Respiration in the Cysts and Trophozoites of *Giardia muris*

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Cysts and trophozoites of the parasitic protozoon *Giardia muris* both showed respiratory activity but respiration in cysts was only 10 to 20% that of trophozoites. The O₂ dependence of respiration in cysts and trophozoites showed O₂ maxima above which respiration decreased. The O₂ concentration at which the respiration rate was greatest was higher for cysts than trophozoites. The effects of various inhibitors on cyst and trophozoite respiration suggested that flavoproteins and quinones play some role in respiration. The substrate specificities and the effects of inhibitors on *G. muris* trophozoites were similar to those observed for *Giardia lamblia*. Metronidazole, the drug most commonly used in the treatment of giardiasis completely inhibited respiration and motility in trophozoites; however, it had no effect on either respiration or viability in cysts. Menadione, a redox cycling naphthoquinone, stimulated then completely inhibited respiration in cysts and trophozoites; a complete loss of cyst viability or trophozoite motility was also observed. The effects of menadione on *G. muris* may indicate that redox cycling compounds have potential as chemotherapeutic agents for the treatment of giardiasis.

INTRODUCTION

Flagellated, parasitic protozoa belonging to the genus *Giardia* infect humans and a wide variety of other vertebrate hosts. The life cycle of this protozoon is simple and direct, and although it requires no intermediate host, it passes through two morphologically distinct forms: the trophozoite and the cyst. The trophozoite colonizes the upper small intestine, and for reasons as yet unknown, rounds up and elaborates a cyst wall. The resulting cyst is shed with the faeces. Transmission of *Giardia* occurs when viable cysts are ingested with food or drink (Meyer & Jarroll, 1980). In humans, transmission is by the faecal–oral and venereal routes. Numerous waterborne outbreaks of the disease related to community water systems have been widely publicized (Craun, 1986). *Giardia lamblia* (syn. *G. intestinalis* and *Lamblia intestinalis*), the human pathogen, is regarded as numerically the most important cause of waterborne infectious disease in the UK and the USA (Meyer & Jarroll, 1980).

Since cysts are responsible for transmission there have been many reports in recent years dealing with methods for killing cysts in water supplies (Jarroll, 1988). The earliest experiments were done mainly with cysts isolated from humans with giardiasis. Such cyst supplies are rare and unreliable. More recently, *Giardia muris* has been proposed as a model for human cysts. *G. muris* can be isolated from mice and represents a distinct species in the genus. It was chosen because it is reliably available in large quantities from laboratory mice, and because its levels of *in vitro* excystation are generally high (>90%). Because *G. muris* cysts are more resistant to chlorine than those of *G. lamblia* they are also suitable for use in monitoring water treatment processes.

Abbreviation: DBMQ, dibromothymoquinone.

Unfortunately, even less is known about the basic physiology and metabolism of *G. muris* than that of *G. lamblia*. In part, this is because trophozoites of *G. muris* have not been cultured *in vitro*. Information on the carbohydrate and energy metabolism of *Giardia* has been limited to that obtained from axenically cultured *G. lamblia* trophozoites (Lindmark, 1980; Weinbach, 1980; Jarroll *et al.*, 1981). What is known of energy metabolism in *G. muris* comes from a single study by Lindmark & Miller (1987) which was confined to a comparison of enzyme activities in cysts and trophozoites of *G. lamblia* and *G. muris*. No pronounced differences in many enzyme activities of carbohydrate and energy metabolism were detected, but the metabolic activity of cyst forms was not assessed. The first suggestion that *Giardia* cysts might be metabolically active came from a report by Bingham *et al.* (1979), which showed that *G. lamblia* cysts were only viable for about 4 d when maintained in water at 37 °C. At a water temperature of 8 °C cysts survived for about 2 months. This temperature-dependent survival suggested that cysts are metabolically active rather than true cryptobiotic forms.

In this paper we determine the rates of O₂ uptake in cysts and trophozoites of *G. muris* and investigate the effects of temperature on the respiration of cysts. We also compare the effects of various substrates and metabolic inhibitors on O₂ uptake in cysts and trophozoites.

METHODS

Growth and maintenance of the organism. *G. muris* was maintained by passage of cysts into 4–6-week-old female CF-W mice as described by Roberts-Thompson *et al.* (1976); 5 d post-infection, cysts were passed in faeces and continued to be passed for up to 3 weeks after infection. Cysts were collected daily and purified on sucrose and Percoll gradients to remove contaminating bacteria according to the procedure described by Sauch (1984). Purified cysts were resuspended in distilled water supplemented with an antibiotic mixture (10%, v/v) containing 20 mg streptomycin sulphate ml⁻¹ and 12 mg benzylpenicillin ml⁻¹. Cysts were maintained at 4 °C prior to use. The usual yield from 30 mice was 1.5×10^8 cysts.

Trophozoites of *G. muris* were obtained by excystation of cysts after acid induction as described by Rice & Schaefer (1981). Enumeration of cysts and trophozoites was done using a Neubauer haemocytometer.

Measurements of O₂ uptake. Measurements of respiration were made in a thermostatically controlled oxygen electrode vessel open for gases (Degn *et al.*, 1980), comprising a 5 ml stirred reaction vessel. Measurements of respiration were made under defined O₂/N₂ ratios as described previously (Lloyd *et al.*, 1982, 1983). Gases used were N₂ and a mixture of 4.9% (v/v) O₂ in N₂ (Matheson, USA). Gas mixtures were obtained using a digital gas mixer (Lloyd, 1985) which produces ratios of the two gases in 5% steps. Calibration of gas mixtures was done as described by Paget *et al.* (1987). Buffer saturated with 4.9% O₂ contained 50 µM-O₂ at 37 °C, 59 µM-O₂ at 27 °C and 71 µM-O₂ at 17 °C (Wilhelm *et al.*, 1977). For the determination of respiratory activities, cysts and trophozoites were suspended in a modified Tyrodes' buffer, as described by Rice & Schaefer (1981).

Trophozoite motility and cyst viability were determined at the end of each experiment. Trophozoite motility was determined microscopically by examination of 500 trophozoites. To calculate cyst viability, cysts were removed from the reaction vessel and excystation was induced as described previously. The number of cysts and trophozoites was counted 1 h after excystation. The percentage excystation was then calculated correcting for the production of two trophozoites per cyst (Rice & Schaefer, 1981).

Chemicals. All substrates, quinacrine, chloroquine, ouabain and NaNO₂ were dissolved in a glucose-free Tyrode's buffer (Rice & Schaefer, 1981). All other inhibitors were dissolved initially in DMSO, which at the concentrations used has no effects on cysts or trophozoites. All chemicals were obtained from Sigma except D-glucose and NaNO₂, which were from BDH. All other chemicals and solvents used were of analytical grade.

RESULTS

Effects of substrates on O₂ uptake in cysts and trophozoites of G. muris

The effect of various substrates on endogenous respiration in the cyst and trophozoite forms of *G. muris* is shown in Table 1. Cysts were unable to utilize sugars and sodium succinate; however, ethanol significantly stimulated respiration. In the trophozoite all substrates with the exception of sodium succinate stimulated respiration. Endogenous respiration in both cysts and trophozoites was inhibited by acetate.

Fig. 1. Reciprocal plot of respiration rate against increasing O_2 concentration (0–50 μM) for *G. muris* cysts respiring endogenous substrate; 1.2×10^7 cysts were used for each determination and all experiments were done at 37 °C. Error bars indicate SEM values, calculated from data obtained in four experiments.

Table 1. *Effects of substrates on respiration in the trophozoite and cyst forms of G. muris*

The effects of substrates on respiration of cysts and trophozoites of *G. muris* were determined using an open oxygen electrode. For each determination, 1.2×10^7 cysts or 2×10^6 trophozoites were used; all experiments were done at 37 °C with a gas phase consisting of 1.01 kPa O_2 in N_2 . Inhibition or stimulation of respiration is expressed as a percentage of the endogenous respiration. Endogenous respiratory rates were 0.48 $\mu M-O_2 \text{ min}^{-1}$ per 10^6 cysts and 2.2 $\mu M-O_2 \text{ min}^{-1}$ per 10^6 trophozoites. Values expressed were typical of those determined from three experiments giving similar results. All substrates were added to a final concentration of 40 mM.

Substrate	Percentage inhibition or stimulation (–) of respiration	
	Trophozoites	Cysts
None	0	0
D-Glucose	76	0
D-Fructose	5	0
Succinate	0	0
Acetate	–29	–24
Ethanol	74	105

O₂ dependence of respiration in cysts and trophozoites

The O_2 dependence of respiration in cysts utilizing endogenous substrate (Fig. 1) shows that respiration increases with increasing O_2 concentration over the range 0–25 $\mu M-O_2$, and at O_2 concentrations greater than 25 $\mu M-O_2$ respiration decreases. In the presence of ethanol the O_2 dependence of respiration followed a similar pattern to that shown in Fig. 1: the results from double reciprocal plots are shown in Table 2. No significant variation was observed in the sensitivities of endogenous or ethanol-supported respiration to elevated O_2 concentration; however, a marked difference was observed in their apparent O_2 affinities.

The dependence on O_2 of D-glucose or ethanol-supported respiration in *G. muris* trophozoites followed a similar pattern to that observed in Fig. 1: results are shown in Table 2. For both substrates, respiration was more sensitive to O_2 than that observed in cysts. Apparent K_m values for O_2 indicate that trophozoites have a higher affinity for O_2 than cysts.

The effect of increasing cyst number on the measured endogenous respiratory rate (Fig. 2) shows that rates of O_2 uptake were proportional to cyst numbers. Interestingly, a significant decrease in the O_2 concentration above which respiration was inhibited was observed as cyst numbers were increased.

The temperature dependence of respiratory activity in *G. muris* cysts is shown in Fig. 3. A decrease in temperature from 37–17 °C produced an 82% reduction in respiratory activity; a Q_{10}

Table 2. *Oxygen kinetics of respiration in G. muris*

Maximal respiratory rates ($V_{\max}$), apparent K_m values and O_2 concentration maxima were obtained from the double reciprocal plots of respiration as a function of O_2 concentrations. All substrates were added to a final concentration of 40 mM; 1.2×10^7 cysts or 2×10^6 trophozoites were used for each determination and all experiments were done at 37 °C. After each experiment viability of cysts and trophozoites was determined by excystment or motility. The duration of each experiment was approximately 100 min, and the interval between each step of increasing O_2 concentration was 10 min. Values are means $\pm$ SEM (number of experiments was four for cysts and three for trophozoites). ND, Not determined.

Substrates	Cysts			Percentage excystation	Trophozoites			
	$V_{\max}$ ($\mu\text{M-O}_2$ min^{-1} per 10^6 cysts)	Apparent K_m for O_2 (μM)	O_2 concentration maximum (μM)		$V_{\max}$ ($\mu\text{M-O}_2$ min^{-1} per 10^6 trophozoites)	Apparent K_m for O_2 (μM)	O_2 concentration maximum (μM)	Percentage motility
None	0.6 $\pm$ 0.05	9.7 $\pm$ 1.0	25 $\pm$ 3	95 $\pm$ 6	ND	ND	ND	ND
D-Glucose	ND	ND	ND	ND	4.05 $\pm$ 0.52	2.0 $\pm$ 0.08	15 $\pm$ 2.1	85 $\pm$ 10
Ethanol	1.23 $\pm$ 0.13	5.8 $\pm$ 0.3	27 $\pm$ 3	86 $\pm$ 8.5	3.85 $\pm$ 0.48	1.8 $\pm$ 0.14	15 $\pm$ 1.7	80 $\pm$ 12

Fig. 2. Reciprocal plots of respiration rate against increasing O_2 concentration for *G. muris* cysts respiring endogenous substrate: the effects of increasing cyst number is shown. Only the linear portion of the graph is shown and conditions used are those described in Fig. 1. Error bars indicate SEM values calculated from data obtained in four experiments. Number of cysts: ●, 4.8×10^6 ; ■, 9.6×10^6 ; ▲, 1.9×10^7 .

Fig. 3. Effect of temperature on the O_2 dependence of respiration; ▲, 37 °C; ■, 27 °C; ●, 17 °C. Conditions used were those described in Fig. 1. Only the linear portion of the graph is shown. Error bars indicate SEM values, calculated from data obtained in four experiments.

value of 0.85 was calculated from these data. A marked decrease in the sensitivity of respiration to O_2 inhibition was observed with decreasing temperature.

Cyst viability or trophozoite motility, determined after exposure to O_2 concentrations up to 50 μM , was never significantly different to controls maintained under N_2 .

Effects of inhibitors on respiration in G. muris cysts

The effects of various inhibitors on respiration and viability in cysts of *G. muris* is shown in Table 3. It can be seen that ethanol-supported O_2 uptake was more sensitive to this range of inhibitors than endogenous respiration; however, the effect of inhibitors on cyst viability was similar. Acetate, a major product of aerobic carbohydrate metabolism, markedly inhibited respiration and cyst viability. Interestingly metronidazole, a drug used in the treatment of giardiasis, had no effect on respiration or viability. $NaNO_2$ completely inhibited respiration and viability in cysts. Other anti-protozoal drugs such as quinacrine and chloroquine had little effect on respiration but significantly reduced cyst viability; similar effects were observed with the ATPase inhibitors quercetin and oubain, and the thiol reagent *p*-chloromercuribenzoate. The

Table 3. *Effects of inhibitors on respiration and viability of the cyst form of G. muris*

Conditions used were as in Table 1, except that viability was determined by the excystment of cysts. Treated cysts were excysted along with a control batch of untreated cysts, and the percentage excystment of this batch was determined to be the 100% value. Inhibition or stimulation of respiration is expressed as a percentage of either endogenous or ethanol-stimulated respiration. Results were typical of those observed in three experiments. Endogenous and ethanol-supported respiratory rates were 0.48 and 0.97 $\mu\text{M-O}_2 \text{ min}^{-1}$ per 10^6 cysts respectively.

Inhibitor	Endogenous respiration			Respiration with ethanol		
	Concentration (mM) giving maximum inhibition	Maximum inhibition (–) or stimulation (%) of O_2 uptake	Percentage viability	Concentration (mM) giving maximum inhibition	Maximum inhibition (–) or stimulation (%) of O_2 uptake	Percentage viability
Acetate	40	26 ± 3	75 ± 6	40	40 ± 3.5	75 ± 8
Metronidazole	6	0	100	6	0	100
NaNO_2	40	20 ± 2	14 ± 1	32	100	5 ± 1
Iodoacetamide	20	100	0	20	100	0
Quinacrine	1.5	5 ± 0.8	28 ± 1	1.5	0	28 ± 1
Chloroquine	1.25	15 ± 0.8	24 ± 2	1.25	100	24 ± 1.5
Quercetin	1.5	4 ± 1	10 ± 1	2.0	70 ± 8	10 ± 1
Oubain	0.22	4 ± 2	8 ± 1	0.14	76 ± 8	8 ± 0.5
Menadione	0.3	–400 then complete inhibition	0	0.3	–350 then complete inhibition	0
DBMQ	2.0	-40 ± 5	15 ± 1	2.0	0	35 ± 4
p-Chloromercuribenzoate	0.035	22 ± 2	0	0.035	15 ± 0.5	0
Bathophenanthroline	1	-20 ± 2	85 ± 6	1	0	100
Salicylhydroxamic acid	5	0	87 ± 6	5	0	87 ± 5
o-Hydroxydiphenyl	2	0	0	2	0	90 ± 5

effects of menadione on endogenous respiration in cysts was complex. Over a period of 30 min, menadione stimulated O_2 uptake; this stimulation was followed by a decrease in respiratory activity, which ceased completely after 1 h. A complete loss of cyst viability was also observed. Menadione had a similar effect on cysts respiring ethanol. DBMQ and bathophenanthroline, a transition metal chelator, also stimulated respiration and significantly reduced cyst viability. Inhibitors of alternative electron transport, salicylhydroxamic acid and *o*-hydroxydiphenyl, had no effect on either cyst respiration or viability.

Effects of inhibitors on respiration in trophozoites of G. muris

The effects of various inhibitors on respiration and motility in trophozoites of *G. muris* are shown in Table 4. The inhibitor sensitivities of respiration and motility in trophozoites utilizing endogenous substrate were similar to those observed for cysts except that metronidazole completely inhibited trophozoite motility and significantly reduced respiration. The effects of inhibitors on trophozoite respiration in the presence of D-glucose were similar to those observed for endogenous respiration; however, D-glucose-supported respiration was more sensitive to chloroquine, metronidazole and $NaNO_2$. The effects of inhibitors on respiration in the presence of ethanol were different: metronidazole, $NaNO_2$ and iodoacetamide completely inhibited respiration and motility; respiration was also more sensitive to chloroquine, oubain and acetate but less sensitive to quinacrine and *p*-chloromercuribenzoate.

DISCUSSION

The results reported in this paper indicate that cysts of *G. muris* have respiratory activity. This is the first report of such activity in the cyst form of any species of *Giardia*. The respiratory activity of *G. muris* cysts was approximately 10 to 20% that of the trophozoite; a marked variation in substrate specificity between these two forms was observed.

The inability of *G. muris* cysts to utilize sugars and succinate cannot be due to low activities of the enzymes of carbohydrate metabolism, as Lindmark & Miller (1987) showed that the activities of these enzymes were no different from those of trophozoites. Thus, the inability of cysts to utilize sugars may be a consequence of the high levels of endogenous substrate (glycogen) present within the cyst (10% of the dry wt: Kulda & Nohynkova, 1978). The presence of endogenous substrates may prevent active sugar uptake or the transport systems may be absent. Respiration was, however, stimulated by ethanol, which can probably diffuse passively into the cysts. The substrate specificity of *G. muris* trophozoites was similar to that observed previously for *G. lamblia* (Lindmark, 1980) and variation in apparent O_2 affinities between cysts and trophozoites would seem to reflect the differences in their respective respiratory rates.

The most striking feature of respiration in cysts and trophozoites of *G. muris* was the presence of a maximum O_2 concentration above which O_2 consumption decreased. The O_2 concentration at which this occurred in cysts was much greater than in trophozoites, indicating that respiration in cysts is more resistant to higher O_2 concentrations. This inhibition of respiration was irreversible; however, loss of respiratory activity did not affect cyst viability or trophozoite motility. A loss of trophozoite motility during long term exposure to 5% O_2 has been observed previously in *G. lamblia* (Gillin & Diamond, 1981). O_2 maxima have been observed in several organisms including the protozoal parasite *Trichomonas vaginalis* (Yarlett *et al.*, 1987) and in the gut-dwelling nematodes *Nippostrongylus brasiliensis* (Paget *et al.*, 1987a, b) and *Ascaridia galli* (Paget *et al.*, 1988). In these organisms O_2 -mediated toxicity was correlated with the production of H_2O_2 generated by electron transport. Whether the inhibition of respiration in *G. muris* is linked to the production of active O_2 species such as H_2O_2 and $O_2^{\cdot -}$ remains to be investigated.

The effects of temperature on respiration in cysts indicated that respiration would be negligible at temperatures below 7 °C; lower temperatures also favour cyst viability (Bingham *et al.*, 1979). These two factors may be linked: at reduced temperatures the rate of endogenous substrate utilization would be decreased which may extend the lifetime of cysts. If the temperature dependency of respiration and viability in *G. muris* and *G. lamblia* cysts is similar then these results may explain the high incidence of giardiasis that occurs in populations where

Table 4. *Effects of inhibitors on respiration and motility in trophozoites of G. muris*

Inhibitor	Concentration (mM)	Endogenous respiration		D-Glucose-supported respiration		Ethanol-supported respiration	
		Maximum inhibition or stimulation (–) of O ₂ uptake (%)	Percentage motility	Maximum inhibition or stimulation (–) of O ₂ uptake (%)	Percentage motility	Maximum inhibition or stimulation (–) of O ₂ uptake (%)	Percentage motility
Acetate	40	22 ± 2	50 ± 1	21 ± 1	48 ± 3	45 ± 4	34 ± 2
Metronidazole	6	55 ± 3	0	76 ± 6	0	100	0
NaNO ₂	15	50 ± 3	0	82 ± 7	0	100	0
Iodoacetamide	20	100	0	100	0	100	0
Quinacrine	1.5	10 ± 2	42 ± 1	8 ± 0.5	53 ± 2	0	74 ± 5
Chloroquine	1.25	19 ± 1	65 ± 2	82 ± 8	11 ± 2	100	9 ± 2
Oubain	0.22	9 ± 1	0	28 ± 3	0	54 ± 3	0
Menadione	0.3	–600 then inhibition	0	ND	ND	–700 then inhibition	0
p-Chloromercuribenzoate	0.035	25 ± 3	62 ± 6	18 ± 1	45 ± 5	12 ± 1	69 ± 3
Salicylhydroxamic acid	5	0	84 ± 5	0	94 ± 4	ND	ND

Conditions used were as in Table 1, except that 10⁶ trophozoites were used for each experiment. Inhibition or stimulation of respiration is expressed as a percentage of endogenous, or substrate-stimulated respiration. The endogenous respiratory rate was 2.2 μM-O₂ min⁻¹ per 10⁶ trophozoites. Respiratory rates in the presence of either D-glucose or ethanol were 2.9 and 3.2 μM-O₂ min⁻¹ per 10⁶ trophozoites. Values are means ± SEM of results from four experiments. ND, Not determined.

water is untreated and supplied from cold streams (Veazie *et al.*, 1978; Wright & Vernon, 1976; Shaw *et al.*, 1977). In such cold waters cysts would remain viable longer, thereby increasing the chance of infection.

The effects of inhibitors on respiration in both cysts and trophozoites was similar. Cyanide-insensitive respiration has been observed in a wide range of free living and parasitic organisms (Lloyd *et al.*, 1983; Paget *et al.*, 1987a). In these organisms, respiration was sensitive to inhibition by *o*-hydroxydiphenyl and salicylhydroxamic acid but this was not found in *G. muris*. It would seem unlikely, therefore, that the respiration of *G. muris* is similar to cyanide-insensitive respiration observed in such organisms.

The effect of iodoacetamide on respiration suggests that glycolysis plays a role in respiration, possibly via the generation of NAD(P)H. The effects of chloroquine and quinacrine may indicate that flavoproteins and, to a lesser extent, quinones play a role in respiration. The evidence for the involvement of iron-sulphur proteins in respiration is less clear. These conclusions are similar to those published previously by Weinbach (1980) and Lindmark (1980) and may be further evidence for a respiratory chain in *G. muris* similar to that described by Weinbach (1980).

Oubain and quercetin, agents that inhibit plasma membrane ATPases (Glick, 1970) both had a pronounced inhibitory effect on ethanol-supported respiration, but not endogenous respiration in cysts of *G. muris*. The inhibition of respiration in cysts and trophozoites by acetate might be explained using the pathway of acetate formation proposed by Lindmark (1980).

Interesting effects were observed with the inhibitors menadione and metronidazole. The drug metronidazole completely inhibited respiration in *G. muris* trophozoites but had no effect on cyst respiration or viability. When metronidazole is bioreduced by an iron-sulphur centre of the pyruvate:ferredoxin oxidoreductase in *T. vaginalis*, a nitro-radical anion is generated and this species is thought to elicit cytotoxicity within the cell (Moreno *et al.*, 1983; Lloyd & Pedersen, 1985; Chapman *et al.*, 1985). NaNO₂ is believed to destroy an iron-sulphur centre of the pyruvate:ferredoxin oxidoreductase in anaerobic bacteria (*Clostridium sporogenes*; Woods *et al.*, 1981) and was shown to inhibit respiration and viability in *G. muris* cysts and trophozoites. If the sites of action of NaNO₂ and metronidazole are similar it seems likely that the inability of metronidazole to inhibit respiration or to reduce viability of cysts is due to impermeability of the cysts to the drug. If the cyst form of *G. lamblia* shows the same resistance to metronidazole, then patients with giardiasis would probably pass viable cysts for several days after treatment with metronidazole.

Menadione, a redox-cycling naphthoquinone (De Groot *et al.*, 1985) has a complex mode of action. When menadione is bioreduced by an electron-donating system, it reacts with O₂, thereby causing an apparent stimulation of O₂ uptake. Its reaction with O₂ generates active O₂ species (De Groot *et al.*, 1985) and this may be linked to the inhibition of respiration and viability. Ames *et al.* (1987) suggested that electron transfer and the generation of active O₂ species may play a role in the action of various anti-protozoal drugs; the effects of menadione would suggest that compounds that generate O₂ radicals, may have potential as chemotherapeutic agents for the treatment of giardiasis.

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