dependencies of respiration and H₂O₂ production for *A. galli* muscle mitochondria were measured in the absence of inhibitors over an increasing range of oxygen concentrations from 2 to 210 μM-O₂. Methods and conditions used were as described in Fig. 2. Error bars indicate S.E.M. values calculated from the data obtained in five experiments.

inhibited respiration by 98% and diminished H₂O₂ production to zero.

Effects of inhibitors on salicylhydroxamic acid-stimulated respiration and H₂O₂ production in *A. galli* muscle-tissue mitochondria

Table 3 shows the effect of some inhibitors on salicylhydroxamic acid-stimulated H₂O₂ production. Addition of antimycin A (100 nmol · mg of protein⁻¹) or 2,3-dimercaptopropanol (1.3 mM) gave 55% inhibition of H₂O₂ production and 16% inhibition of respiration; azide (1.5 mM) inhibited respiration by 16%, but did not significantly diminish H₂O₂ production.

Oxygen affinities of H₂O₂ production in uncoupled mitochondria isolated from various tissues of *A. galli* in the presence or absence of antimycin A

The oxygen-dependencies of respiration and H₂O₂ production over the range of 2–210 μM-O₂ for mitochondria isolated from *A. galli* muscle tissues in the absence of antimycin A are shown in Fig. 3. In the presence of antimycin A (100 nmol · mg of protein⁻¹) the oxygen-dependencies of H₂O₂ production and oxygen uptake in *A. galli* muscle mitochondria were similar to those observed in Fig. 3; results from the double-reciprocal plots are shown in Table 4. It can be seen that, in the presence or absence of antimycin A, the apparent O₂ affinities for H₂O₂ production and respiration were not significantly different; however, the inhibitor produced a marked increase in the O₂ inhibition threshold for H₂O₂ production.

The O₂-dependence of respiration and H₂O₂ production in gut-plus-reproductive-tissue mitochondria

Table 4. Oxygen kinetics of H₂O₂ production and respiration in various tissues of *A. galli*

Maximum respiratory and H₂O₂ production rates, apparent K_{m,O_2} values and O₂-inhibition thresholds were obtained from double-reciprocal plots showing respiration and H₂O₂ production as a function of oxygen concentration. Conditions used were as described in Table 2, except that total protein concentrations of 4.2 and 5.1 mg were used for (a) muscle- and (b) gut-plus-reproductive-tissue mitochondria respectively

Inhibitor	Respiration			H ₂ O ₂ production			
	Maximum observed rate ($\mu\text{M} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein ⁻¹) (A)	Apparent K_{m,O_2}	O ₂ -inhibition threshold (μM)	Maximum observed rate ($\mu\text{M} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein ⁻¹) (B)	Apparent K_{m,O_2}	O ₂ -inhibition threshold (μM)	(B)/(A) (%)
(a) Muscle tissue							
None	6.4 ± 0.7	4.0 ± 0.5	105 ± 8	0.32 ± 0.04	5.1 ± 0.7	63 ± 7	4.9
Antimycin A (100 nmol · mg of protein ⁻¹)	5.8 ± 0.4	6.6 ± 0.8	146 ± 11	0.24 ± 0.05	7.1 ± 0.9	146 ± 11	4.2
(b) Gut + reproductive tissue							
None	4.7 ± 0.5	6.3 ± 0.5	138 ± 9	0.24 ± 0.05	6.5 ± 0.6	138 ± 9	5.1

was similar to that observed in Fig. 3. The results from the double-reciprocal plot are shown in Table 4. The rates of H₂O₂ production expressed as a percentage of respiration and the O₂ inhibition threshold for H₂O₂ were markedly greater than those observed for muscle-tissue mitochondria. However, these values were similar to those determined for muscle-tissue mitochondria in the presence of antimycin A. Again the apparent O₂ affinities for H₂O₂ production and respiration were not significantly different.

Catalase activity in mitochondrial preparations

Endogenous catalase, present in mitochondrial preparations competes with MP-11 to trap H₂O₂. Observed levels of H₂O₂ production determined by this method must be corrected to allow for the endogenous catalase activity of these fractions. Mitochondrial fractions isolated from muscle or gut plus reproductive tissues contained catalase at 17 ± 2 and 12 ± 2 units · mg of protein⁻¹ respectively. By using the data obtained previously by Paget *et al.* (1987a), the observed rates of H₂O₂ production in muscle mitochondria were estimated to be only 25–30% of the maximum possible value. Corresponding values in gut-plus-reproductive-tissue mitochondria accounted for approx. 30–35%.

DISCUSSION

The low environmental oxygen concentrations experienced by *A. galli* *in situ* (0–20 $\mu\text{M-O}_2$; Atkinson, 1980) and the disproportionate concentration of cytochrome oxidase-positive mitochondria within peripheral tissues of this worm (Fry & Beesley, 1985) suggests that, *in vivo*, the cortex tissues (including the gut plus reproductive tissues) of the worm are at best supplied with oxygen at extremely low levels. Comparisons of the apparent K_m values for oxygen between whole worms utilizing endogenous substrates and uncoupled isolated mitochondria provided with succinate or L- α -glycero-

phosphate shows a more-than-20-fold discrepancy; this suggests the existence of a substantial oxygen gradient through the worm. By using the model systems and the equations of Hill (1929), an ambient O₂ concentration of 240 μM would be required to provide $[\text{O}_2] > 2 \mu\text{M}$ to all tissues of a worm of 0.15 mm diameter. Thus the metabolic properties of the mitochondria from *A. galli* would be expected to be quite different from those from smaller, more aerobic, nematodes such as *N. brasiliensis* (Paget *et al.*, 1987a,b).

The comparability of apparent K_m values for oxygen of muscle- and gut-plus-reproductive-tissue mitochondria observed in the presence of antimycin A would seem to indicate that alternative electron transport in both types of mitochondria is mediated by similar terminal oxidase(s) having an apparent K_m for oxygen $\approx 5 \mu\text{M-O}_2$. This value is similar to that previously shown for the alternative oxidase in mitochondria isolated from *N. brasiliensis* (Paget *et al.*, 1987b), but higher than those for mitochondria from mammalian sources (Jobsis & Rosenthal, 1978; Jones, 1978).

A most interesting feature of respiration in isolated mitochondria of *A. galli* is the presence of an oxygen-inhibition threshold. The addition of antimycin A increased this threshold, whereas the presence of salicylhydroxamic acid decreased it. Thus the salicylhydroxamic acid-sensitive pathway is less sensitive to inhibition by higher oxygen concentrations than is the antimycin A-sensitive pathway. The oxygen inhibition threshold observed in whole *A. galli* was at oxygen concentrations greater than air saturation. *In vivo*, therefore, *A. galli* would be unlikely to experience O₂-mediated toxicity. O₂ toxicity has been investigated in the nematodes *Turbatrix aceti* and *Caenorhabditis elegans* (Blum & Fridovich, 1983), where evidence for the formation of O₂ radical species (O₂⁻, OH[·]) was obtained at hyperbaric (more than atmospheric) O₂ concentrations. The present study has revealed that H₂O₂ is produced by *A. galli*. Whether formation of H₂O₂ is accompanied by

other reduced O₂ products (Sawyer & Valentine, 1981) in *A. galli* is not known. The effects of inhibitors on respiration and H₂O₂ production in mitochondria isolated from muscle tissues of *A. galli* shows that the rate of H₂O₂ 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. This accountability suggests the presence of only two major sites of H₂O₂ production; one presumably associated with the classical respiratory pathway and located between the bc₁ complex and cytochrome *c* oxidase, the other with the alternative pathway. In contrast, gut-plus-reproductive-tissue mitochondria of *A. galli* possess only one site of H₂O₂ production, and similarities in the oxygen dependence of respiration and H₂O₂ production in these mitochondria suggests that an alternative oxidase is the most likely site of H₂O₂ production. If the site of H₂O₂ production is an *o*-type cytochrome, as proposed by Köhler (1985) and Atkinson (1980) and as confirmed in unpublished photochemical action spectra (T. A. Paget, M. Fry & D. Lloyd, unpublished work), the stoichiometry of H₂O₂ production to oxygen uptake by the alternative pathway should be 1:1 (Webster & Orii, 1977; Köhler & Bachmann, 1980). From our data the actual stoichiometry of H₂O₂ production to O₂ uptake was 0.14:1. The difference between the experimental and theoretical stoichiometries can be explained if the alternative portion of respiration is comprised of more than one pathway, and the other pathway(s) produce water as the end product. It is possible that an H₂O₂ oxidoreductase (Webster, 1975; Webster & Orii, 1977) could impart a physiological role for H₂O₂ metabolism during survival over periods of anoxia.

The site at which electrons are apportioned between the main and alternative electron-transport chains is at present not clearly defined. The stimulation of H₂O₂ production by low concentration of salicylhydroxamic acid and the sensitivity of the stimulated rates of H₂O₂ production to antimycin A may indicate a close association of the two systems, which are either branched or parallel systems with functional linkage(s). Comparable effects have been observed in *N. brasiliensis* (Paget *et al.*, 1987b), where low concentrations of salicylhydroxamic acid stimulated respiration and H₂O₂ production. A stimulation of respiration by low concentrations of salicylhydroxamic acid in *A. castellanii* (Edwards & Lloyd, 1977) was associated with increased electron flux through the classical electron-transport chain. That other bc₁ complex inhibitors produce effects similar to those observed with antimycin A suggests the site of H₂O₂ production to be located between the bc₁ complex and cytochrome *c* oxidase. The relationships of these pathways of electron transport to the energy-generating fumarate reductase pathway (Fry & Brazeley, 1984) remain to be investigated.

Increasing concentrations of salicylhydroxamic acid (> 2.5 mM) inhibit the alternative pathway, but also lead to complete inhibition of H₂O₂ production. This may indicate that, at higher concentrations, salicylhydroxamic acid can also interact with the classical respiratory pathway in a way that may interfere with the formation of H₂O₂ from O₂^{-•} and radicals, which may act as intermediates (Bors *et al.*, 1980). Mechanisms of

inhibition by salicylhydroxamic acid have been discussed by Siedow & Bickett (1981).

The stimulation of H₂O₂ production by low concentrations of salicylhydroxamic acid may provide an approach to the treatment of some helminth infections, although this would seem more feasible in nematodes smaller and more aerobic than *A. galli*.

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