Studies on metabolism of lung flukes genus *Paragonimus*

III. Occurrence of organic acids in uterine eggs, larvae and adults

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There have been many contributions in the studies of carbohydrate metabolism in parasitic helminths in recent years (von Brand, 1950, 1952, 1960; Bueding, 1949 a, 1962 ; Read, 1961; Smith, 1965), and it seems that one evidence for the involvement of the metabolic pathways is demonstrated by the presence of intermediate compounds and products of the sequences. However, very little is so far known concerning the information in the lung flukes of the genus *Paragonimus*. Therefore, the present study was carried out to detect some organic acids associated with carbohydrate metabolism of the lung flukes.

Materials and Methods

Preparation of Samples :

Adults, uterine eggs, rediae and cercariae of *Paragonimus westermani* (Kerbert, 1878), and metacercariae of *Paragonimus miyazakii* Kamo, Nishida, Hatsushika et Tomimura, 1961 were washed with deionized water and weighed as previously described (Hamajima, 1966 a, b).

Analyses of Volatile Fatty Acids :

A 10-g portion of the adult lung flukes was homogenized with the aid of glass homogenizer in an ice bath, and the homogenate was treated in a centrifugal precipitation tube with 10 ml of freshly prepared 5 % metaphosphoric acid and deproteinized. The suspension was centrifuged at 5,000 r.p.m. for 10 minutes. The supernatant was decanted, and the precipitate was washed with 5 ml of water. The mixture of the supernatant and washings was neutralized to pH 8.5 with 5 *N* potassium hydroxide and concentrated *in vacuo* to a volume of 3 ml at 40°C. The solution was adjusted to pH 3 with phosphoric acid-sodium phosphate buffer. The acidic solution was transferred to a distilling flask, and volatile fatty acids were immediately separated by steam distillation from the solution. The distillate (150 ml) was collected in a flask immersed in a dry ice-acetone bath, and titrated with 0.1 *N* ammonium hydroxide for paper chromatographic analysis and 0.1 *N* sodium hydroxide for gas chromatographic analysis. The respective neutralized distillates were concentrated *in vacuo* at 50°C in test tubes, and the former was made up to a known volume (0.5 to 1 ml). The latter, after evaporating, was dried over anhydrous calcium sulfate under reduced pressure.

For paper chromatographic analysis, a 10 to 50-μl portion of the ammonium salts solution was spotted on Toyo Roshi No. 50 filter paper for one-dimensional ascending chromatography and developed by tert-butanol-28 % ammonia solution-water (80 : 4 : 16, v/v), n-butanol-ethanol-

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28 % ammonia solution-water (75 : 5 : 4 : 16, v/v) and dimethylethylcarbinol-ethanol-28 % ammonia solution (75 : 5 : 4 : 16, v/v) at room temperature. The spots corresponding to the ammonium salts of the volatile fatty acids from the lung flukes were detected by using bromthymol blue (BTB) as indicator, by comparison with those of the authentic samples. BTB solution was previously adjusted to pH 6 with 0.1 *N* sodium hydroxide, and sprayed on the filter paper.

To prepare the sample for the gas chromatographic analysis, some acids were separated from the sodium salts by adding a 0.3-ml portion of 30 % (w/v) aqueous phosphoric acid, and the extract was poured into the micro-column (0.3× 10 cm) placing 3 cm of Celite on 3 cm of anhydrous sodium sulfate. The residue was rinsed well with ether and the ether solution replased in the micro-column. The volatile fatty acids were eluted with 1 ml of ether under slight pressure. The effluent was used for the sample of the gas-liquid chromatography as descrided by James and Martin (1952) and Annison (1954). The apparatus was a Shimazu Model GC-1B gas chromatograph attached with a hydrogen flame ionization detector. Inlet pressure and flow rate of nitrogen carrier gas were 2 kg/cm^2 and 30 ml/min. The column used was 3 m long and of 4 mm internal diameter, and was packed with a mixture of 25 % diethylene glycol succinate polyester and 2 % phosphoric acid on Shimalite c (60–80 mesh). The temperature of the column in 155°C was employed. The volatile fatty acids of the lung flukes were identified by comparing with the retention times of known acids under the same conditions.

Analyses of Keto Acids :

A 20 to 100-mg portion of the materials was homogenized with 1 to 5 ml of water by a motor driven glass homogenizer in an ice bath. According to the procedure of Katuki and Kaneyuki (1958), 1 to 5 ml of homogenate was treated in a centrifugal precipitation tube with an equal volume of 10 % metaphosphoric acid in an ice bath with occasional stirring. The treated homogenate was centrifuged at 5,000 r.p.m. for 10 minutes, and 0.2 to 1 ml of freshly prepared solution of 2,4-dinitrophenylhydrazine (100 mg 2, 4-dinitrophenylhydrazine dissolved in 100 ml of 2 *N* hydrochloric acid) was added to a 0.8 to 4-ml portion of the supernatant. This reaction mixture was kept at 30°C for 30 minutes, and 2, 4-dinitrophenylhydrazine derivatives (DNPH derivatives) of keto acids were extracted in a tube with 1.6 to 8 ml of ethyl acetate for 5 minutes. The ethyl acetate extract containing the DNPH derivatives was re-extracted with 0.4 to 2 ml of carbonate-bicarbonate solution (50 g $NaCO_3$ and 5 g $NaHCO_3$ dissolved in 1 *l* of water). The upper layer was removed, and the carbonate-bicarbonate solution was chilled in an ice bath, acidified in the cold with 0.1 to 0.5 ml of 6 *N* hydrochloric acid and re-extracted with 1.6 to 8 ml of ethyl acetate. The ethyl acetate extract containing DNPH derivatives was dried completely in a colored desiccator over sulfuric acid under reduced pressure. The dryness was carried out as quickly as possible in room temperature. The dried DNPH derivatives were dissolved in a known volume (0.1 to 0.5 ml) of acetone. Using a standardized micropipett, a 50-μl portion was spotted on Toyo Roshi No. 50 filter paper which was washed with 1 *N* bicarbonate solution for one-dimensional ascending paper partition chromatography. The DNPH derivatives of keto acids were separated chromatographically with n-butanol-ethanol-water (5 : 1 : 4, v/v), n-butanol saturated with 3 % ammonia solution and dimethylethylcarbinol-ethanol-water (5 : 1 : 4, v/v) at room temperature. The spots corresponding to DNPH derivatives of keto acids from lung flukes were detected by comparison with those of the authentic samples as fluorescent areas when viewed under an ultraviolet light.

Analyses of the Other Organic Acids :

A 50 to 500-mg portion of the materials was treated in a boiling-water bath in a few minutes and was homogenized with 1 to 10 ml of water by a motor driven glass homogenizer in an ice bath. The homogenate was maintained at 80°C for 15 minutes, and centrifuged at 5,000 r.p.m. for 10 minutes. The supernatant and washings were passed through a column (1×

15 cm) of ion-exchange resin (Amberlite IR-120, 100 mesh, in the H^+ form). The organic acids were washed from the resin with 30 ml of water, concentrated *in vacuo* at 60°C. The residue was dissolved in a known volume (0.05 to 0.5 ml) of water. A 50-μl portion was spotted on Toyo Roshi No. 50 filter paper for one-dimensional ascending chromatography. The organic acids were separated chromatographically with ethanol-28 % ammonia solution-water (80 : 4 : 16, v/v), ether-acetic acid-water (13 : 3 : 1, v/v) and ether-formic acid-water (5 : 2 : 1, v/v) at room temperature, and the spots corresponding to organic acids from the lung flukes were detected by using BTB indicator, by comparison with those of the authentic samples. In addition to the above analyses lactic acid was detected by the method of Barker and Summerson (1941).

Results and Discussion

1. *Detection of Volatile Fatty Acids:*

Table 1 shows Rf values of volatile fatty acids in the adult lung flukes. Seven spots corresponding in Rf value to formic, acetic, propionic,

Table 1. Volatile fatty acids in adult *Paragonimus westermani*

Spot	Acid	tert-Butanol 80 28% Ammonia solution 4 Water 16		n-Butanol 75 Ethanol 5 28% Ammonia solution 4 Water 16		Dimethylethylcarbinol 75 Ethanol 5 28% Ammonia solution 4 Water 16	
		Rf value	A	Rf value	A	Rf value	A
1	Acetic acid	0.36	+	0.18	+	0.10	+
2	Formic acid	0.40	+	0.13	+	0.14	+
3	Propionic acid	0.44	+	0.27	+	0.21	+
4	n-Butyric acid	0.53	+	0.38	+	0.33	+
5	α-Methylbutyric acid	0.59	+	0.48	+	0.42	+
6	n-Valeric acid	0.64	+	0.53	+	0.52	+
7	n-Caproic acid	0.75	+	0.64	+	0.69	+

A = Adults. + = Positive.

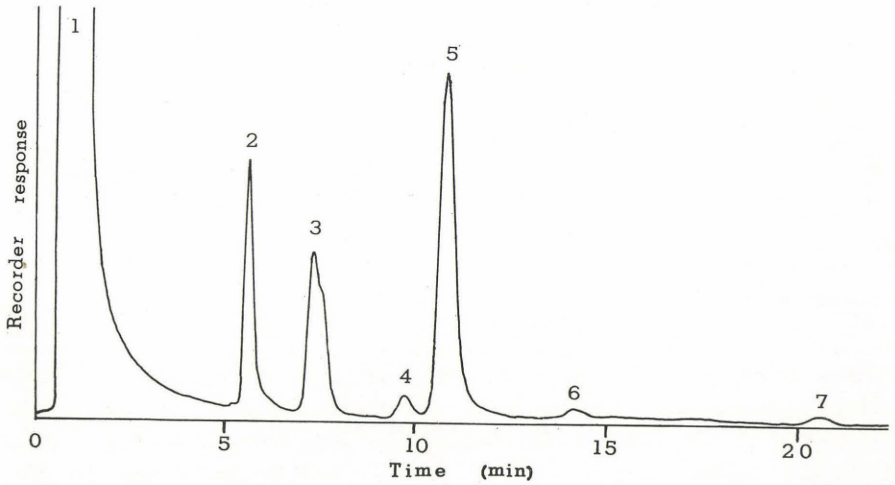

Fig. 1. Gas chromatogram of volatile fatty acids in adult *Paragonimus westermani*
Peak identification: 1. solvent: ethyl ether; 2. acetic acid; 3. propionic acid; 4. n-butyric acid; 5. α-methylbutyric acid; 6. n-valeric acid; 7. n-caproic acid.

n-butyric, α-methylbutyric, n-valeric and n-caproic acids were detected by paper chromatography in comparison with the authentic samples. Figure 1 shows gas chromatogram of the volatile fatty acids in the adult lung flukes. Six peaks corresponding in retention time to acetic, propionic, n-butyric, α-methylbutyric, n-valeric and n-caproic acids were detected by gas chromatography in comparison with the authentic samples. In the present study, the formic acid in the adult lung flukes was found as a spot on the paper chromatograms, but the acid could not be located by gas chromatography because it was very difficult to detect formic acid with a hydrogen flame detector, if one did not inject a large quantity of the acid into the column. The third peak on the gas chromatogram in Figure 1 has contained a shoulder distinctly, but the identity of the substance for a shoulder on the main peak of propionic acid was not established. The amounts of six volatile fatty acids were ranging between 0.5 to 10 μg per gram of wet weight of fresh tissue.

There have been many contributions in detection of volatile fatty acids produced by helminth parasites. Formic, acetic, propionic, trimethylacetic, n-butyric, isobutyric, n-valeric, isovaleric, α-methylvaleric, dl-α-methylbutyric, cis-α-methyl crotonic, trans-α-methyl crotonic (tiglic), n-caproic and isocaproic acids have been found in various species, e. g. as trematode, in *Fasiola hepatica* by Weinland and Brand (1926) and Mansour (1959); as cestode, in *Echinococcus granulosus* by Agosin (1957); as nematode, in *Ascaris lumbricoides* by Flury (1912), Wakabayashi (1942), Bueding and Yale (1951), Bueding (1952, 1953), Moyle and Boldwin (1952), Yoshizawa (1954), Entoner and Gonzalez (1959), Harpur and Waters (1960), Ellison *et al.* (1960), Ueno (1960 a) and Saz and Gerzon (1962), in *Trichinella spiralis* by von Brand *et al.* (1951), in *Gnathostoma spinigerum* by Ando (1957), in *Heterakis gallinae* by Glocklin and Fairbairn (1952), in *Metastrongylus elongatus* by Murakoshi and Niimura (1964), in *Litomosoides carinii* by Bueding (1949 b), and in *Haemonchus placei* by Dicken *et al.* (1960); and as acanthocephalan, in *Moniliformis dubius* by Laurie (1959). In the present study, formic, acetic, propionic, n-butyric, α-methylbutyric, n-valeric and n-caproic acids were detected in the adult *P. westermani*. Some acids of them are the substances associated with the scheme demonstrated by Saz and Weil (1960).

2. *Detection of Keto Acids and the Other Organic Acids:*

Tables 2 and 3 show keto acids and the other organic acids found in the uterine eggs, larval and adult lung flukes. In the samples of the uterine eggs, larval and adult lung flukes, two positive spots of DNPH derivatives as keto acids were detected by paper chromatography in comparison with the authentic samples. One of them was a spot corresponding in Rf value to α-ketoglutaric acid and the other spot to pyruvic acid (Table 2). On the other hand, in the uterine eggs and adult lung flukes, five positive spots corresponding in Rf value to citric, fumaric, lactic, malic and succinic acids were detected by paper chromatography in comparison with the authentic samples (Table 3). However, in the larvae, only lactic and trace of succinic acids were detected by paper chromato-

Table 2. Keto acids in *Paragonimus westermani* uterine eggs, *P. westermani* rediae and cercariae, *Paragonimus miyazakii* metacercariae and *P. westermani* adults

Spot	Acid	n-Butanol 5 / Ethanol 1 / Water 4					n-Butanol saturated with 3% Ammonia solution			Dimethylethylcarbinol 5 / Ethanol 1 / Water 4			
		Rf value	UE	RC	MC	A	Rf value	UE	A	Rf value	UE	MC	A
1	α-Ketoglutaric acid	0.11	+	+	+	+	0.07	+	+	0.10	+	+	+
2	Pyruvic acid	0.66	+	+	+	+	0.60	+	+	0.73	+	+	+

UE = Uterine eggs. RC = Rediae and cercariae. MC = Metacercariae. A = Adults. + = Positive.

Table 3. The other organic acids in *Paragonimus westermani* uterine eggs, *P. westermani* rediae and cercariae, *Paragonimus miyazakii* metacercariae and *P. westermani* adults

Spot	Acid	Ethanol 80 28% Ammonia solution 4 Water 16					Ether 13 Acetic acid 3 Water 1			Ether 5 Formic acid 2 Water 1			
		Rf value	UE	RC	MC	A	Rf value	UE	A	Rf value	UE	MC	A
1	Citric acid	0.10	+	−	−	+	0.30	+	+	0.45	+	−	+
2	Malic acid	0.19	+	−	−	+	0.36	+	+	0.52	+	−	+
3	Succinic acid	0.28	+	+	+	+	0.75	+	+	0.78	+	+	+
4	Fumaric acid	0.36	+	−	−	+	0.97	+	+	0.94	+	−	+
5	Lactic acid	0.46	+	+	+	+	0.71	+	+	0.82	+	+	+

− = Negative. Other abbreviations are the same as in Table 2.

graphy. And also analyses for lactic acid by procedure of Barker and Summerson (1941) were positive in the samples of the uterine eggs, larval and adult lung flukes, respectively.

It is well known that organic acids excluding volatile fatty acids have been detected in various species. Namely, lactic acid associated with the glycolysis has been detected in six species, i. e. as trematode, in *Schistosoma mansoni* by Bueding (1950) and *Clonorchis sinensis* by Read (1961); and as nematode, in *L. carinii* by Bueding (1949 b), *Dracunculus insignis* by Bueding and Oliver-Gonzales (1950), *Setaria digitata* by Hiwatashi (1958) and *Dirofilaria uniformis* by von Brand *et al.* (1963). More, lactic, pyruvic and succinic acids have been found in four species, i. e. as cestode, in *E. granulosus* by Agosin (1957), in *Hymenolepis diminuta* by Read (1961), and in *Moniezia benedeni* by Read (1961); and as nematode, in *H. gallinae* by Glocklin and Fairbairn (1952) and Fairbairn (1954). Moreover, lactic, pyruvic, oxaloacetic, malic, fumaric, succinic and α-ketoglutaric acids associated with the glycolysis and the latter half of the Krebs' cycle have been found in two species, i. e. as nematode, in *A. lumbricoides* by von Brand (1935), Yoshizawa (1954), Ueno (1960 b) and Ueno *et al.* (1960); and as acanthocephalan, in *M. dubius* by Graff (1964, 1965). Similarly, in the present study some of the above mentioned organic acids were detected in the larvae of the lung flukes. Furthermore, lactic, pyruvic, citric, α-ketoglutaric, succinic, fumaric and malic acids associated with the glycolysis and the Krebs' cycle have been found in two species, i. e. as trematode, in *F. hepatica* by Mansour (1959) and Bryant and Williams (1962); and as nematode, in *H. placei* by Dicken *et al.* (1960). Likewise, in the present study these organic acids were detected in the uterine eggs and adults of the lung fluke.

Summary

The organic acids in the uterine eggs, larvae and adults of the lung flukes of the genus *Paragonimus* were detected mainly by one-dimensional paper chromatography and gas chromatography. As volatile fatty acids, formic, acetic, propionic, n-butyric, α-methylbutyric, n-valeric and n-caproic acids were detected in the adults. On the other hand, as keto acids and the other organic acids, α-ketoglutaric, citric, fumaric, lactic, malic, pyruvic and succinic acids were detected in the uterine eggs and adults. However, in the larvae of the lung flukes, α-ketoglutaric, lactic, pyruvic and succinic acids were detected.

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肺吸虫の代謝に関する研究
III. 子宮卵，幼虫および成虫における有機酸

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一次元ペーパークロマトグムフ法およびガスクロマトグラフ法により，子宮卵，幼虫および成虫における有機酸の検出をおこなつた。その結果，揮発性脂肪酸として，成虫より，ギ酸，酢酸，プロピオン酸，ラク酸，α-メチルラク酸，バレリアン酸およびカプロン酸を検出した．一方，子宮卵および成虫より，α-ケトグルタール酸，クエン酸，フマール酸，乳酸，リンゴ酸，ピルビン酸およびコハク酸を検出した．さらに，幼虫からは，α-ケトグルタール酸，乳酸，ピルビン酸およびコハク酸のみを検出した．