SUCCINATE DEHYDROGENASE ACTIVITY IN UNEMBRYONATED EGGS, EMBRYONATED EGGS, MIRACIDIA AND METACERCARIAE OF SOME FLUKES

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The authors examined the activity of succinate dehydrogenase (SD) in *Fasciola hepatica* and *Paragonimus westermani* with the stages in development from cell division egg to metacercaria. As a result of this experiment, in the unicellular stages and miracidium stages of *F. hepatica* and *P. westermani*, a very slight SD activity was detected. However, in the miracidium formation stages of these flukes, the activity was very strong. Thus, it was clear that the reduction of methylene blue decreased suddenly from the highest value when metamorphosis occurred. It was suggested that respiratory metabolism rises with the progress of development and ability to decolorize methylene blue in creases. In the metacercaria stages of *F. hepatica*, *P. westermani*, *Metagonimus yokogawai*, the ability to decolorize methylene blue was persistent, but it was not so strong. During the time, so called hibernation time, when metacercaria invades into final host, metacercaria keeps metabolism of respiration as low as possible, and saves consumption of energy for maintenance of life. Metacercaria has biological function for parasitism.

Biological studies have been made on the development of the flukes as specimens. Accordingly, all the life history has been clarified in these flukes up to this time. Especially, morphological studies have been performed in detail on the forms of the asexual stage of flukes. At present, it is clear that the flukes take a process of development starting with egg through miracidium, sporocyst, redia, and cercaria to metacercaria. And succinate dehydrogenase

has been already found in some flukes (Huang and Chu, 1962; Bryant and Williams, 1962; Barry *et al.*, 1968; Hamajima, 1972, 1973).

MATERIALS AND METHODS

1) Liver fluke eggs

Bile samples were harvested from the gallbladder of a cow infected with *Fasciola hepatica*. Eggs were obtained from them by the sedimentation method.

II) Lung fluke eggs

Metacercariae of *Paragonimus westermani* were isolated from *Eriocheir japonicus*, a kind of fresh-water crab, collected in Nagasaki Prefecture, and used to cause lung fluke infection in a dog. Adult worms were harvested from the lungs of the infected dog. Then fluke eggs were obtained from the uterus of these adult worms.

III) Cultivation and observation of eggs

Collected eggs were washed well, treated with 5% antiformin, and washed again thoroughly with water.

The state of development of these eggs was observed every 5 days during the period of incubation. Materials were collected at the time of observation.

IV) Metacercaria

Succinate dehydrogenase activity in the stage of metacercaria was compared with some species of flukes. For this

purpose, metacercariae of *Metagonimus yokogawai* were harvested from *Gnathopogon elongatus elongatus*, a kind of fresh-water fish, collected from the Tone River, and those of *Heterophyes heterophyes* were isolated from *Tribolodon hakonensis*, another kind of fresh-water fish, produced in Hida, Oita Prefecture.

V) Estimation of succinate dehydrogenase activity

The Thunberg tube was used to estimate succinate dehydrogenase (hereinafter referred to as SD) activity. The method of Tom and Wilson was applied to the measurement of ability to decolorize methylene blue.

The reaction mixture and Thunberg tube method is shown in Fig. 1. The substrate is sodium succinate. The test suspension is containing about 10^4 eggs and 1 % of worms prepared in each stage of development. Eggs were collected by centrifugation, placed in the

Main chamber	Test suspension (containing about 10^4 eggs and 1% larval worms)	0.5 ml
	Buffer solution (pH 9.0)	2.5 ml
Side chamber	1/50,000 M methylene blue	1.0 ml
	1/20 M sodium succinate (or distilled water as control)	1.0 ml
Exhaust	4 mg Hg, by vacuum pump for about 3 minutes	
Constant temperature	30°C Allow sample to stand at 30°C for 10 minutes in thermostat water-bath.	
Mixing	Place the content of the side chamber into the main chamber and mix well by stirring.	
Estimation	Estimate -log T value by a Hitachi photoelectrocolorimeter at 660 m μ immediately after and 3, 6, and 9 minutes after mixing; regard the value of distilled water as zero.	
Buffered solution	1/10 M KH ₂ PO ₄ , 1/20 M NaB ₄ O ₇ , pH 9.0	
Methylene blue	Prepare dilution from 1/5,000 M solution immediately before use.	

Fig. 1. Outline of the Thunberg tube method.

glass homogenizer with addition of buffer solution, pH 9.0, and triturated in cooled condition. Such amount of buffer solution was added to the resulting sample as to make the total amount of the resulting suspension 5 ml. Then, 0.5 ml of this suspension was put in the main chamber of the Thunberg tube as the test suspension. Again, 2.5 ml of buffer solution was added to the content of the main chamber. In the side arm were placed 1.0 ml of 1/50,000 M methylene blue and then 1.0 ml of 1/20 M sodium succinate. After grease was painted, the tube was set with such care not to mix the content of the main chamber with that of the side arm. Then ventilation was induced by the vacuum pump for about 3 minutes (at 4 mg Hg). The tube was allowed to stand in the thermostat at 30°C for 10 minutes, so that it might react and remain at a constant level of temperature. Then the content of the side arm was mixed with that of the main chamber. The intensity of decoloration of methylene blue was estimated by the photo-electrocolorimeter immediately after 3, 6, and 9 minutes after mixing of the contents.

RESULTS AND DISCUSSION

Experiment I. [Succinate dehydrogenase activity in unembryonated eggs, embryonated eggs, miracidia and metacercariae of liver fluke.]

It was not always possible to prepare homogeneous materials for measurement of the velocity of development of individual liver fluke eggs. Therefore, trials were repeated for this purpose. The results obtained are indicated in Table 1 and Fig. 2.

a) Unicellular stage

The extract obtained from 10⁴ eggs before cell division immediately after collection from the bile was examined

TABLE I
Ability to decolorize (liver fluke)

Stage	-Log T			
	0	3	6	9
Unicellular stage	0.578	0.550	0.541	0.541
Multicellular stage	0.553	0.531	0.525	0.496
Stage of miracidium formation (A)	0.593	0.553	0.516	0.490
Stage of miracidium formation (B)	0.660	0.603	0.590	0.580
Miracidium stage	0.563	0.547	0.547	0.544
Metacercaria stage	0.437	0.435	0.420	0.415

for SD activity. As a result, the activity was first 0.578, as expressed in terms of -log T, 0.55 after 3 minutes, 0.541 after 6 minutes, and 0.541 after 9 minutes. In short, changes in SD activity were very slight.

b) Multicellular stage

Eggs were incubated at 30 C for 5 days. A number of multicellular eggs were observed under the microscope. The SD activity of these eggs showed a mild increase in ability to decolorize over a period from 6 to 9 minutes, as compared with the same activity of unicellular eggs.

c) Stage of miracidium formation (A)

Some eggs presented the miracidium formation during an incubation period of 10 days when it was observed by microscopy. As shown in Fig. 2-C, the ability to decolorize of the SD activity was 0.593, as expressed in terms of -log T, immediately after mixing the two contents, 0.553, or 0.04 lower than the preceding value, after 3 minutes, and 0.553 after 6 minutes. In other words, the activity was not reduced, and decoloration taken place in the same inclination was still sharp after 9 minutes. The suspension retained only a very indistinct color of methylene blue after 2 hours.

Fig. II Ability to decolorize (liver fluke)

(a) : Unicellular stage	(b) : Multicellular stage
(c) : Stage of miracidium formation (A)	(d) : Stage of miracidium formation (B)
(e) : Miracidium stage	(f) : Metacercaria stage

d) Stage of miracidium formation (B)

Some eggs were noticed to have formed miracidia. Eggs at 15 days of incubation are shown in Fig. II. Different from those at 10 days of incubation, they exhibited SD activity rapidly for the first 3 minutes, a little reduced activity after that, and a sudden dec-

rease in this activity over a period from 3 to 9 minutes.

e) Miracidium stage

A miracidium formed in the egg needs photo-energy to leave the egg. Therefore, the eggs were taken out of the incubator and exposed to light in order to collect miracidia. After some time, a number of miracidia could be recog-

nized by the naked eye. When the cultures was confined to contain miracidia released from eggs by microscopy, it was allowed to freeze rapidly in a freezing mixture composed of solid carbon dioxide and acetone, so that the miracidia might stop their movement. Then the frozen culture was thawed and centrifuged. The resulting material was used for the measurement. As shown in Figs. c and d, SD activity was very strong in eggs forming a miracidium. It was, however, extremely low in the miracidia formed, as shown in Fig. e.

f) Metacercaria stage

Cercariae released from *Lymnaea* snails were allowed to be settled on a sheet of cellophane, and the resultant metacercariae used as materials. Like miracidia, the metacercariae exhibited an ability hardly changeable to decolorize methylene blue. This ability, as expressed in terms of $-\log T$, was recorded to be 0.437 - 0.435 - 0.42 - 0.415 with the lapse of time. In Fig. II, comparison is made on the ability expressed in terms of $-\log T$ to decolorize methylene blue measured sequentially at certain intervals over a period from the egg to the metacercaria of the liver fluke.

The results about the difference of ability to decolorize (SD activity) with

Fig. III Difference of ability to decolorize (liver fluke)

- (a) : Unicellular stage
- (b) : Multicellular stage
- (c) : Stage of miracidium formation (A)
- (d) : Stage of miracidium formation (B)
- (e) : Miracidium stage
- (f) : Metacercaria stage

the stage of development are indicated in Table II and Fig. III.

Experiment II. [Succinate dehydrogenase activity in unembryonated eggs, embryonated eggs, miracidia and metacercariae of lung fluke.]

The lung fluke shows essentially the same stages of development as the liver

TABLE II
Difference of ability to decolorize (liver fluke)

Stage	0-3	3-6	6-9	Ave.
Unicellular stage	0.028	0.009	0.000	0.012
Multicellular stage	0.022	0.006	0.029	0.019
Stage of miracidium formation (A)	0.040	0.037	0.026	0.034
Stage of miracidium formation (B)	0.057	0.013	0.010	0.027
Miracidium stage	0.016	0.000	0.003	0.006
Metacercaria stage	0.002	0.015	0.005	0.007

TABLE III
Ability to decolorize (lung fluke)

Stage	-Log T			
	0	3	6	9
Unicellular stage	0.535	0.505	0.497	0.497
Multicellular stage (A)	0.735	0.645	0.610	0.540
Multicellular stage (B)	0.650	0.620	0.590	0.570
Multicellular stage (C)	0.715	0.670	0.620	0.590
Multicellular stage (D)	0.540	0.520	0.470	0.480
Stage of miracidium formation	0.485	0.475	0.425	0.375
Miracidium stage	0.590	0.570	0.555	0.550
Metacercaria stage	0.500	0.460	0.445	0.435

Fig. IV Ability to decolorize
(lung fluke)

- (a') : Unicellular stage
- (b') : Multicellular stage (A)
- (c') : Multicellular stage (B)
- (d') : Multicellular stage (C)
- (e') : Multicellular stage (D)
- (f') : Stage of miracidium formation
- (g') : Miracidium stage
- (h') : Metacercaria stage

fluke, but it is not parasitic on the same site as the latter. Essentially the same experiment as the preceding one was performed on lung flukes and gave the results presented in Table III and Fig. IV. As is clear from this figure, it was confirmed that lung flukes had shown SD activity similar to that of liver flukes.

a') Unicellular stage

Eggs were collected directly from the uterus of lung flukes and used as samples of the unicellular stage. The results obtained from them are shown in Table III and Fig. IV a'. They showed perfectly the same tendency as those obtained from liver flukes of the unicellular stage.

b') Multicellular stage

Eggs were incubated at 30°C for 5 days. The results obtained from them are presented in Table III and Fig. IV b'. SD activity was shown very remarkably over a period beginning immediately after the mixing of both contents at lasting for 3 minutes. During this period, the activity expressed in $-\log T$ value was very strong, ranging from 0.735 to 0.645, with a difference of 0.09.

c') Multicellular stage (A)

Eggs were incubated for 10 days. The results obtained from them are shown in Table III and Fig. IV c'. The ability to decolorize of these eggs was similar to that of liver fluke eggs incubated for 10 days.

d') Multicellular stage (B)

Miracidia were formed, without exception, in the liver fluke eggs incubated for 15 days, but not in any of the lung fluke eggs incubated for 15 days. As shown in Fig. IV d', SD activity was considerably stronger in the multicellular stage of eggs (B) than in that of eggs (A).

e') Multicellular stage (C)

Miracidia, which were not observed in stage d', failed to appear in this

stage either when eggs were incubated for 20 days. As shown in Fig. IV e', the ability to decolorize, expressed to be $0.54 \rightarrow 0.52 \rightarrow 0.47 \rightarrow 0.46$. It decreased a little over a period from 6 to 9 minutes after the mixing of both contents. SD activity, however, was manifested distinctly.

f') Stage of miracidium formation

A large number of eggs forming miracidia were found in cultures on the 23rd day of incubation. As shown in Fig. IV f', SD activity was very strong. It was particularly strong over periods from 3 to 6 minutes and from 6 to 9 minutes. Therefore, SD activity was persistent.

g') Miracidium stage

Fig. IV g' indicates the result obtained from the miracidium stage. Materials for this stage were prepared by the same procedure as employed in the experiment on the liver fluke. As shown in Fig. IV g', eggs with formed miracidia exhibited the highest SD activity. Nevertheless, they presented such a remarkable decrease in ability to decolorize as liver fluke.

h') Metacercaria

Fig. IV h' shows the results obtained from metacercariae of the lung fluke collected from a species of small freshwater crabs as materials. The metacercariae of the lung fluke exhibited a little stronger SD activity than those of the liver fluke, and stronger SD activity than the eggs of unicellular stage, but they presented a low value of ability to decolorize. This ability was persistent, although it remained low.

The difference of ability to decolorize (SD activity) with the stages of development is indicated in Table IV and Fig. V.

TABLE IV
Difference of ability to decolorize
(lung fluke)

Stage	0-3	3-6	6-9	Ave.
Unicellular stage	0.030	0.008	0.000	0.013
Multicellular stage (A)	0.090	0.035	0.004	0.043
Multicellular stage (B)	0.030	0.030	0.020	0.027
Multicellular stage (C)	0.045	0.050	0.030	0.042
Multicellular stage (D)	0.020	0.050	0.010	0.027
Stage of miracidium formation	0.010	0.050	0.050	0.037
Miracidium stage	0.020	0.015	0.005	0.013
Metacercaria stage	0.040	0.015	0.010	0.022

Fig. V Difference of ability to decolorize
(lung fluke)

(a') : Unicellular stage
 (b') : Multicellular stage (A)
 (c') : Multicellular stage (B)
 (d') : Multicellular stage (C)
 (e') : Multicellular stage (D)
 (f') : Stage of miracidium formation
 (g') : Miracidium stage
 (h') : Metacercaria stage

TABLE V
Comparison of metacercaria stage
ability to decolorize of flukes

Stage	-Log T			
	0	3	6	9
<i>Fasciola hepatica</i>	0.437	0.435	0.420	0.415
<i>Paragonimus westermani</i>	0.500	0.460	0.445	0.435
<i>Metagonimus yokogawai</i>	0.575	0.575	0.560	0.540

Fig. VI Comparison of metacercaria stage
ability to decolorize of flukes.

(k) : *Fasciola hepatica*
 (l) : *Paragonimus westermani*
 (m) : *Metagonimus yokogawai*

Experiment III. [Succinate dehydrogenase activity in metacercariae of *Metagonimus yokogawai*]

It was observed that there was a decrease in SD activity when metamorphosis occurred to the liver and lung flukes in the course of development. Metacercariae of *Metagonimus yokogawai* were collected from scales of deers or *Leuciscus hakonensis*. The ability to decolorize methylene blue was measured in them and results obtained are indicated in Table V and Fig. VI. It was persistent, though low in value, in the metacercariae of both species.

Succinate dehydrogenase is important for oxidative metabolism. The activity is indicative of presence of tricarboxylic acid cycle and succinate oxidation. In this experiment, these results indicate that succinate dehydrogenase is present in unembryonated eggs, embryonated eggs, miracidia and metacercariae of these flukes as found in various life cycle stages of several trematode species (unembryonated eggs, embryonated eggs of *P. westermani*, Hamajima 1972, 1973; miracidia of *F. hepatica*, Bryant and Williams 1962; rediae and cercariae of *Paragonimus ohirai*, Hamajima 1973; metacercariae of *Paragonimus miyazaki*, Hamajima, 1972, 1973; adults of *F. hepatica*, Barry *et al.* 1968 and Bryant and Williams 1962; adults of *Schistosoma mansoni*, Huang and Chu 1962; adults of *P. westermani*, Hamajima 1973). In addition to the above mentioned, Bruce *et al.* (1969) have shown a result of the experiment, suggesting presence of the dehydrogenase in cercariae of *S. mansoni*. Furthermore, in this experiment of *F. hepatica* and *P. westermani*, the rate of methylene blue reduction was higher in the miracidium formation stage eggs than in the unicellular stage eggs, miracidia and metacercariae. On

the other hand, a lack of cytochrome oxidase activity was reported for *Paragonimus* unembryonated eggs (Hamajima 1973), and similar results were obtained from low O₂ uptake and utilization of substrates in the homogenates of unembryonated eggs, miracidia, rediae and cercariae, and metacercariae of *Paragonimus* (Hamajima 1972). And also, metacercariae of *Paragonimus* resisted to cyanide inhibition (Hamajima 1973). Thus these results indicate that the physiological role of succinate oxidation is minor important in metacercaria stage. Moreover, it is suggested that the energy metabolism probably proceeds to the direction of the formation of pyruvate, phosphoenol pyruvate and succinate by malic enzyme, phosphoenol pyruvate carboxylase and fumarate reductase. On the contrary, the result obtained in this experiment for succinate dehydrogenase seems to indicate that the physiological role of succinate oxidation is more important to the miracidium formation stage eggs than unicellular stage eggs, miracidia and metacercariae because of the development and differentiation required large amount of energy. The activity in metacercaria of lung fluke was strong as compared with other flukes. This will be caused that metacercaria of lung fluke lives in *Eriochaer japonicus*.

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