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Glycolytic and oxidative metabolism in the larvae of a nematode, *Anisakis* sp.

FUSANORI HAMAJIMA*, SAWAKO NISHIHARA** AND CHIEMI YASUHIRO**

*Department of Parasitology, Faculty of Medicine, Kyushu University, Fukuoka, Japan

**Department of Home Life Science, Fukuoka Woman's University, Fukuoka, Japan

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Introduction

The adults of *Anisakis* are parasitic in the marine mammalian such as the sperm whale (*Physter catodon*), the blackfish (*Globicephala scammoni*) and the blue white dolphin (*Stenella caeruleo-alba*). And its larvae have been known as the parasite of the sea fish such as the horse mackerel (*Trachurus japonicus*), the mackerel (*Scomber japonicus* and *Scomber tapeinocephalus*), the herring (*Clupea pallasi*) and etc.

Up to the present, many cases of human infection with this nematode have been reported in the countries where the people eat raw sea fish. Very ltttle attention, however, has been paid to this nematode, and to its metabolism, in particular. Therefore, the present study was carried out to demonstrate glycolytic and oxidative metabolism in the *Anisakis* larvae.

Materials and Methods

Anisakis larvae were obtained from the internal organs of freshly killed horse mackerels that were captured from East China Sea. This nematode was identical with type I of *Anisakis* larvae (Oshima, 1966; Kobayashi, 1967). They were immersed at 27°C in Ringer's solution for 12 hours to wash the surface of the body and the inside of the intestine. Sterilization of the worms was carried out with the aid of the Ringer's solution containing 60 mg of penicillin G (K salt) and 300 mg of dihydrostreptomycin per 100 ml. The materials were washed at least five times with deionized water, and placed on filter paper to absorb excess moisture. They were weighed precisely. Homogenates were prepared with the aid of a motor-driven glass homogenizer in an ice bath for 5 minutes, by using various kinds of medium depend on the experiment.

For the determination of glycolytic activity, lactate produced by the homogenate of the *Anisakis* larvae was estimated by the method of Barker & Summerson (1941). In this experiment, the homogenate in 0.03 *M* phosphate buffer (pH 7.2) was used. Oxygen uptakes were determined by the standard Warburg technique (Umbreit *et al.*, 1951). The center well of the Warburg vessels contained a folded comblike cutting filter paper and 0.3 ml of 20% KOH solution. The homogenate in 0.25 *M* sucrose (pH 7.3) was used. The presence of succinate dehydrogenase of the Krebs' cycle was determined by the visual measurement of 90% methylene blue reduction by employing the Tunberg technique (Umbreit *et al.*, 1951). The homogenate in *M*/15 phosphate buffer

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(pH 7.2) was used. Cytochrome oxidase activity was essentially determined by the spectrophotometric method of Cooperstein & Lazarow (1951). The homogenate in 0.03 *M* phosphate buffer (pH 7.4) was centrifuged at 3,000 r.p.m. for 5 minutes. The supernatant fluid was used. Measurement were made on a Hitachi spectrophotometer at 550 mμ. Succinoxidase was assayed by the method of Schneider & Potter (1943). The homogenate in deionized water was used. The nitrogen content of the preparations was determined by a micro-Kjeldahl procedure. All reagents employed were of the highest available purity.

Results and Discussion

1. Glycolysis

As shown in Table 1, glycogen markedly stimulated the lactate formation, and glucose, galactose, fructose, lactose, maltose or trehalose slightly stimulated the formation, respectively. However, mannose and glucosamine did not stimulate the formation above the level of the endogenous.

Table 1 Effect of substrates on the lactate productions by homogenates of *Anisakis* larvae

Substrate	Lactic acid produced	
	μ moles	QL(N)
Endogenous	1.07	1.17
Glycogen	8.91	9.74
Glucose	4.75	5.18
Galactose	3.56	3.89
Fructose	2.57	2.81
Lactose	2.57	2.81
Maltose	2.18	2.38
Trehalose	1.88	2.05
Mannose	0.53	0.58
Glucosamine	0.11	0.12

The reaction mixtures contained 60 μ moles of KH_2PO_4, 15 μ moles of $MgCl_2$, 45 μ moles of KCl, 1.5 μ mole of ATP, 0.15 μ mole of NAD, 60 μ moles of nicotinamide, 0.15 ml of 3% glycogen or 90 μ moles of substrate (glucose, galactose, fructose, lactose, maltose, trehalose, mannose or glucosamine) and 0.45 ml of 25% homogenate in 0.03 *M* phosphate buffer (pH 7.2) in final volume of 1.5 ml. Reactions were run for 60 minutes after equilibration at 37°C *in vacuo*. The amounts of lactate production are shown in μ moles lactic acid produced per gram in wet weight per hour. QL (N) is μl gas volume released under standard condition from lactic acid produced per milligram nitrogen per hour. The values presented are the averages of three times determinations.

It is known that lactic acid is produced as one end product of glycolysis (e.g. in nematodes; *Litomosoides carinii*: Bueding, 1949; *Ascaris lumbricoides*: Bueding & Yale, 1951, and Rathbone & Rees, 1954; *Trichinella spiralis*: von Brand *et al.*, 1952, Goldberg, 1958, and Agosin & Aravena, 1959; *Dirofilaria uniformis*: von Brand *et al.*, 1963; in trematodes; *Schistosoma mansoni*: Bueding, 1950; *Fasciola hepatica*: Mansour, 1959; *Paragonimus westermani*: Hamajima, 1967; in cestodes; *Hymenolepis diminuta*: Read, 1951; *Echinococcus granulosus*: Agosin, 1957). Therefore, in this experiment it would appear that the Embden-Meyerhof pathway could act as an energy supply from several carbohydrates, although the amount of the lactate formed by the homogenate (0.05 μ moles/mg.N/h.: endogenous rate) was of smaller quantity than that of *T. spiralis* larvae reported by Agosin & Aravena (1959) (0.72 μ moles/mg.N/h.: endogenous rate).

2. Tricarboxylic acid cycle

As shown in Table 2, citrate, isocitrate, α-ketoglutarate or succinate, considerably stimulated the oxygen consumption in the homogenate. And pyruvate, cis-aconitate or malate slightly stimulated the consumption. On the contrary, in the higher concentration (30 μ moles), oxaloacetate or fumarate did not stimulate the consumption above the level of the endogenous. However, the mixture of pyruvate at the higher concentration and oxaloacetate at the lower concentration (3 μ moles), and fumarate at the lower concentration stimulated the consumption above the levels of the pyruvate and endogenous.

From the results mentioned above, the QO_2 of the homogenate of *Anisakis* larvae (6.71μl/mg.N/h.: succinate QO_2) was much less than those reported by Agosin & Repetto (1963) in scolices of *E. granulosus* (94μl/mg.N/h.: succinate QO_2) and by Warren (1965) in *An-*

Table 2 Effect of substrates on the oxygen consumption in the homogenates of *Anisakis* larvae

Substrate	O_2 uptake	
	μl	QO_2(N)
Endogenous	95.78	4.67
Pyruvate	112.26	5.47
Oxaloacetate	91.70	4.47
Oxaloacetate*+Pyruvate	133.60	6.51
Citrate	152.70	7.45
cis-Aconitate	111.02	5.41
Isocitrate	124.12	6.05
α-Ketoglutarate	192.20	9.37
Succinate	137.58	6.71
Fumarate	88.88	4.33
Fumarate*	173.72	8.47
Malate	123.72	6.03

The reaction mixture contained 30 μ moles of KH_2PO_4, 12 μ moles of $MgCl_2$ and $MnCl_2$, 3 μ moles of ATP, 0.3 μ moles of NAD and NADP, 60 μ moles of nicotinamide, 30 μ moles of substrate (pyruvate, oxaloacetate, citrate cis-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate or malate) or 3 μ moles of substrate (oxaloacetate* or fumarate*), 485 μ moles of sucrose and 1.0 ml of 25% homogenate in 0.25 M sucrose (pH 7.3) in final volume of 3.0 ml. The gas phase was air. Reactions were run for 120 minutes after equilibration at 37°C. The quantities of the oxygen consumption are shown in μl oxygen consumed per gram in wet weight per hour. QO_2(N) is μl oxygen consumed per milligram nitrogen per hour. The values presented are the averages of triplicate determinations.

cylostoma caninum larvae (548μl/mg.N/h.: succinate QO_2). But it seemed to indicate the presence of the Krebs' cycle oxidation in the *Anisakis* larvae as found in various species of the parasite (e.g. in nematodes; *Nematodirus filicollis*, *Nematodirus spathiger*, *Ascaridia galli* and *Neoaplectana glaseri*: Massey & Rogers, 1950; *T. spiralis*: Goldberg, 1957; *A. caninum*: Warren, 1965; *A. lumbricoides*: Oya *et al.*, 1965; in trematodes; *P. westermani*: Tada *et al.*, 1961, and Hamajima, 1967; *F. hepatica*: Bryant & Williams, 1962; in cestodes; *E. granulosus*: Agosin & Repetto, (1963). Pardee & Potter (1948) and Matsuyama (1958) reported that oxaloacetate inhibited the activity of succinate dehydrogenase. Thus, on the basis of the results mentioned above it seemed that oxaloacetate at the higher concentration inhibited activity of the dehydrogenase in this experiment. On the other hand, even if fumarate at the higher concentration did not stimulate the consumption as shown in Table 2, the fumarate afforded the greatest stimulation of the lactate formation (30.06 μmoles/gm. wet weight/h.). Judging from the results, it was considered that, in the case of the higher concentration of the fumarate, the reaction probably proceeded to the formation of lactate via the decarboxylation by malic enzyme. The result in this experiment was similar to that in *Ascaris* muscle (Oya, 1959).

3. Succinoxidase system

As shown in Table 3 and Figure 1, succinate stimulated the reduction of methylene blue by the homogenate, while 6×10^{-3} M malonate inhibited the reduction. On the other hand, the supernatant fluid of the homogenate oxidized the reduced cytochrome c and 0.01 M sodium cyanide added to the reaction mixture inhibited the oxidation. Thus, the results of this experiment indicated the presence of succinate dehydrogenase and cytochrome oxidase activity in the *Anisakis* larvae as found in various species of the parasite (e.g. in nematodes; *T.*

Table 3 Effect of succinate or malonate on the methylene blue reduction by the homogenate of *Anisakis* larvae

Substrate	Reaction mixture	Time for 90% reduction (minutes)	% Decrease due to addition
Succinate	Complete	3	67
	Plus malonate (24 μ moles)	6	33
	Endogenous	9	—

The complete system contained 0.267 μ mole of methylene blue, 40 μ moles of succinate, 1.0 ml of 25% homogenate in M/15 phosphate buffer (pH 7.2) in final volume of 4.0 ml. Reactions were run at 37°C *in vacuo*.

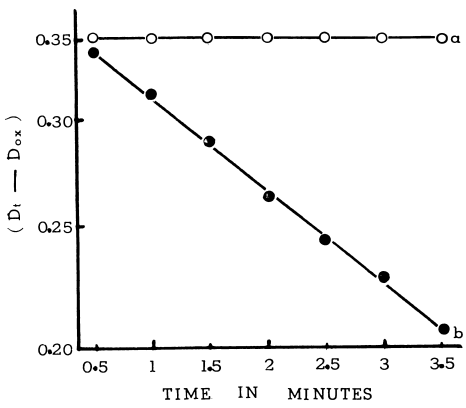

Fig. 1 Graph (on log scale) showing the oxidation of ferrocytochrome c by the supernatant fluid of the homogenate of *Anisakis* larvae.

The complete system contained 0.17 μmole of cytochrome c, 4 μl of polyethylene glycol, 0.2 ml of supernatant fluid of 10% homogenate in 0.03 M phosphate buffer (pH 7.4) and 0.03 M phosphate buffer (pH 7.4) in final volume of 4.0 ml. Reactions were run at 21°C. Dt is the extinction at time t. Dox is the extinction after complete oxidation by the addition of potassium ferricyanide (4 μmoles). Assay time is 3.5 minutes.

a Plue sodium cyanide (40 μmoles).

b Complete system.

spiralis: Goldberg, 1957; *Strongyloides papillosus*: Costello & Grollman, 1958; *A. lumbricoides*: Costello *et al.*, 1963; *Necator americanus*: Fernando, 1963; *A. caninum*: Warren, 1965; in *trematodes*; *S. mansoni*: Bueding & Charms, 1952; *Paragonimus miyazakii* and *Paragonimus ohirai*: Hamajima, 1965; *P. westermani*: Hamajima, 1967; in cestodes; *H. diminuta*: Read, 1952; *Hymenolepis nana*: Goldberg & Nolf, 1954). And the effect of succinate, cytochrome c, malonate or cyanide on the succinate dehydrogenase-cytochrome oxidase activity was examined in the homogenate (Table 4). In this experiment, succinate or cytochrome c stimulated the oxygen consumption above the level of the endogenous. The consumption was markedly increased when both the succinate and cytochrome c were added in the reaction mixture. The addition to reaction mixture of 6×10^{-3} M malonate or 10^{-4} M

Table 4 Effect of succinate, cytochrome c malonate or cyanide on the succinoxidase activity in the homogenates of *Anisakis* larvae

Reaction mixture	O_2 uptake	
	μl	$QO_2(N)$
Complete	230.88	11.26
Minus succinate and cytochrome c	79.40	3.87
Minus succinate	106.28	5.18
Minus cytochrome c	100.20	4.89
Plus malonate (18 μ moles)	161.36	7.87
Plus potassium cyanide (0.3 μ mole)	132.84	6.48

The complete system contained 10 μ moles of KH_2PO_4 (pH 7.4), 60 μ moles of succinate, 0.04 μ mole of cytochrome c, 1.2 μ mole of $AlCl_3$, 1.2 μ mole of $CaCl_2$ and 1.0 ml of 25% homogenate in final volume of 3.0 ml. The gas phase was air. Reactions were run for 60 minutes after equilibration at 37°C. The quantities of oxygen consumption are shown in μl and $QO_2(N)$ as present in Table 2. The values presented are the averages of triplicate determination.

potassium cyanide, however, inhibited the consumption in the homogenate below the level of the complete system. Thus, the stimulation or inhibition of the consumption in the homogenate by succinate or malonate indicated the presence of succinate dehydrogenase, and the stimulation or inhibition of the consumption by cytochrome c or cyanide indicated the presence of terminal respiratory enzymes that are linked to heavy metals. Therefore, the results of the experiment indicted the presence of a succinoxidase system in the *Anisakis* larvae as reported in the *A. caninum* larvae by Warren (1965).

The dry weight of the *Anisakis* larvae was 26.43% of the wet weight, and 2.05% of the wet weight was nitrogen.

Summary

In the present study, the glycolytic and oxidative metabolism was investigated in the *Anisakis* larvae.

1. Several carbohydrates associated with the Embden-Meyerhof pathway or Krebs' cycle

stimulated the lactate formation or oxygen consumption in the homogenate.

2. Succinate stimulated the methylene blue reduction by the homogenate.

3. Reduced cytochrome c was oxidized by the supernatant fluid of the homogenate.

4. Succinate or cytochrome c stimulated the oxygen consumption in the homogenate.

5. The oxygen consumption was markedly increased when both succinate and cytochrome c were added in the reaction mixture.

6. Malonate and cyanide inhibited the oxygen consumption in the homogenate.

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アニサキス幼虫における解糖および呼吸代謝

浜　島　房　則
(九州大学医学部寄生虫学教室)
西　原　沢　子　安広千恵美
(福岡女子大学家政学部)

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