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Studies on Metabolism of Lung Flukes Genus *Paragonimus* IV. Reactions of Glycolysis in Homogenates of Eggs, Larvae and Adults

FUSANORI HAMAJIMA

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

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Introduction

Lung flukes of the genus *Paragonimus* have three different hosts during their life cycle. Some aspects of glycolysis in different stages of the flukes are of interest in comparative biochemistry. The presence of glycolysis has been previously reported in various species of parasites (von Brand, 1950, 1960 ; Rathbone and Rees, 1954 ; Goldberg, 1958 ; Agosin and Aravena, 1959 ; Hamajima *et al.*, 1969 ; Bueding, 1950 ; Murakoshi *et al.*, 1962 ; Hamajima, 1965, 1967 ; Read, 1951). However, few studies concerned with glycolytic activity in lung flukes have been done. The present study was carried out to verify the presence of glycolysis by the incorporation of radioactivity into the glycolytic intermediates and lactate formation when P^{32} as inorganic phosphate and some carbohydrates were incubated with homogenates of various stages of lung flukes in the life cycle. The formation of these intermediate metabolites and the end product indicats that the glycolytic pathway is present. This paper reports the results of these experiments with some comments.

Materials and Methods

Adults of *Paragonimus westermani* (Kerbert, 1878), *Paragonimus ohirai* Miyazaki, 1939, and *Paragonimus miyazakii* Kamo, Nishida, Hatsushika et Tomimura, 1961 were obtained from the lungs of the dogs sacrificed 6 months after inoculation of the metacercariae, and washed with Ringer's solution. Unembryonated eggs were prepared from evacuated eggs in the Ringer's solution of adult lung flukes. Mature rediae and cercariae were liberated from livers of snails, *Assiminea parasitologica* Kuroda, 1958, experimentally infected with *P. ohirai*. Metacercariae except the outer cyst wall were isolated from crabs, *Eriocheir japonicus* de Haan, 1835, naturally infected with *P. westermani*, *Sesarma* (*Holometopus*) *dehaani* H. Milne Edwards, 1853, infected with *P. ohirai*, and *Potamon dehaani* (White, 1847), infected with *P. miyazakii*. The materials were washed at least five times with deionized water, and placed on filter paper to absorb excess moisture before weighing. Homogenates were prepared using various kinds of media with the aid of a motor-driven glass homogenizer with a loose-fitting teflon pestle suspended in an ice bath for 3 minutes.

The determination of the presence of the glycolytic pathway was made as follows : the homogenate of *Paragonimus* was transferred to the reaction mixture (see Fig. 1, Tables 1 and 2) and incubated for 60 minutes at 37°C under anaerobic condition. Then, the reaction was terminated with the addition of hot absolute ethanol to yield a final concentration of 80 %(v/v). The reaction

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mixture was centrifuged at 3,000 g for 10 minutes. The resulting supernatant was passed through a column (1×15 cm) of ion-exchange resin (Amberlite IR-120, 100 mesh, in the H^+ form). The radioactive metabolic intermediates were washed from the resin with 30 ml of water and evaporated *in vacuo* at 60°C. The residue was dissolved in 0.072 ml of water. An aliquot of 0.01–0.02 ml was spotted on Toyo Roshi No. 50 filter paper for one-dimensional chromatographic separation. Solvent used for the separation was a mixture of 28 % ammonia solution-Isoamyl alcohol-Isopropyl alcohol-72 % lactic acid-10 % trichloroacetic acid (TCA)-water (0.15 : 5 : 15 : 0.5 : 0.5 : 10, v/v) (Sekiguchi, 1955). Paper chromatography was carried out at 0°±2°C. The presence of spots corresponding to radioactive metabolic phosphate esters was determined by radioautography. The spots were confirmed by the methods of Hanes and Isherwood (1949), Forsyth (1948) and Partridge (1949). The spots of radioactive metabolic phosphate esters were identified by comparison with Rf values of the authentic samples, and also by the reactions for phospho-molybdate complex, aldose and ketose. For analysis of the end product, the reaction was terminated with the addition of TCA to yield a final concentration of 5 % (v/v). Lactate production was estimated by the method of Barker and Summerson (1941). The nitrogen content of the preparations was determined by a micro-Kjeldahl procedure of Parnas and Wagner (1921). All reagents employed were of the highest available purity.

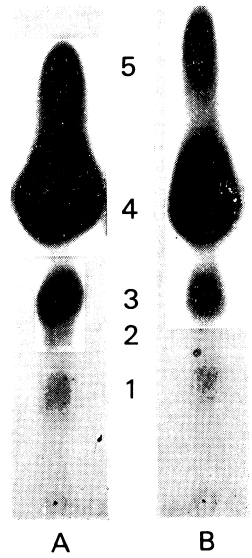

Fig. 1 Radioautogram of phosphate esters formed by the homogenate of adult *Paragonimus ohirai*.

A, Radioautogram when glycogen was used as a substrate.

B, Radioautogram when glucose was used as a substrate.

Spot identification : 1. Fructose 1,6-diphosphate ; 2. Glucose 1-phosphate ; 3. Glucose 6-phosphate ; 4. Orthophosphate ; 5. Unknown.

The reaction mixture contained 24 μmoles of citrate (pH 7.2 with KOH), 15 μmoles of $MgCl_2$, 45 μmoles of KCl, 1.5 μmoles of ATP, 0.15 μmole of NAD, 60 μmoles of nicotinamide, 0.15 ml of 3 % glycogen or 90 μmoles of glucose, 3 μCi of P^{32} and 0.3 ml of 24 % homogenate in 0.01 *M* citrate buffer (pH 7.2) containing 0.3 μg of KH_2PO_4. The reaction was carried out for 60 minutes at 37°C under anaerobic condition.

Results

As shown in Figure 1, for *P. ohirai* adults five positive spots were observed on the radioautogram (A) when glycogen was used as a substrate. These four spots corresponded in Rf value to fructose 1, 6-diphosphate (A, 1), glucose 1-phosphate (A, 2), glucose 6-phosphate (A, 3) and orthophosphate (A, 4) in comparison with the authentic samples. The one spot (A, 5) was unknown. The radioautogram (B) revealed four radioactive spots when glucose was used as a substrate. These three spots corresponded in Rf value to the spots for fructose 1, 6-diphosphate (B, 1), glucose 6-phosphate (B, 3) and orthophosphate (B, 4) in comparison with the authentic samples. The one spot (B, 5) was unknown. The spots for fructose 1, 6-diphosphate (A, 1 and B, 1), glucose 1-phosphate (A, 2), glucose 6-phosphate (A, 3 and B, 3), orthophosphate (A, 4 and B, 4) and unknown (A, 5 and B, 5) corresponded to the areas of blue spots produced by the reaction for phospho-molyb-

Table 1 Effect of FDP on lactate production by homogenates of *Paragonimus* eggs, larvae and adults

Species	Lactic acid produced μmoles					
	Eggs		Metacercariae		Adults	
	FDP	Endogenous	FDP	Endogenous	FDP	Endogenous
P. westermani	13.4	2.1	9.1	2.7	28.0	2.5
P. miyazakii	16.0	2.8	6.2	1.6	28.4	2.4
P. ohirai	24.7	2.2	13.2 28.0*	1.9 2.0*	39.5	2.3

* Rediae and cercariae

The reaction mixture contained 60 μmoles of KH_2PO_4 (pH 7.2 with KOH), 15 μmoles of $MgCl_2$, 45 μmoles of KCl, 1.5 μmole of ATP, 0.15 μmole of NAD, 60 μmoles of nicotinamide, 0.3 μmole of FDP, 75 μmoles substrate (FDP), 0.45 ml of 24 % homogenate in 0.03 *M* phosphate buffer (pH 7.2) and water in final volume of 1.5 ml. The reaction was run for 60 minutes at 37°C under anaerobic condition. The amount of the lactate production is shown in μmoles lactic acid produced per gram wet weight per hour.

date complex. Moreover, these spots for glucose 1-phosphate (A, 2) and glucose 6-phosphate (A, 3 and B, 3) yielded brown color of the reaction for aldo hexose. Furthermore, this spot for fructose 1, 6-diphosphate (A, 1 and B, 1) produced red color of the reaction for keto hexose.

As shown in Table 1, fructose 1, 6-diphosphate (FDP) markedly stimulated the lactate formation when the homogenates of eggs, rediae and cercariae, and adults of *P. westermani*, *P. miyazakii* and *P. ohirai* were employed. While slight stimulation of lactate was observed by the use of the homogenate of metacercariae of these species. The highest lactate formation was obtained in the homogenates of eggs, rediae and cercariae, and adults of *P. ohirai*.

For the homogenate of *P. westermani*, lactate formation was slightly stimulated by glucosamine, glucose, fructose, maltose, glycogen, galactose and mannose (Table 2). Lactose, however, did not stimulate lactate formation above the level of the endogenous rate. In the homogenate of *P. miyazakii*, lactose, maltose, glucosamine and glucose markedly stimulated lactate formation. It was slightly stimulated by mannose, fructose, galactose and glycogen. In the homogenate of *P. ohirai*, maltose, glucosamine and glucose stimulated lactate formation, while glycogen, lactose, fructose, galactose and mannose stimulated its formation to a lesser degree.

Discussion

As shown in Figure 1, the homogenate of adult *P. ohirai* seems to incorporate P^{32} into glucose 1-phosphate, glucose 6-phosphate and fructose 1, 6-diphosphate. Therefore, the presence of phosphorylase, phosphoglucomutase, hexokinase, and phosphofructokinase in the homogenate of adult *P. ohirai* is suggested.

The results shown in Table 1 indicate that lactate was formed in the reaction mixture, suggesting that aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, pyruvate kinase and lactate dehydrogenase are present in the homogenates of eggs, larvae and adults of *Paragonimus*.

In Table 2, it is indicated that lactate was formed in the reaction mixture. This constitutes evidence of the presence of all enzymes associated with the glycolytic pathway. These results are in agreement with the observations for other trematode species by Murakoshi *et al.* (1962).

It is known that some phosphate esters are produced as intermediate metabolites of glycolytic pathway in worms; e.g. in nema-

Table 2 Effect of substrates on lactate production by homogenates of *Paragonimus* adults

Species	Substrate	Lactic acid produced μmoles	QL (N)
P. westermani	Endogenous	0.2	0.5
	Glucosamine	1.3	2.9
	Glucose	1.2	2.7
	Fructose	1.0	2.2
	Maltose	1.0	2.2
	Glycogen	0.8	1.8
	Galactose	0.6	1.3
	Mannose	0.5	1.1
	Lactose	0.2	0.5
P. miyazakii	Endogenous	0.3	0.6
	Lactose	2.1	4.3
	Maltose	2.1	4.3
	Glucosamine	2.0	4.2
	Glucose	2.0	4.1
	Mannose	1.6	3.4
	Fructose	1.6	3.4
	Galactose	1.2	2.6
	Glycogen	1.2	2.6
P. ohirai	Endogenous	0.2	0.4
	Maltose	2.9	5.9
	Glucosamine	2.7	5.4
	Glucose	1.6	3.3
	Glycogen	1.1	2.2
	Lactose	1.1	2.2
	Fructose	1.1	2.1
	Galactose	1.0	2.0
	Mannose	0.9	1.9

The reaction mixture contained 60 μmoles of KH_2PO_4 (pH 7.2 with KOH), 15 μmoles of $MgCl_2$, 45 μmoles of KCl, 1.5 μmole of ATP, 0.15 μmole of NAD, 60 μmoles of nicotinamide, 0.15 ml of 3 % glycogen or 90 μmoles of substrates (glucose, glucosamine, galactose, fructose, lactose, maltose, mannose) and 0.45 ml of 24 % homogenate in 0.03 *M* phosphate buffer (pH 7.2) in final volume of 1.5 ml. Reactions were carried out for 60 minutes after equilibration at 37°C under anaerobic condition. The amount of the lactate production is expressed as μmoles lactic acid produced per gram wet weight per hour. QL (N) is μl gas volume released under the standard condition from lactic acid produced per milligram nitrogen per hour. The values presented are the averages of three determinations.

todes, *Strongyloides ratti* (Jones, Swartzwelder and Abadie, 1955), *Ancylostoma caninum* (Warren and Guevara, 1963), in trematodes, *P. westermani* (Hamajima, 1966), in cestodes, *Hymenolepis diminuta* (Read, 1951). It is also known that lactate is one end product of glycolysis, e.g. in nematodes, *Litomosoides carinii* (Bueding, 1949), *Ascaris lumbricoides* (Bueding and Yale, 1951; Rathbone and Rees, 1954), *Trichinella spiralis* (von Brand *et al.*, 1952, Goldberg, 1958, and Agosin and Aravena, 1959), *Dirofilaria uniformis* (von Brand *et al.*, 1963), in trematodes, *Schistosoma mansoni* (Bueding, 1950), *Fasciola hepatica* (Mansour, 1959), *P. westermani* (Hamajima, 1967), in cestodes, *H. diminuta* (Read, 1951), *Echinococcus granulosus* (Agosin, 1957). Therefore, the production of intermediate metabolites and the end product in this experiment has demonstrated the presence of the Embden-Meyerhof pathway in *Paragonimus*. As a consequence, it can be deduced that several carbohydrates could act as an energy supply via the Embden-Meyerhof pathway, although the amount of lactate formed by the homogenate of adult *P. westermani* (0.02 μmole/mg. N/h. : endogenous rate) was much less than that of *T. spiralis* larvae reported by Agosin and Aravena (1959) (0.72 μmole/mg. N/h. : endogenous rate) and of *Anisakis* larvae reported by Hamajima *et al.* (1969) (0.05 μmole/mg. N/h. : endogenous rate).

Summary

The glycolytic reaction was investigated in the homogenates of eggs, larvae, and adults of *Paragonimus westermani*, *Paragonimus miyazakii* and *Paragonimus ohirai*.

1. The homogenate of adults incorporated P^{32} into some intermediate metabolites associated with glycolysis.

2. Fructose 1, 6-diphosphate stimulated lactate formation in the homogenates of eggs, larvae, and adults.

3. The highest formation of lactate was found in the homogenate of *P. ohirai*, the adult in particular, followed by eggs, rediae and cercariae. Low formation of lactate was seen in the homogenate of metacercariae.

4. Several carbohydrates associated with glycolysis stimulated the lactate formation in the homogenates of adults.

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肺吸虫の代謝に関する研究
IV. 虫卵，幼虫および成虫のホモジネートにおける解糖作用

浜島　房則
（九州大学医学部寄生虫学教室）

P^{32} の中間代謝産物へのとり込みおよび基質による乳酸形成の刺激によつて，肺吸虫の虫卵，幼虫および成虫のホモジネートにおける解糖作用を検討した．その結果，P^{32} は大平肺吸虫の解糖系における中間代謝産物，グルコース 1-燐酸，グルコース 6-燐酸およびフラクトース 1，6-ジ燐酸にとり込まれた．また，フラクトース 1，6-ジ燐酸は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，レジア，セルカリア，メタセルカリアおよび成虫の乳酸形成量の増加を刺激した．その形成量は大平肺吸虫の成虫において多く，宮崎肺吸虫のメタセルカリアにおいて少なかつた．さらに，グリコーゲン，グルコース，グルコースアミン，フラクトース，ラクトース，マルトース，ガラクトースおよびマンノースはウェステルマン肺吸虫，宮崎肺吸虫および大平肺吸虫における成虫の乳酸形成を刺激した．以上のことから，肺吸虫に解糖系の存在することを明らかにした．