Fasciola hepatica miracidia are dependent on respiration and endogenous glycogen degradation for their energy generation

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SUMMARY

It is generally accepted that free-living stages of parasitic helminths are dependent on aerobic degradation of endogenous energy sources for their energy generation. This concept, however, is not the result of extensive experimental evidence, but originated mainly intuitively as oxygen is widely available in their habitat and these stages generally have a small size. *Schistosoma mansoni*, the sole parasitic helminth whose energy metabolism has been studied throughout its life-cycle indeed has aerobically functioning free-living stages. However, large differences exist in energy metabolism between adult stages of distinct parasitic helminths, and caution should be taken in predicting that all free-living stages of all parasitic helminths have the same, aerobic energy metabolism. Hence, this report studied the energy metabolism of *Fasciola hepatica* miracidia and demonstrated that *F. hepatica* miracidia are also dependent on aerobic degradation of their endogenous glycogen stores by glycolysis and on Krebs cycle activity for energy generation. However, in contrast to *S. mansoni*, *F. hepatica* miracidia cannot function anaerobically, as inhibition of the respiratory chain blocked motility and carbohydrate degradation, and finally resulted in death of the miracidia. Therefore, this report demonstrated that differences exist between miracidia of distinct species, in pre-adaptation of their energy metabolism to the occasional hypoxic conditions within their next host.

Key words: trematode, *Fasciola hepatica*, miracidium, carbohydrate metabolism.

INTRODUCTION

Parasitic stages of helminths do not use oxygen as the final electron acceptor and possess a predominantly anaerobic energy metabolism, excreting products of a partial degradation of glucose (Von Brand, 1979). This glucose is obtained from the host and is the major source of energy for these parasitic stages. Free-living stages, on the other hand, do not gather food and are, therefore, completely dependent on the endogenous energy stores they acquired in the previous host. Oxygen, however, is mostly present in the environment of free-living stages. This free access to oxygen, combined with a small size, which ensures rapid diffusion of oxygen throughout the organism, would enable free-living stages to live economically. For that reason free-living stages are supposed to have an aerobic energy metabolism, possibly relying on Krebs cycle activity and oxidative phosphorylation for the generation of energy to obtain as much energy as possible from their endogenous glycogen stores. This thrifty metabolism would very well serve their only mission: becoming a multiplying parasitic stage in the next host.

Caution should be taken, however, in predicting the aerobic or anaerobic nature of the energy metabolism of parasitic helminths, because metabolic studies have been confined almost exclusively to the adult parasitic stages in the final vertebrate host and to the free-living or larval stages immediately preceding these. Mainly for reasons of symmetry it is believed that the metabolism of other free-living stages is aerobic, and that of the parasitic stages in the intermediate hosts is anaerobic. *Schistosoma mansoni* is, however, the only trematode whose energy metabolism has been extensively studied throughout its entire life-cycle (Bueding, 1950; Coles, 1973; Von Kruger *et al*. 1978; Van Oordt, Tielens & Van den Bergh, 1989; Tielens *et al*. 1991, 1992).

The energy metabolism of the liver fluke, *Fasciola hepatica* has been studied extensively for many years (Mansour, 1959; Barrett, Coles & Simpkin, 1978; Lloyd, 1986; Tielens, 1994). These studies were mainly restricted, however, to the adult stage in the final host and to the directly preceding larval stages. It is known that the juvenile *F. hepatica* immediately after its excystment from the metacercarial cyst

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possesses a mainly aerobic energy metabolism, which during its growth gradually transforms into the well-known anaerobic metabolism of the adult liver fluke. Virtually nothing is known of the metabolism of other stages in the life-cycle of *F. hepatica*. If, however, miracidia indeed have an aerobic energy metabolism whereas the parasitic stages in the snail have an anaerobic one, then a second aerobic to anaerobic transition occurs in the energy metabolism of *F. hepatica* during its life-cycle (Tielens, 2000). In the present study we investigated the energy metabolism of miracidia of *F. hepatica*.

MATERIALS AND METHODS

Chemicals and biomaterials

D-[6-^{14}C]glucose was purchased from Amersham (Bucks, UK). Hexokinase and glucose-6-phosphate dehydrogenase were from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade. Eggs of *F. hepatica* were isolated by sieving bile from infected sheep livers, and were subsequently extensively washed with copper-free tap water. Collected eggs were stored in the dark at room temperature in copper-free tap water for 3–8 days. Hatching of miracidia was induced by illumination of the eggs, and they were subsequently purified by isolation from the illuminated side-arm of an Erlenmeyer flask (Rau, Bourns & Ellis, 1972).

Radioactive incubations and analysis of excreted end-products

Radioactive incubations and analysis of excreted end-products were performed as described before (Tielens *et al*. 1991). In short, *F. hepatica* miracidia ($0{\cdot}5–2 \times 10^4$) were incubated for 4 h at room temperature in 2 ml of copper-free tap water containing 10 mM Hepes (pH 7·4), streptomycin (200 mg/l), penicillin (200 IU/ml) and 1 mM D-[6-^{14}C]glucose (50 μCi). Secreted end-products in the parasite-free medium were analysed as described previously (Van Oordt *et al*. 1989).

Measurement of oxygen and glycogen consumption

Oxygen consumption was measured as described before (Tielens *et al*. 1991). In short, oxygen consumption of *F. hepatica* miracidia was determined with a Clark-type oxygen electrode maintained at room temperature by a circulating water jacket. Miracidia were resuspended in fresh copper-free tap water at a density of $0{\cdot}5–3 \times 10^4$ miracidia/ml. Oxygen utilization was recorded for 15 min in a total volume of 2 ml. The glycogen content of the miracidia was determined as described before (Tielens, Van den Heuvel & Van den Bergh, 1990), and glucose was determined using a standard procedure with hexokinase and glucose-6-phosphate dehydrogenase (Deutsch, 1983).

RESULTS

Aerobic carbohydrate metabolism

The carbohydrate metabolism of *F. hepatica* miracidia was investigated by incubation of isolated miracidia in water containing 1 mM [6-^{14}C]glucose. Analysis of excreted end-products demonstrated that these miracidia produced radioactively labelled CO_2, but no other radioactively labelled end-products could be detected (not shown). Hence, under aerobic conditions *F. hepatica* miracidia degraded glucose exclusively to CO_2 and water by glycolysis and subsequent Krebs cycle activity.

F. hepatica miracidia can, to a limited extent, use external glucose for their energy metabolism, but analysis of the glycogen content of miracidia demonstrated that miracidia degraded at the same time large amounts of their internal glycogen content (Table 1). In fact, internal glycogen degradation exceeded the degradation of external glucose enormously, as only 1 to 2 % of the degraded glucose was derived from externally supplied glucose. Hence, miracidia do not consume external glucose in significant amounts even when glucose is present in their environment.

Oxygen consumption and respiratory chain activity

As our studies on the carbohydrate metabolism suggested that *F. hepatica* miracidia are strongly dependent on aerobic degradation of their endogenous glycogen stores, we investigated their oxygen consumption in relation to their carbohydrate metabolism. In theory, every mole of glucose derived from glycogen that is degraded to CO_2 consumes 6 moles of oxygen. Therefore, the expected rate of oxygen consumption for the experimentally determined rate of glycogen degradation (4·5 nmol/h/1000 miracidia) is 27 nmol/h/1000 miracidia. Oxygen consumption analysis with a Clark-type electrode (Fig. 1) demonstrated that 1000 miracidia used 20 ± 9 nmol O_2/h ($n = 5$), which correlates well with the predicted oxygen consumption based on glycogen degradation.

Carbohydrate metabolism coupled to respiration

In contrast to the free-living stages of *F. hepatica*, miracidia and metacercariae, the parasitic adult liver flukes are almost completely dependent on an anaerobic energy metabolism (Tielens, Van den Heuvel & Van den Bergh, 1984). Since some free-living stages of parasites are pre-adapted to their

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Table 1. Carbohydrate metabolism of *Fasciola hepatica* miracidia

(Five independent incubation experiments containing $1–5 \times 10^4$ miracidia were performed in copper-free water containing 10 mM Hepes (pH 7·4), streptomycin (200/mg/l), penicillin (0·2 IU/L) and 1 mM D-[6-^{14}C]glucose (50 μCi). The end-products were analysed in the parasite-free medium as described in the Materials and Methods section. All values were corrected for blank incubations and mean values are given with standard deviation. Shown are 3 representative experiments under standard conditions (1, 2 and 3) and the average of 5 incubations in 4 independent experiments. Experiments 1 and 2, and also 3 and 4, were parallel incubations performed with miracidia from the same batch.)

Experiment	Addition	Miracidia (1000 mc/inc.)	Starting glycogen content (nmol glucose units/1000 mc)	Glycogen degradation (nmol glucose units h/1000 mc)	Labelled CO_2 production (pmol/h/ 1000 mc)	External glucose degradation (% of internal glycogen degradation)
1	—	54·6	152	7·6	35	0·47
2	—	42·2	282	6·7	51	0·76
3	—	15·4	444	7·2	113	1·57
$\Sigma(n = 5)$	—	33·6 ± 16·3	280 ± 165	4·5 ± 3·5	75 ± 44	1·6 ± 1·2
4	Cyanide*	14·2	329	5·7	2	0·03

*The incubation (4) of miracidia in the presence of cyanide (1 mM) resulted in rapid immobilization of the miracidia followed by death of the organisms.

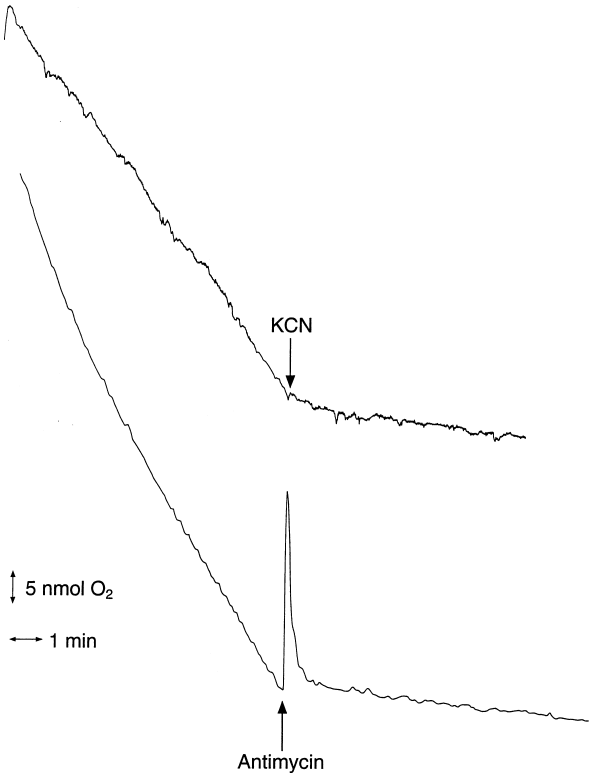

Fig. 1. Oxygen consumption by intact *Fasciola hepatica* miracidia. Oxygen consumption was measured using a Clark-type electrode. Cyanide (final concentration 1 mM) or antimycin A (final concentration 1 μg/ml) was added at the indicated time-point.

next parasitic environment (Tielens, 1994), we investigated whether *F. hepatica* miracidia are also capable of fermenting carbohydrates. Therefore, we analysed the excreted end-products from miracidia incubated with D-[16-^{14}C]glucose and 1 mM cyanide (Table 1). Although the production of radioactive-labelled CO_2 was almost completely blocked no other excreted end-products could be detected. This demonstrates that glucose degradation is coupled to respiration and that *F. hepatica* miracidia cannot ferment carbohydrates. This inability to degrade glucose without the use of their respiratory chain correlates with the observed block in motility, and death of the organisms. Interestingly, the glycogen content of miracidia incubated in the presence of cyanide, which are metabolically inactive, decreased significantly during incubation and was comparable to aerobic glycogen utilization (Table 1). However, the glycogen present in miracidia after incubation with cyanide is likely to be hydrolysed to glucose units by uncontrolled glycogen phosphorylase activity after the death of the miracidia. This was confirmed by the absence of degradation of external glucose, which demonstrates that the flux through glycolysis is nil when respiration is inhibited. Hence, the observed reduction in glycogen during incubation of miracidia in the presence of cyanide does not correlate with an active carbohydrate metabolism.

DISCUSSION

This study demonstrated that *F. hepatica* miracidia did not degrade significant amounts of external glucose compared to the degradation of endogenous glycogen. Hence, the carbohydrate metabolism of *F. hepatica* miracidia is dependent on endogenous glycogen stores. On the other hand, the relatively minute amounts of external [6-^{14}C]glucose that were degraded by the miracidia, could be used to monitor the degradation of the endogenous glycogen reserves, as the [6-^{14}C]glucose in the incubation medium had a very high specific activity. *F. hepatica* miracidia degraded this [6-^{14}C]glucose only to radioactively labelled CO_2. This shows that *F. hepatica* miracidia degrade carbohydrates by glycolysis and Krebs-cycle activity, which is in agreement with earlier histochemical studies (Humiczewska, 1975).

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Since aerobic degradation of carbohydrates to CO_2 by glycolysis and Krebs cycle activity is dependent on respiratory chain activity, we analysed the oxygen consumption of *F. hepatica* miracidia, and these oxygraph studies demonstrated that 1000 miracidia consumed 20 ± 9 nmol of oxygen/h. In addition, specific inhibitors of the respiratory chain, antimycin A and cyanide, almost completely blocked this oxygen consumption. Therefore, it can be concluded that the 20 ± 9 pmol oxygen consumed/h/1000 miracidia is used by the respiratory chain. This rate of oxygen consumption in *F. hepatica* miracidia correlates well with their rate of glycogen degradation ($4{\cdot}5 \pm 3{\cdot}5$ nmol/h/1000 miracidia). Hence, these results strongly suggest that endogenous glycogen is the main, if not the sole, energy source for miracidia, as significant degradation of other energy stores, like lipids or amino acids, would have resulted in a much higher rate of overall oxygen consumption compared to the predicted oxygen consumption based on the observed glycogen degradation. Although triacylglycerols, a main energy store in other eukaryotes, could be detected in miracidia (not shown), these lipids apparently do not function as a significant source for energy generation. This is in agreement with the earlier results obtained on *S. mansoni* miracidia (Tielens *et al*. 1991).

Although analysis of the total amount of glycogen in miracidia after hatching demonstrated large differences in glycogen content between distinct batches of miracidia, these differences are probably due to differences in storage time and/or age of the eggs between the distinct batches. However, in most experiments 1000 miracidia contained between 100 and 300 nmol glucose units of glycogen, which is a sufficient amount to supply the miracidia with energy for at least 20 h. Hence, *F. hepatica* miracidia contain a sufficient amount of energy to support their search for a new host for multiple hours, after which they still contain a considerable amount of energy to penetrate this next host. Therefore, the observed glycogen content and the observed rate of its degradation correlate well with the reported mean life-span of *F. hepatica* miracidia of about 1 day (Andrews, 1999).

The aerobic energy metabolism correlates with the reported 6 times higher level of ubiquinone in *F. hepatica* miracidia compared to rhodoquinone, two electron transporters which are used in the aerobic respiratory chain or during anaerobic malate dismutation, respectively (Van Hellemond *et al*. 1996). On the other hand, the significant amount of rhodoquinone in *F. hepatica* miracidia (14% of the total amount of quinones) (Van Hellemond *et al*. 1996), suggests that they are in this respect pre-adapted to the hypoxic environment that occasionally occurs within their next host. However, *F. hepatica* miracidia apparently cannot function anaerobically, as inhibition of the respiratory chain induced a rapid block of motility followed by death of the miracidia. Furthermore, analysis of excreted end-products demonstrated that in the presence of cyanide, *F. hepatica* miracidia did not degrade any [6-^{14}C]glucose, and therefore, cannot switch to a fermentative energy metabolism. Although a significant amount of rhodoquinone is present in *F. hepatica* miracidia, this alone results apparently not in suitable conditions for anaerobic malate dismutation, and probably other components of the metabolic machinery are not (yet) adapted to the putative occasional anaerobic functioning inside the snail host. Furthermore, miracidia of *F. hepatica* appear not to possess other fermentation pathways like, for instance, lactate production. This is in agreement with the very low lactate production observed in *F. hepatica* adult flukes, both under aerobic as well as anaerobic conditions (Tielens *et al*. 1982). Hence, miracidia of *F. hepatica* differ from those of *S. mansoni* and also from the juvenile *F. hepatica* immediately after emergence out of the metacercarial cyst, which can both function anaerobically, and are thus pre-adapted to the anaerobic conditions of the new environment within their next host. These stages of *S. mansoni* and *F. hepatica* are already adapted to immediate anaerobic functioning although the mechanisms that induce this anaerobic functioning are completely different in the two organisms. In *S. mansoni* cercariae the transition to anaerobic functioning (lactate production) is induced immediately by the high glucose concentration in their new environment, the final vertebrate host. In *F. hepatica*, on the other hand, the transition to anaerobic functioning (malate dismutation) occurs slowly and is forced by the growth of the fluke which limits the amount of oxygen available to the inner-most tissues of the parasite (Tielens, 1994).

This report demonstrates that the energy metabolism of *F. hepatica* miracidia resembles that of *S. mansoni* miracidia in 2 aspects: (*i*) miracidia are self-supporting organisms that use endogenous glycogen as their main energy source, (*ii*) under aerobic conditions miracidia have an aerobic energy metabolism in which glycogen is degraded to CO_2 by glycolysis and Krebs-cycle activity coupled to energy generation via oxidative phosphorylation. However, in contrast to *S. mansoni* miracidia, those of *F. hepatica* are not facultative anaerobes. Hence, differences between miracidia of distinct species exist in the extent they are pre-adapted to the anaerobic energy metabolism forced upon them by the occasionally occuring anaerobic conditions within their next host.

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