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Paper Chromatographic Analysis on the Carbohydrate Metabolism of the Lung Fluke, *Paragonimus westermani* and the Pancreatic Fluke, *Eurytrema pancreaticum*

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INTRODUCTION

Metabolic studies on the parasites are of great interest in comparative physiology, and important for the understanding of host-parasite relationship, rational diagnosis and treatment of the parasites.

There have been many contributions in the studies of carbohydrate metabolism in parasitic helminthes, but only a little informations are available concerning the intermediary metabolism of carbohydrate in trematodes.

Recently, radioactive isotope labeled carbohydrate has served to elucidate the various metabolic schemes of a certain trematodes. In the chinese liver fluke, *Clonorchis sinensis*, considerable amount of exogenous ^{14}C-glucose was absorbed (Hahn et al., 1961), and the distribution of this labeled substance was demonstrated by autoradiography (Hahn et al., 1962: Seo & Lee, 1971). Kang et al. (1969) showed the evidences of anaerobic glycolysis and Krebs cycle activity in this fluke by means of chromatography combined with au toradiography.

Bryant & Williams (1962) found relatively high percentage of the labeled carbon in glycolytic intermediates and end products in *Fasciola hepatica*. Seo et al. (1964a) studied on the quantitative analysis of the metabolic process of exogenous ^{14}C-glucose by *F. hepatica*, and similar experiments were carried out by *Eurytrema pancreaticum* (Seo et al., 1964b), and *Paramphistomum cervi* (Seo et al., 1965a). Park et al. (1967) clarified the uptake and distribution of ^{14}C-succinate in *E. pancreaticum* and it was also studied by *C. sinensis* (Hahn 1971) by means of macro- and microautoradiography. Thersell et al. (1968) observed the fate and distribution of ^{14}C-glucose by using autoradiography, paper electrophoresis and paper chromatography by *F. hepatica*. By the lung fluke, *Paragonimus westermani*, Hamajima (1966a, b & 1967) had proved the intermediary metabolites of carbohydrate and protein by means of chromatography.

From these studies, it was suggested that the evidences of glycolytic pathways and

tricarboxylic acid cycle were presented by these flukes.

However there were only a little informations about the intermediary metabolism of ^{14}C-glucose and the evidences concerning the aerobic or anaerobic glycolysis of trematodes, especially in *P. westermani* and *E. panceaticum.*

In the present study, attempts are made to know the fate of ^{14}C-glucose within the flukes and their incubation medium by *P. westermani* and *E. pancreaticum.* For this purpose, paper chromatography combined with the radioactivity counting by means of Geiger-Mueller counter was applied to detect the intermediary metabolites of the labeled glucose.

MATERIALS AND METHODS

Collection of Worms:

Adult worms of *P. westermani* were obtained from the lungs of freshly killed dogs which had been sacrificed 6 months after infection with metacercariae. Intact and healthy worms were selected and immediately immersed in Tyrode solution. Adult flukes of *E. pancreaticum* were taken alive from the pancreatic ducts of newly slaughtered cattles at the local abattoir. They were brought to laboratory in saline solution. After washing the flukes several times with saline, they were kept in sterilized Tyrode solution at 37°C until incubation began.

Isotope Used:

D-glucose-^{14}C(U), speicfic activity 3.9mCi /mM, was obtained from Nuclear Chicago Corporation, U. S. A. This labeled glucose was dissolved in Tyrode solution to the concentration of 1μCi/ml. This solution was designated incubation medium.

Incubation Procedures:

The procedure of incubation was essentially the same as that of Smith & Moses (1960), Bryant & Williams (1962), and Kang et al. (1969).

Intact and healthy worms of both species, wet weight about 500mg, were transferred separately to 25ml Erlenmeyer flask containing 5ml of incubation medium and incubated for 1 hour in Dubnoff metabolic shaking incubator at 37°C. After the incubation, each group of flukes was killed by the addition of boiling absolute alcohol to yield a final concentration 5ml of incubation medium and incubated for 1 hour in Dubnoff metabolic shaking incubator at 37°C. After the incubation, each group of flukes was killed by the addition of bolling absolute alcohol to yield a final concentration of 80% (v/v) alcohol. Then the homogenates were prepared by homogenization of those flukes in 2ml of Tyrode solution using motor driven glass homogenizer and the debris was removed by centrifugation.

Paper Chromatographic Techniques:

Chromatography was run according to Lederer & Lederer(1961) and Block et al. (1958).

The soluble radioactive intermediates in aliquots of the prepared supernatants were separated by one or two dimensional descending paper chromatography. Ten to twenty μl of the supernatants were applied to a spot of 0.3cm in diameter, 10cm from bottom and right edge of 46×57cm sheets of Whatman No. 1 filter paper, by means of micropipette, and dried rapidly in a current of warm air. The same volume of the solution from the incubation medium was also spotted on the paper, after the end of incubation.

As solvent systems, for organic acids 1) ethyl cellosolve/conc. ammonia/water(80 : 5 : 15), 2) ethanol/conc. ammonia/water (80 : 5 : 15) were used; and for amino acids 1) butanol/propionic acid/water(45.9 : 23.4 : 30.6), 2) phenol/water(72 : 28) were employed. Each solvent was allowed to flow for a distance of 35cm, and for this purpose chromatography

was carried out for 10 to 12 hours at room temperature.

Finally the chromatograms were developed with spraying of 1) bromocresol green, 2) bromocresol blue, 3) bromothymol blue, 4) ammoniacal silver nitrate, and 5) silver nitrate/alkaline phenol for the visualization of the acid compounds, and with ninhydrin for the visualization of amino acids.

The spots appeared on the chromatograms were identified by their chromatographic positions and Rf values in comparison with the co-chromatography of some authentic materials in the same solvent systems. The suspicious spots as well as conspicuous spots on the chromatogram were also confirmed by radioactivity checking of their elutes with Geiger-Mueller counter.

EXPERIMENTAL RESULTS

In the present study, the chromatograms of organic acid fraction from the lung fluke showed the occurrence of spots which had Rf value corresponding to intermediary metabolites of carbohydrate metabolism, such as citric acid, malic acid, succinic acid, fumaric acid, lactic acid and pyruvic acid (Table 1). Identification of these acids was based upon the consistency with which the spots appeared at their respective locations and close agreement of their Rf values with those of known authentic materials, as well as their color reaction to spraying reagents. The yellow spots of organic acid were visualized in the blue or purple background by the dyes of brome series and white spots appeared in brown or black-brown background by silver nitrate series.

On the contrary to *P. westermani*, the spots corresponding to intermediary metabolites of carbohydrate were not detected on the chromatogram of organic acid fraction from *E. pancreaticum*, and the adjacent elutes of the

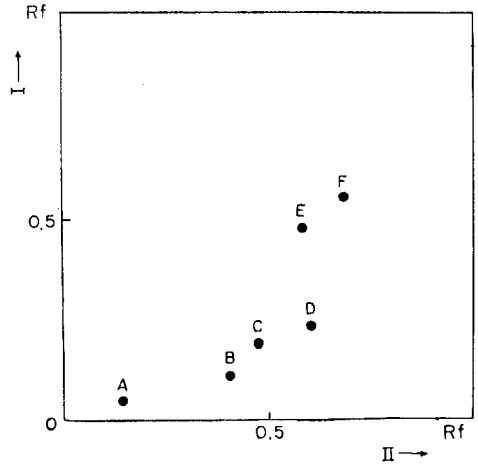

Fig. 1. Two dimensional chromatogram of organic acids from *Paragonimus westermani*. Solvents for Ist direction, ethanol/conc. ammonia/water(80 : 5 : 15) and for IInd direction, ethyl cellosolve/conc. ammonia/water(80:5 : 15). Spots coincide with; A-citric acid, B-malic acid, C-succinic acid, D-fumaric acid, E-lactic acid and F-pyruvic acid.

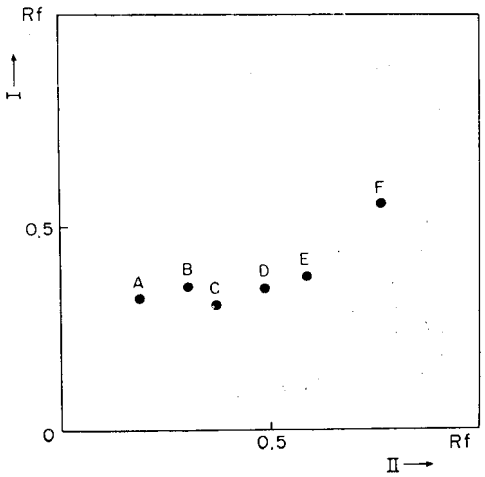

Fig. 2. Two dimensional chromatogram of amino acids from *Eurytrema pancreaticum*. Solvent systems for Ist direction, butanol/propionic acid/water (45.9 : 23.4 : 30.6), and for IInd direction, phenol/water(72 : 28). Spots correspond to, A-aspartic acid, B-glutamic acid, C-serine, D-threonine, E- alanine and F-valine.

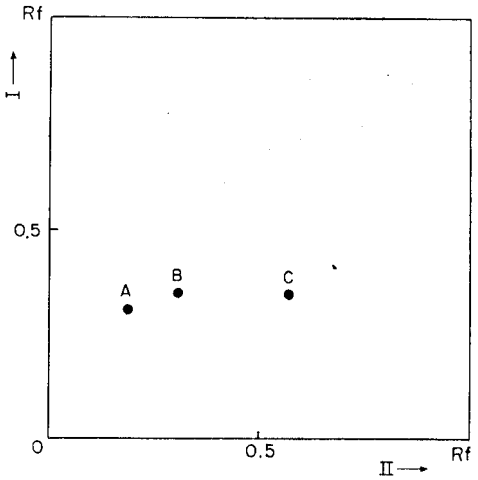

Fig. 3. Two dimensional chromatogram of amino acids recovered from the incubation medium of *Paragonimus westermani*. Solvent systems are butanol/propionic acid/water (45.9 : 23.4 : 30.6) for Ist direction, phenol/water(72 : 28), for IInd direction. A-aspartic acid, B-glutamic acid, and C-alanine.

Table 1. The organic acids recovered from the organic acid fractions of flukes.

Acids	*P. westermani*	*E. pancreaticum*
Citric acid	+	−
Malic acid	+	−
Succinic acid	+	−
Fumaric acid	+	−
Lactic acid	+	−
Pyruvic acid	+	−

Solvent systems:
1) Ethyl cellosolve/conc. ammonia/water (80 : 5 : 15)
2) Ethanol/conc. ammonia/water(80 : 5 : 15)

spot did not show the radioactivity.

In addition to these metabolic intermediates, amino acids formed by the degradation of ^{14}C-glucese were also found in the chromatograms of *P. westermani*(Table 2). These were alanine, aspartic acid, glutamic acid, serine, threonine and valine. These amino acids were also detected in the chromatograms of amino acid fraction by *E. pancreaticum*.

Table 2. The amino acids recovered from the flukes.

Amino acids	*P. westermani*	*E. pancreaticum*
Alanine	+	+
Aspartic acid	+	+
Glutamic acid	+	+
Serine	+	+
Threonine	+	+
Valine	+	+

Solvent systems:
1) Butanol/propionic acid/water(45.9:23.4:30.6)
2) Phenol/water(72:29)

Table 3. The amino acids recovered from the incubation media.

Amino acids	*P. westermani*	*E. pancreaticum*
Alanine	+	−
Aspartic acid	+	−
Glutamic acid	+	−
Precipitation	+	+

Solvent systems: same as Table 2.

From the chromatograms of incubation medium by *P. westermani*, the spots similar properties of alanine, aspartic acid, glutamic acid were observed (Table 3). The addition of absolute alcohol to the aliquots of medium in which the flukes had been incubated was observed to cause a precipitate. This phenomenon suggests a positive qualitative reaction for protein.

By *E. pancreaticum*, the chromatograms of incubation medium revealed no detectable spots but the precipitates were produced when the boiling absolute alcohol was added to the incubation medium.

DISCUSSION

To demonstrate the occurrence of glycolytic

types of carbohydrate degradation, according to von Brand (1966), three lines of study have been employed. The firstline of investigation is the search for glycolytic enzymes within the tissue of parasites, the second line is the search for phosphorylated intermediates of glycolysis. In addition to the above two lines, the third one is that the labeled glucose has served to elucidate the pathways of formation of many metabolic endproducts.

In these regards, the glycolytic enzymes and Krebs cycle enzymes have been known to present in extracts of *P. westermani* (Lee, 1967: Park & Seo, 1967: Lee & Seo. 1967). In addition, presence of substances associated with glycolytic pathway and Krebs cycle was declared by Tada et al. (1961) and Hamajima (1964, 1965, 1966b & 1967): and they claimed that carbohydrate degradation occurred by these schemes in this fluke. By the experiment of Hamajima(1966 b), the phosphorylated intermediates of glycolysis were detected in the eggs, larvae and adults of *P. westermani* in chromatograms. Particularly in the adults, according to him, α-ketogluraric, citric, fumaric, lactic, malic, pyruvic and succinic acids were detected as ketoacids and other organic acids.

In the present study, it has proved that the fluke utilized exogenous ^{14}C-glucose and produced the intermediates of glycolysis and Krebs cycle by chromatography. And this result was well corresponded to the experiments of Tada et al. (1961) and Hamajima (1967). From the above evidences, it was presumed that this fluke possesses a Embden-Meyerhoff scheme as well as Krebs cycle.

Park (1971) performed the autoradiography of *P. westermani* in order to know the uptake and distribution of exogenous ^{14}C-glucose and observed that the radioactive grains were accumulated mainly in the parenchyme and subcuticular musculature, however, the radioactivity was slightly revealed in the regions of ovary, testes and vitelline follicles. Hamajima (1966) has also reported the detection and the measured amounts of eighteen amino-acids in the adult lungfluke of *P. westermani*.

Amino acids found in the chromatogram of *P. westermani* were supposed to be derived from the transamination of intermediary metabolites of carbohydrates. Transaminase activity of this fluke was recovered by Min & Seo (1966). The appearance of labeled amino acids in incubation medium may imply that the flukes often given off energy-rich compound, which could be utilized by their host, as von Brand (1966) stated.

A series of investigation have been performed to prove the Krebs cycle intermediates by *F. hepatica* (Bryant & Williams, 1962: Thorsell et al., 1968), and *C. sinensis*(Kang et al., 1969). In comparison with the reports of these, authors, the experimental results of present study by *P. westermani* closely corresponded to their observation.

On the glycolytic enzymes and Krebs cycle enzymes of *E. pancreaticum*, a little information was available(Lee, 1967: Park & Seo, 1967: Lee & Seo, 1967). They noted activities of lactic acid dehydrogenase, malic acid dehydrogenase and phosphatases in this fluke. The uptake and distribution of ^{14}C-succinate in the vitro media by this fluke was observed by Park et al. (1967) on the autoradiograms obtanied through direct contact methods, which have shown the radioactivity chiefly in reproauctive organs. However, little is known about the intermediary metabolites of carbohydrate degradation, glycolytic pathways and oxidative process by *E. pancreaticum*.

Seo et al. (1964b) studied the metabolic fate of ^{14}C-glucose in *E. pancreaticum* and declared that only the average values, 0.33 and 0.64 per cent of glucose utilized by the flukes

from the medium ^{14}C-glucose participated in the oxidation into respiratory CO_2 and the synthetic process into glycogen respectively. And also noted that the glycogen could be formed by *E. pancreaticum* from non-carbohydrate sources such as a certain amino acids, glycerol and fatty acids. In contrast with the above figures in pancreatic fluke, according to Seo et al. (1964), in case of *Fasciola hepatica* the average 33 and 37 percent of the utilized glucose for the oxidation to respiratory CO_2 and incorporation into to glycogen respectively were originated from the medium ^{14}C-glucose. In present experiment, none of the intermediary metabolite of carbohydrates was discovered. From the above previous works and the present study, it would be suggested that non-carbohydrate sources of nutrient had played a main role in the energy metabolism by this fluke, *E. pancreaticum*. From the analysis of ^{14}C-acetate utilized by this fluke, Seo et al. (1965b) suggested that the fatty acid could play a part of oxidative process into respiratory CO_2 and the synthetic process into glycogen.

Lee(1965) observed the metabolism of ^{14}C-lactate by *E. pancreaticum* and *F. hepatica*, and emphasized that the lactate played a role of major part of the oxidative metabolism in *F. hepatica*, whereas minor part of lactate participated in *E. pancreaticum*. On the other hand, the uptake and distribution of a certain ^{14}C-amino acids such as ^{14}C-proline(u) and ^{14}C-glycine(u), in *E. pancreaticum* were well described by means of autoradiographic methods (Yoon et al., 1964: Seo et al., 1966).

It could be thought from the result of present experiment that organic compounds such as acetate or amino acids might play a greater role in energy metabolism rather than carbohydrates, in *E. pancreaticum*. The uptake and distribution of a certain ^{14}C-amino acids were well described by *E. pancreaticum* (Yoon et al., 1964: Seo et al., 1966).

It can be expected that further trials of metabolic studies in *E. pancreaticum* will elucidate the oxidative process and synthetic process in more detail.

SUMMARY

In order to know the metabolic fate of glucose, radioactive ^{14}C-glucose-(U) was given to the lung fluke, *Paragonimus westermani* and to the pancreatic fluke, *Eurytrema pancreaticum* in Tyrode medium for the detection of intermediary metabolites.

For the identification of these products, paper chromatography in combination with radioactivity counting by Geiger-Mueller counter was applied.

The results obtained were as follows:

1) The labeled metabolites detected in the organic acid fraction of *P. westermani* corresponded to pyruvic acid, lactic acid, malic acid, succinic acid, citric acid, and fumaric acid, while in the organic acid fraction of *E. pancreaticum* showed no distinct spots of these organic acids.

2) In the amino acid fraction of *P. westermani*, 6 kinds of amino acid derived from the degradation of ^{14}C-glucose, such as alanine, aspartic acid, glutamic acid, valine, threonine and serine were found.

These amino acids were also identified in the amino acid fraction of *E. pancreaticum*.

3) Labeled amino acids such as alanine, aspartic acid and glutamic acid were observed in the chromatograms of incubation medium for *P. westermani*, while in case of *E. pancreaticum*, none of these acid were recovered.

4) The precipitation which suggests a positive reaction for protein occurred when boiling absolute alcohol was added to the incubation medium for both species of flukes.

——國文抄錄——

肺吸虫(*Paragonimus westermani*)및 膵蛭(*Eurytrema pancreaticum*)의 葡萄糖代謝에 關한 硏究

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安 鍾 豪·徐 丙 鳳

吸虫類의 糖代謝를 究明하기 위하여 ^{14}C-glucos(U) 含有培地內에 肺吸虫(*P. westermani*) 및 膵蛭(*E. pancreaticum*)을 一定時間 保存培養한 다음 그들 虫體의 homogenates를 만들어 Paper chromatography를 實施하고 糖代謝의 中間代謝産物을 chromatogram 上에서 및 放射能測定에 依하여 同定하였다.

實驗에 使用된 肺吸虫은 被囊幼虫을 개에 感染시켜 그 肺에서 成虫을 얻었으며 膵蛭은 屠畜場에서 소의 膵臟으로 부터 採取하였다. 培地로서는 Tyrode 液에 ^{14}C-glucose가 그 1μCi/ml 添加된것을 5ml 씩 使用하였고 여기에 肺吸虫 및 膵蛭을 500mg 씩 산채로 넣고 37°C에서 한時間 Dubnoff metabolic shaking incubator에서 振盪시켰다.

保存培養이 끝난 即時 培地의 alcohol 濃度가 80% 되도록 alcohol을 加하여 虫體를 죽이고 虫體는 Tris-buffer 溶液으로 잘 씻은후 glass homogenizer에 磨碎하고 遠沈하였다. 遠沈이 끝난 후 沈渣는 버리고 上淸液을 따서 chromatography의 sample로 하였다. 이 sample의 10~20μl를 Whatman No. 1 濾紙에 直徑이 0.3cm 되도록 附着시키고 一次元및 二次元 descending chromatography를 實施하였다.

溶媒가 10~12時間동안 約 35cm를 進展하면 濾紙를 꺼내어 乾燥한 후 發色試藥으로 染色하였다. 이와같이 하여 얻은 chromatogram은 本來 알고있는 有機酸 및 아미노酸의 chromatogram과 Rf 및 色을 比較하여 同定하였고 이를 chromatogram 上의 斑點에서 放射能檢出與否도 아울러 調査하였다.

그 結果 肺吸虫에서는 pyruvic acid, lactic acid, citric acid, malic acid, succinic acid, fumaric acid 等의 有機酸과 alanine, aspartic acid, glutamic acid, serine, threonine과 같은 아미노酸이 檢出되었으나 膵蛭에서는 有機酸은 發見되지 않았고 위의 아미노酸만이 檢出되었다.

또 肺吸虫 培地의 chromatogram에서는 alanine, aspartic acid, glutamic acid 等의 아미노酸이 發見되었으나 膵蛭의 培地에서는 이들이 檢出되지 않았다. 培地에 absolute alcohol을 加했을때 沈澱이 생기는 蛋白質陽性反應은 두 虫體의 培地에서 모두 觀察되었다.

위의 實驗結果로 미루어 볼때 肺吸虫內에서는 高等動物에서와 같은 嫌氣性解糖 및 好氣性 酸化過程이 營爲되는것을 알 수 있었으나 膵蛭에서는 判明되지 않았다.

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