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Studies on Metabolism of Lung Flukes Genus *Paragonimus*

V. Reactions of the Tricarboxylic Acid Cycle in Homogenates of Eggs, Larvae and Adults

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Introduction

Lung fluke of genus *Paragonimus* requires three different hosts in the life cycle. Respiratory metabolism of the fluke in various developmental stages is of great interest in comparative biochemistry. Although many papers on tricarboxylic acid cycle (TCA cycle) in parasites have already appeared (von Brand, 1966), similar studies on *Paragonimus* have been published rather few (Tada *et al.*, 1961; Hamajima, 1967). The purpose of the present report is to compare effects of several intermediates of TCA cycle as well as some inhibitors on oxygen consumption and methylene blue reduction of various developmental stages of the lung fluke which lives in different environmental conditions such as water, snail, crab, and mammal.

Materials and Methods

Adults of *Paragonimus westermani* and *Paragonimus miyazakii* were obtained from the lungs of dogs sacrificed 6 months after infection. They were washed repeatedly with Ringer solution prior to experiments. As the adult worms which were incubated in Ringer solution at 37°C laid many eggs, the eggs were collected by centrifugation and used as a material of unembryonated eggs. Embryonated eggs were obtained after the incubation of unembryonated eggs at 27°C in water for two weeks. Mature rediae and cercariae of *Paragonimus ohirai* were obtained from the livers of experimentally infected snails, *Assiminea parasitologica*. Metacercariae of *P. miyazakii* were collected from naturally infected crabs, *Potamon dehaani*. The materials were washed at least five times with deionized water, placed on filter paper to take out excess water, and weighed. Preparations were suspended in 0.25 *M* sucrose or *M*/15 phosphate buffer (pH 7.2) and then homogenized at 0°C by a motor-driven glass homogenizer with a loose-fitting teflon pestle for 3 minutes.

Oxygen uptake was determined by Warburg technique (Umbreit *et al.*, 1951). The reaction mixture consisted of 30 μmoles of KH_2PO_4 (pH 7.3 with KOH), 12 μmoles of $MgCl_2$ and $MnCl_2$, 3 μmoles of ATP, 0.15 μmole of NAD and NADP, 60 μmoles of nicotinamide, 30 μmoles of substrate, 6 μmoles of acetate, 485 μmoles of sucrose, 1.0 ml of 24 % homogenate in 0.25 *M* sucrose and water in final volume of 3.0 ml. The gas phase was air. The reactions were run for 60 minutes after equilibration at 37°C.

The quantities of O_2 utilized were expressed as μl O_2 consumed per gram of the materials in wet weight per hour. The Q_{O_2} (N) is μl O_2 consumed per milligram nitrogen per hour. In some experiments on O_2 consumption, pyruvate was used in combination with the other kind of substrate; for instance, combination of 30 μmoles of pyruvate and fumarate or 30 μmoles of pyruvate and

3 μmoles of oxaloacetate.

Presence of dehydrogenases associated with TCA cycle was determined by Thunberg technique (Umbreit *et al.*, 1951) in which the time needed for 90% reduction of methylene blue was indicative of dehydrogenase activity. The reaction system consisted of 0.267 μmole of methylene blue, 40 μmoles of substrate, 1.0 ml of 10% homogenate in *M*/15 phosphate buffer (pH 7.2) and *M*/15 phosphate buffer (pH 7.2) in final volume of 4.0 ml. The reactions were run at 37°C under anaerobic condition. The nitrogen content of preparations was determined by a micro-Kjeldahl procedure. All reagents employed were of the highest purity so far available.

Results

As shown in Table 1, succinate added as a substrate stimulated O_2 uptake of unembryonated eggs of *P. westermani* comparing with endogenous respiration. On the contrary, combination of pyruvate and fumarate as substrates suppressed the respiration. Different results were obtained in embryonated eggs (eggs with formed miracidia) of *P. westermani*; the O_2 utilization was markedly stimulated in combination of pyruvate and fumarate but not in succinate. Completely similar results to unembryonated eggs of *P. westermani* were seen in rediae and cercariae of *P. ohirai*, i.e. stimulation by succinate and suppression by combination of pyruvate and fumarate (Table 2).

In case of *P. miyazakii*, combination of pyruvate and fumarate strongly inhibited O_2 uptake of the metacercariae (Table 2). Besides pyruvate and fumarate, its respiration of the metacercariae was not stimulated by all substrates excepting succinate. In cases of the substrates which suppressed the respiration, however, production of gas was recognized at the beginning of the reaction in spite of absorption of CO_2 by 10% KOH in the center well of the vessels (Fig. 1).

Table 1 Effect of substrates on the O_2 uptake in the homogenate of *Paragonimus westermani* unembryonated eggs and eggs with miracidia

Substrate	O_2 uptake (μl)	
	Unembryonate eggs	Eggs with miracidia
Endogenous	290	464
Pyruvate+Fumarate	252	583
Succinate	344	479

Table 2 Effect of substrate on the O_2 uptake in the homogenate of *Paragonimus ohirai* rediae and cercariae, and *Paragonimus miyazakii* metacercariae

Substrate	O_2 uptake (μl)	
	Rediae & Cercariae	Metacercariae
Endogenous	584	109
Pyruvate+Fumarate	272	−476
Succinate	872	229
Isocitrate	—	32
α-Ketoglutarate	—	98
Malate	—	21

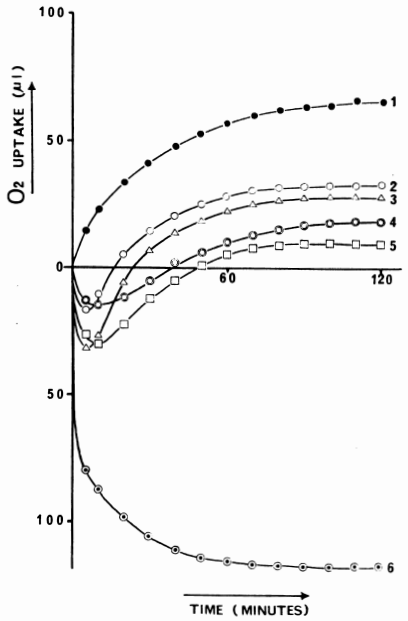

Fig. 1 Effect of substrates on the O_2 uptake by homogenates of *Paragonimus miyazakii* metacercariae.
Substrate: 1. Succinate; 2. Endogenous; 3. α-Ketoglutarate; 4. Isocitrate; 5. Malate; 6. Pyruvate+Fumarate.

High O_2 uptake was recognized in adult worms of *P. miyazakii* as it is shown in Table 3. Among nine kinds of TCA cycle intermediates, succinate showed the strongest effect on O_2 uptake being almost three times as much as that of endogenous respiration. No acceleration of O_2 uptake was seen exceptionally in case of 10^{-2} *M* oxaloacetate. However, 10^{-3} *M* oxaloacetate* used in combination with pyruvate accelerated the respiration of the worms.

Table 3 Effect of substrates on the O_2 uptake in the homogenate of *Paragonimus miyazakii* adults

Substrate	O_2 uptake	
	μl	Q_{O_2}(N)
Endogenous	484	44
Pyruvate	809	74
Citrate	609	55
cis-Aconitate	858	78
Isocitrate	714	65
α-Ketoglutarate	629	57
Succinate	1,361	124
Fumarate	931	85
Malate	835	76
Oxaloacetate	451	41
Oxaloacetate*+Pyruvate	830	76

* 3 μmoles

Methylene blue reduction of the worms was enhanced by addition of intermediates of TCA cycle such as succinate, α-ketoglutarate, isocitrate and malate, to the reaction system (Table 4). On the other hand, malonate inhibited the activity in all of the materials tested, and arsenite inhibited that in adults as it was presumed. Comparing the endogenous dehydrogenase activities of the worms in various developmental stages, the highest activity was recognized in metacercariae and the lowest in rediae and cercariae, while stimulation by the substrates was seen most remarkably in adult worms.

Discussion

Several reports have been published on respiratory metabolism of parasites with special regards to TCA cycle (von Brand, 1966). Costello and Brown (1962) reported that succinate stimulated O_2 uptake in homogenate of *Ascaris suum* unembryonated eggs. On the other hand, presence of some enzymes associated with TCA cycle was found in *Fasciola hepatica* miracidia by Bryant and Williams (1962). Recently, Becker (1968) demonstrated change in respiratory metabolism of *Schistosoma mansoni* sporocyst. Bruce *et al.* (1969) substantiated also utilization of intermediate substances of TCA cycle in *S. mansoni* cercariae and schistosomules using radio isotope technique. In addition, Thomas and Gallicchio (1967) reported that *Clinostomum campanulatum* metacercariae

Table 4 Effect of substrates on the methylene blue reduction by the homogenates of *Paragonimus westermani* unembryonated eggs and adults, *Paraonimus ohirai* rediae and cercariae, and *Paragonius miyazakii* metacercariae

Substrate	Time for 90% reduction (minutes)			
	Eggs	Rediae & Cercariae	Metacercariae	Adults
Succinate	60	100	10	1
Succinate+Malonate*	220	260	20	7
α-Ketoglutarate	70	120	12	4
α-Ketoglutarate+Arsenite*	—	—	—	20
Isocitrate	120	160	15	10
Malate	90	150	18	25
Endogenous	160	180	20	30

* 24 μmoles

possess the components of TCA cycle. In the present experiments, similar results were obtained from the differences of O_2 uptake and utilization of substrates observed to various extent in the homogenates of unembryonated eggs, miracidia, rediae and cercariae, and metacercariae of *Paragonimus*. Low level of O_2 uptake in the homogenates of unembryonated eggs, embryonated eggs, rediae and cercariae, and metacercariae might be due to gas yielded at the beginning of reaction as reported by Oya (1959) in *Ascaris* muscles. It is suggested here that the reaction probably proceeded to the formation of pyruvate and phosphoenolpyruvate via decarboxylations by malic enzyme and phosphopyruvate carboxylase as shown by Saz and Lescure (1969) in *Ascaris* worms.

Tada *et al.* (1961) and Hamajima (1967) have reported that TCA cycle seems to be present in adults of *P. westermani*. In addition, evidence for the presence of TCA cycle enzymes in *Ancylostoma caninum* adults has been adduced from data on the worms' ability to oxidize pyruvate and succinate (Warren, 1965). In the present experiments, similar result was obtained from the difference of O_2 uptake and utilization of substrates in the homogenate of *Paragonimus* adults. On the contrary, Ward and Fairbairn (1970) found that *Hymenolepis diminuta* lacks TCA cycle in adult worms. Furthermore, Oya *et al.* (1965), and Prichard and Schofield (1968) reported that TCA cycle may be of minor importance in *Ascaris* muscle and in *F. hepatica* adult. The Qo_2 of the adults of *Paragonimus* obtained in the present experiments was higher than that in scolices of *Echinococcus granulosus* (Agosin and Repetto, 1963) and in larvae of *Anisakis* (Hamajima *et al.*, 1969), but lower than that in adults of *A. caninum* (Warren, 1965). The differences of Qo_2 stated above might be caused by different localization and eating habits of the parasites. In case of adult of *P. miyazakii*, 10^{-2} *M* oxaloacetate did not accelerate O_2 uptake, but 10^{-3} *M* oxaloacetate* combined with pyruvate accelerated the respiration. This phenomenon might be explained by the fact that oxaloacetate inhibited activity of succinate dehydrogenase (Pardee and Potter, 1948).

Presence of dehydrogenases relating to TCA cycle has been reported in various kinds of parasites (von Brand, 1966). Costello and Brown (1962) found that the Thunberg method provided evidence for the presence of isocitrate, α-ketoglutarate, succinate and malate dehydrogenases in *Ascaris* unembryonated eggs. Furthermore, malate dehydrogenase was found in *S. mansoni* cercariae (Pino *et al.*, 1966). In addition, isocitrate, α-ketoglutarate, succinate and malate dehydrogenases were demonstrated in *E. granulosus* scolices (Agosin and Repetto, 1963), in *Trichinella spiralis* larvae (Goldberg, 1957) and in *Anisakis* larvae (Hamajima *et al.*, 1969). On the other hand, isocitrate, α-ketoglutarate, succinate and malate dehydrogenases have been reported from adults of various species; e.g. in trematodes, *P. westermani* (Hamajima, 1967), *F. hepatica* (Prichard and Schofield, 1968), *Schistosoma japonicum* (Huang and Chu, 1962) and *S. mansoni* (Oya *et al.*, 1970); in cestodes, *H. diminuta* (Read, 1952) and *Hymenolepis nana* (Weitz and Schardein, 1964); in nematodes, *A. suum* (Bueding *et al.*, 1955) and *A. caninum* (Warren, 1965). In the present experiments, identical results were obtained from the differences of methylene blue reduction and utilization of substrates in the homogenates of unembryonated eggs, rediae and cercariae, metacercariae, and adults of *Paragonimus*. And it is strongly suggested that there are presences of dehydrogenases associated with TCA cycle in *Paragonimus*.

In conclusion, although the present studies suggested possibility for the presence of TCA cycle in all developmental stages of *Paragonimus* from the observations in the stimulating effects of the intermediates on O_2 uptake or methylene blue reduction, it remains still unsolved whether TCA cycle is operative physiologically in eggs and larvae of *Paragonimus*.

Summary

The present studies were undertaken in order to make comparison of respiratory metabolism relating to TCA cycle in various developmental stages of lung flukes, *Paragonimus westermani*, *P. miyazakii* or *P. ohirai*. The effects of the intermediates of TCA cycle added to the reaction mixture on oxygen consumption and methylene blue reduction were determined by Warburg and Thunberg techniques, respectively. Although the substrates so far tested generally stimulated oxygen consumption of all developmental stages of the flukes, suppressive effects also were seen in some substrates according to the developmental stages. The highest and the lowest oxygen consumptions were recognized in adult and metacercaria. Succinate, α-ketoglutarate, isocitrate, and malate accelerated the reduction of methylene blue by the flukes. Arsenite and malonate inhibited the reduction. The results thus obtained suggested possibility for the presence of TCA cycle in the respiratory metabolism of all developmental stages of *Paragonimus*.

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肺吸虫の代謝に関する研究
V. 虫卵，幼虫および成虫ホモジネートにおけるクエン酸回路

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本研究は，ワールブルグ検圧計およびツンベルグ法による酸素消費量およびメチレンブルー還元によつて，ウェステルマン肺吸虫，宮崎肺吸虫および大平肺吸虫の虫卵，幼虫および成虫にクエン酸回路があるかどうかを明らかにするためになされた．その結果，クエン酸回路上の基質，コハク酸は虫卵，レジア・セルカリアおよびメタセルカリアホモジネートの呼吸を刺激した．また，ピルビン酸およびフマール酸の同時添加はミラシジウム形成卵ホモジネートの呼吸を刺激した．これに反し，これらの両基質の同時添加は虫卵，レジア・セルカリア，メタセルカリアの呼吸を刺激しなかつた．また，コハク酸はミラシジウム形成卵の呼吸を刺激しなかつた．さらに，イソクエン酸，α-ケトグルタル酸およびリンゴ酸はメタセルカリアホモジネートの呼吸を刺激しなかつた．しかし，成虫ホモジネートの呼吸はクエン酸回路上のすべての基質によつて刺激された．最高の酸素消費量は成虫においてみられ，最低はメタセルカリアにおいてみられた．また，ミラシジウム形成卵の場合を除いて，コハク酸は他の基質のそれよりも強く呼吸を刺激した．コハク酸，α-ケトグルタル酸，イソクエン酸およびリンゴ酸は虫卵，レジア・セルカリア，メタセルカリアおよび成虫ホモジネートによるメチレンブルー還元を促進した．しかし，マロン酸はコハク酸脱水素酵素，亜ヒ酸はα-ケトグルタル酸脱水素酵素の活性を阻害した．