[Jap. J. Parasit., Vol. 24, No. 5, 318-331, 1975]

Schistosome Miracidial Immobilization Caused by Tissue Extracts Prepared from Various Species or Strains of Snails*

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(Received for publication August 1, 1975)

There is little information about miracidial immobilization caused by tissue extracts of snails to schistosome miracidia. Benex and Lamy (1959) observed that tissue extracts from the snail *Planorbis* (*Planorbarius*) *corneus*, were capable of immobilizing *Schistosoma mansoni* miracidia and suggested that snails which are refractory to schistosome infection may possess immobilizing substances. On the other hand, Michelson (1964) reported that substances which immobilize *S. mansoni* miracidia were observed in the tissue extracts of *Biomphalaria glabrata* infected with this trematode, whereas the extract from non-infected *B. glabrata* did not affect the miracidia. Extracts prepared from 11 strains of snails refractory to *S. mansoni* infection (with the exception of two in stances) failed to immobilize the miracidia. He mentioned that his data did not support the hypothesis proposed by Benex and Lamy of a universally distributed immobilizing substance which may account for the resistance of many snail species to *S. mansoni* infection. Michelson (1963) also demonstrated that immobilization of *S. mansoni* miracidia occurred in extracts from *B. glabrata* either infected with *Daubaylia potomaca* (cephalobid nematode) or inoculated with *S. mansoni* eggs.

As far as the authors know, no other report of miracidial immobilization by snail tissue extracts employing other schistosome miracidia is known. It is still not clear whether extracts from some species of snails possess immobilizing activity which immobilize miracidia or whether the extract which affects incompatible miracidia fails to immobilize compatible miracidia. In the present work, an attempt has been made to ascertain whether the miracidial immobilization takes place in tissue extracts from wider species or strains of snails, as well as to learn the nature of immobilizing substances relative to the host-parasite relationship in miracidia and molluscs. This paper deals with observations made on the immobilization of schistosome miracidia using tissue extracts prepared from various species or strains of snails and with experiments on some characteristics of immobilizing activity in snail tissues.

Materials and Methods

Snails used. Twenty-three species or strains of snails either refractory or susceptible to each schistosome species were employed. The locality of snails collected and the period of maintenance in the laboratory are listed in Table 1. Only uninfected adult size

* This investigation was supported by the World Health Organization.

Table 1 Snail species or strains used for the preparation of tissue extracts

Species or strains	Locality	Period of maintenance in laboratory
Oncomelania nosophora	Yamanashi, Japan	about 2 years
O. quadrasi	Philippines	about 3 years
O. formosana	Yueh-mei, Formosa	about 3 years
O. chiui	A-li-lao, Formosa	about 7 years
Pomatiopsis lapidaria	Michigan, U.S.A.	2 weeks-5 months
Biomphalaria glabrata M-line	Puerto Rico & Brazil	about 15 years
B. glabrata Puerto Rico	Puerto Rico	about 15 years
B. glabrata Brazil	Bahia, Brazil	about 7 years
B. glabrata St. Lucia	St. Lucia	about 1 year
B. pfeifferi	Lake Victoria, Tanzania	about 6 years
Bulinus truncatus	Egypt	about 6 years
B. globosus	Rodesia	about 17 years
B. guernei	Gambia	about 5 years
B. natalensis	Lake Sibaya, Zululand	about 3 years
Bulinus sp. Ethiopia	Markaka, Ethiopia	about 2 years
Indoplanorbis exustus	Bombay, India	about 5 years
Helisoma trivolvis	Michigan, U.S.A.	about 6 years
H. campanulatum	Michigan, U.S.A.	about 2 years
Lymnaea stagnalis	Michigan, U.S.A.	about 3 years
L. emarginata	Michigan, U.S.A.	about 3 years
Pseudosuccinea columella	Michigan, U.S.A.	about 2 years
Physa gyrina	Michigan, U.S.A.	4 months-1 year
Physa sp. Egypt	Cairo, Egypt	about 15 years

laboratory cultured snails (except *Pomatiopsis lapidaria*) were used.

Snail extracts. The procedures for the preparation of tissue extracts are essentially the same as described by Michelson (1964). Snail tissue extracts were prepared as follows: Snails were crushed gently and the shell was removed from the soft tissue. The whole soft tissue or particular tissues were dissected free from the whole tissue and washed by immersion in distilled water for a few seconds and blotted with filter paper. The pooled tissues from 3 to 20 snails were weighed on a balance to the nearest 1 mg. These were then ground in a glass homogenizer at about 1500 rpm for 5 minutes in 0.07 M NaCl solution or distilled water which was added to the final concentration, 5 % (w/v) unless otherwise indicated. After the homogenate was stored at 5°C for 18 to 20 hours, the sediment was removed by centrifugation (500 *g*) at 5°C for 15 minutes and the supernatant was used for the test. The snail extract was stored in a refrigerator at 5°C and always used within 4 days after preparation (except the test for low temperature).

Miracidia used. *Schistosoma japonicum* miracidia used in the test were hatched from eggs obtained from mice infected with the Japanese strain of *S. japonicum*. Either livers or intestines from infected mice were homogenized with 0.85 % NaCl solution for several minutes. The supernatant of the homogenate was removed by centrifugation and the sediment washed with saline 2 to 3 times. The sediment with about 5 ml saline was placed on the filter paper in a funnel. The saline was aspirated, leaving remnants containing eggs adhering on the moist filter

paper. The moist filter paper was cut in several pieces and put into a 250 ml Erlenmeyer flask. The flask was filled with conditioned water (aerated chlorine-free tap water) and exposed under a fluorescent lamp for miracidial hatching. The miracidia which accumulated at the top of the flask were used for the immobilization test *S. mansoni* miracidia were obtained from eggs in livers of mice infected with the Puerto Rican strain of *S. mansoni*. The eggs were recovered in the same manner as described above and the technique for obtaining miracidia was similar. *Schistosoma haematobium* miracidia were hatched from the eggs collected by macerating the intestine of the hamster infected with the Egyptian strain of *S. haematobium*. The procedure for hatching miracidia was the same as described in the case of *S. japonicum* miracidia. The miracidia of *Schistosomatium douthitti* were obtained from eggs in the tissue of intestines of mice infected with that trematode. The same procedure as described above was applied for hatching miracidia. *Gigantobilharzia huronensis* miracidia were hatched from eggs collected from feces or the intestine of canaries, using the same technique employed to obtain *S. japonicum* miracidia. In all cases, miracidia ware transferred from the upper part of the hatching flask to a small vessel and kept at room temperature for 30 minutes to 1 hour. During this conditioning period, some (about 25%) miracidia lost their ability to swim and showed an "immobilization-like response", after 30 minutes in the test system. Only actively moving miracidia which showed normal progressive movement were selected and removed from the vessel by pipetting them out under a stereoscopic microscope for the immobilization test.

Miracidial immobilization test. The following procedures were applied for the miracidial immobilization test. A suspension using 10 to 20 actively moving miracidia were placed in 0.1 ml conditioned water in a depression plate after which 0.1 ml of test media were added. The depression was covered with a glass slide and incubated at 26°C. The miracidia were observed under a stereoscopic microscope at 30 minutes, 1, 2 and 4 and/or 8 hours after incubation. The miracidial immobilization was thereby determined by observing the percentage of cessation of both progressive and rotating movement by the miracidia in the various extracts or test media used.

Results

A. *Basic experiments for miracidial immobilization test.*

1. Influence of various media on the movement of miracidia.

Michelson (1964) used aqueous snail extracts prepared with charcoalfiltered water for the miracidial immobilization test. In the preliminary experiment by the senior author, the mobility of *S. japonicum* miracidia was affected even in conditioned water. About 20% of the miracidia ceased their progressive and rotating movement after 1 hour, and approximately 30% after 2 hours. Obervation were therefore made on the influence of various concentrations of NaCl, *Lymnaea* physiological salt solution (Carriker, 1946) and other media on the movement of miracidia of the 4 species, *S. japonicum*, *S. mansoni*, *S. haematobium* and *S. douthitti* (Figures 1, 2, 3 and 4). High concentration of NaCl (0.14 M) generally immobilized miracidia completely or at least to a high degree within 2 to 4 hours of exposure. Miracidia were influenced to a lesser degree by low concentration of NaCl (0.035 M or 0.018 M) within 4-hour exposure. At the end of 8 hours, however, miracidia of all species were affected to a rather high extent by all media used. As to the response of miracidia to NaCl solution, *S. haematobium* miracidia showed higher sensitivity than other species in high concentration of the solution, whereas *S. masoni* miracidia seemed to be less sensitive than others. These results suggest that observations of miracidial immobilization should be made within 4 hours of exposure. Low concentration of

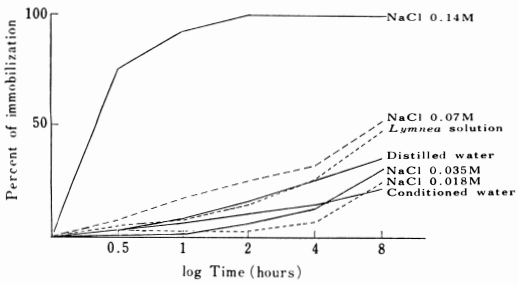

Fig. 1 Influence of various media on the immobilization of *S. japonicum* miracidia

Lymnea solution represents *Lymnaea* physiological salt solution (Carriker 1946) and Conditioned water represents aerated chlorine-free tap water.

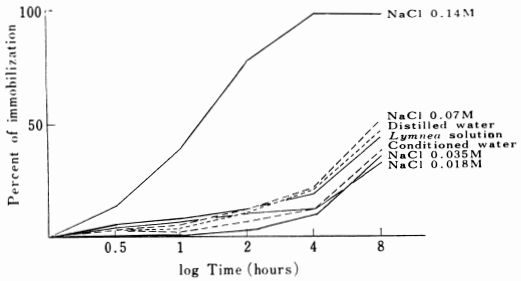

Fig. 2 Influence of various media on the immobilization of *S. mansoni* miracidia

NaCl may be useful as the medium for miracidial immobilization tests. In the present study, a 0.07 M NaCl solution which was diluted to a 0.035 M as a final concentration was used for preparing snail extracts.

2. Immobilization of miracidia by vairous dilutions of whole tissue extract.

In the course of this work observations were made on the immobilization of *S. japonicum* miracidia by the extract from whole tissues of *B. glabrata*, and a model titration test with 4 to 6 trials were done on several dilutions of the extract at various periods of exposure. In the majority of cases, the mean percentage of miracidial immobilization in each dilution of the extract increased with the time of exposure. There were no significnat differences, however, among the percent observed within the first 2 hours of

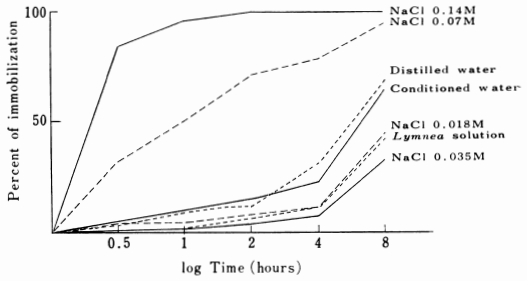

Fig. 3 Influence of various media on the immobilization of *S. haematobium* miracidia

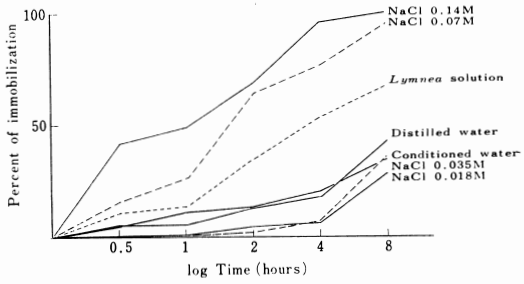

Fig. 4 Influence of various media on the immobilization of *S. douthitti* miracidia

exposure (Table 2). In low dilutions of the extract, percentages of immobilization were signi ficantly higher in 4-hour exposure than at other periods of exposure. Although the range of the percent of immobilization varies among the values observed in different dilutions, the ranges observed were slightly more narrow in 2 to 4-hour exposures as compared with those in other periods of observation. But in 4-hour exposure, miracidial movements were affected by distilled water or even by conditioned water to a rather high degree in some other experiments. It is suggested that a 2-hour exposure is suitable for testing miracidial immobilization when using snail tissue extracts. In the present work, the observations of the test were taken usually for 1, 2 and 4 hours; however, the result of 2-hour exposure is the most significant for obtaining comparative data.

3. Influence of tissue concentration and

Table 2 Immobilizing activity of whole tissue extract from *B. glabrata* to *S. japonicum* miracidia in various periods of observation

Dilution of extract*	No. of test	Mean percent miracidia immobilized and its confidence limits (95%)			
		30 min.	1 hr.	2 hrs.	4 hrs.
1 : 2	6	97.9± 1.6	100	100	100
1 : 4	6	81.5± 1.6	93.8± 2.4	98.4± 1.2	100
1 : 8	6	68.0± 7.2	72.5±10.0	88.4± 6.4	97.6± 1.6
1 : 16	6	53.4± 4.0	66.4± 3.2	78.2± 3.2	88.7± 5.2
1 : 32	6	42.5±10.0	50.0± 6.4	63.7± 6.4	81.3± 2.8
1 : 64	6	25.5±18.8	32.4±14.0	44.2± 9.2	66.2± 2.8
1 : 128	6	8.9±10.8	14.6±11.6	26.0±12.0	28.2±15.6
1 : 256	4	3.4± 5.1	5.2± 2.9	8.7± 2.2	13.8± 1.4
NaCl 0.035M control	6	1.5± 2.0	3.0± 2.0	9.9± 2.4	15.5± 3.2
Average standard deviation for 5–95% immobilization observed		11.4	9.3	8.6	8.8

* 5 % original extract was diluted with 0.035 M NaCl solution.

extraction media on the miracidial immobilization of whole tissue extracts.

Two different concentrations of extracts (5% and 10% w/v) prepared from whole tissue of *B. glabrata* were tested on the immobilization of *S. japonicum* miracidia. At the same time, saline (0.07 M NaCl) and distilled water were used as an extraction media for these concentrations of tissue extracts. In two or four replicates of four series of experiments, no significant difference between saline and distilled water extraction was observed on the immobilizing activity of two different concentrations of extracts. The activity of 10% extracts was slightly higher than that of 5% extracts in either saline or distilled water extraction. In the present study, however, 5% tissue extracts were usually used for the test, because observations of miracidial movement under a microscope were disturbed by turbidity of high concentration extracts such as 10% (w/v).

B. *Screening experiments with whole tissue extracts.*

1. Immobilization of human schistosome miracidia by whole tissue extracts from various species or strains of snails.

(1) Miracidia of *S. japonicum*: Whole tissue extracts prepared from 22 different species or strains of snalis belonging to 9 genera were used in the miracidial immobilization test. Repeated tests using 2 to 5 extracts from each species or strain of snail were made upon *S. japonicum* miracidia. As shown in Table 3, the extracts from 9 species or strains of snails representing 4 genera showed complete immobilization of the miracidia. In general, the extract from susceptible snails to *S. japonicum* infection failed to immobilize the miracidia to any great extent in comparison to the extracts from either refractory species or the snails which had not been tested to determine susceptibility to the trematode infection. The presence (more than 50%) of a miracidial immobilizing activity is suggested in snail tissue extracts from four species or strains of the genus *Biomphalaria*, four of five species of *Bulinus*, one species of *Indoplanorbis*, two species of *Helisoma*, one species of *Pseudosuccinea* and two species of *Physa*. A doubtful reaction (30–50%) or no immobilization occurred when miracidia

Table 3 Immobilization of miracidia of human schistosomes by whole tissue extracts prepared from various species or strains of snails

Tissue extracts* prepared from	Mean percent of immobilization produced in 2 hours by miracidia of		
	S. japonicum	*S. mansoni*	*S. haematobium*
Oncomelania nosophora	21.4(9-43)**†	11.9(7-15)	23.5(18-34)
Oncomelania quadrasi	42.1(29-65)**	24.4(10-26)	15.6(9-28)
O. formosana	19.5(12-29)**	17.8(15-21)	23.7(18-34)
O. chiui	37.2(21-53)**	11.4(9-13)	40.4(31-57)
Pomatiopsis lapidaria	26.8(25-29)**	12.5(11-15)	10.3(3-23)
Biomphalaria glabrata M-line	100 (100)	13.7(3-33)**	32.7(16-39)
B. glabrata Puerto Rico	100 (100)	11.1(9-21)**	—
B. glabrata St. Lucia	100 (100)	13.9(8-22)**	30.9(21-41)
B. pfeifferi	100 (100)	20.2(17-26)**	46.7(19-51)
Bulinus truncatus	46.9(43-51)	15.1(9-22)	15.6(13-22)**
B. guernei Gambia	67.4(52-78)	17.1(12-22)	27.3(14-29)**
B. globosus Ghana	99.3(99-100)	11.7(6-17)	29.9(11-47)**
B. natalensis	100 (100)	21.9(17-24)	33.3(20-46)**
Bulinus sp. Ethiopia	100 (100)	15.9(3-29)	17.9(11-32)**
Indoplanorbis exustus	100 (100)	14.5(5-17)	23.8(18-30)
Helisoma trivolvis	100 (100)	19.7(18-22)	59.2(56-62)
H. campanulatum	100 (100)	20.0(17-21)	30.8(20-41)
Lymnaea stagnalis	23.5(13-39)	6.0(5- 8)	13.8(9-18)
L. emarginata	56.9(30-68)	13.2(8-16)	28.6(20-34)
Pseudosuccinea columella	68.9(46-97)	22.5(20-25)	—
Physa gyrina	93.6(78-97)	12.1(6-24)	11.1(3-20)
Physa sp. Egypt	91.2(71-100)	7.7(6-12)	24.4(8-35)
NaCl solution control	14.1(5-30)	12.8(5-24)	24.0(6-41)

* 5 % original extracts were diluted 1 : 2.

** Figures indicate that each species or strain is susceptible to each species of miracidia used.

† Figures in parentheses indicate the range in percent of immobilization observed.

were placed in extracts from the species of *Oncomelania*, *Lymnaea* or *Pomatiopsis*.

(2) Miracidia of *S. mansoni*: Miracidia immobilization tests with *S. mansoni* miracidia were performed by using extracts from the same species or strains as used in the test on *S. japonicum* miracidia. All of the extracts from various species or strains of snails showed a low percentage of immobilization of *S. mansoni* miracidia in repeated tests with at least more than two extracts from each sanil (Table 3). It is indicated that the species or strains of snails employed in the experiment gave no evidence that miracidial immobilizing activity against *S. mansoni* miracidia was present.

(3) Miracidia of *S. haematobium*: Miracidial immobilization tests were repeated with 2 or 3 extracts from each of 20 different species or strains of snails on miracidia of *S. haematobium*. A rather low percentage of miracidia immobilization was observed in almost all the extracts used in the test (Table 3). Some cases produced as high or more than 50 percent of immobilization. However, it should be noted that the concurrent control using NaCl solution showed an influence on the miracidia movement to a rather high

Table 4 Immobilization of miracidia of other mammalian and bird schistosomes by whole tissue extracts prepared from various species or strains of snails

Tissue extracts* prepared from	Mean percent of immobilization produced in 2 hours by miracidia of	
	S. douthitti	*G. huronensis*
Oncomelania nosophora	11.6(4-21)†	24.0(16-31)
O. quadrasi	4.4(2-18)	39.4(35-42)
O. formosana	13.0(9-15)	23.7(18-34)
Pomatiopsis lapidaria	19.6(15-28)	15.1(7-23)
Biomphalaria glabrata M-line	100 (100)	50.0(46-63)
B. glabrata St. Lucia	100 (100)	—
B. pfeifferi	98.3(96-100)	100 (100)
Bulinus truncatus	10.3(7-13)	24.4(15-33)
B. guernei Gambia	45.0(30-58)	6.3(4- 9)
B. globosus Rodesia	94.7(89-100)	36.1(22-50)
B. natalensis	68.4(55-82)	—
Bulinus sp. Ethiopia	20.8(8-38)	48.6(35-69)
Indoplanorbis exustus	100 (100)	12.5(8-17)
Helisoma trivolvis	100 (100)	18.3(2-33)
H. campanulatum	97.5(83-100)	8.3(6-10)
Lymnaea stagnalis	14.3(11-15)**	66.0(50-81)
L. emarginata	17.8(10-25)**	78.1(57-100)
Pseudosuccinea columella	48.8(32-66)	6.3(5- 7)
Physa gyrina	65.4(49-82)	10.0(4-16)**
Physa sp. Egypt	60.0(48-72)	7.7(4-11)
NaCl solution control	18.9(3-31)	13.8(9-18)

* 5% original extracts were diluted 1 : 2.

** Figures indicate that each species or strain is susceptible to each species of miracidia used.

† Figures in parentheses indicate the range in percent of immobilization observed.

degree (about 40% in some cases). It seemed that all extracts used in this experiment failed to immobilize *S. haematobium* miracidia.

2. Immobilization of other mammalian and avian schistosome miracidia.

(1) Miracidia of *Schistosomatium douthitti*: Immobilization tests with *S. douthitti* miracidia were demonstrated on whole tissue extracts from 20 different species or strains of snails belonging to 9 genera. The tests were repeated with 2 to 4 extracts from each species or strain of snail. As shown in Table 4, complete or nearly complete immobilization of the miracidia was observed on the extract from 7 species or strains of snails. The extracts from two species of *Lymnaea* which were susceptible to *S. douthitti* infection and species of the family Amnicolidae showed a low percent of immobilization as compared with extracts from other genera of snails except some species of *Bulinus*.

(2) Miracidia of *Gigantobilharzia huronensis*: Extracts were prepared from 18 different species of snails and miracidial immobilization tests with *G. huronensis* were conducted. Only the extracts from 4 species of snails showed some possibility of immobilizing *G. huronensis* miracidia (Table 4).

C. *Miracidia immobilizing activity of extracts from tissue part of various snails.*

Table 5 Immobilizing activity of extracts prepared from various tissue parts of various species of snails to schistosome miracidia

Snails used	Extract* prepared from	Mean percent of immobilization produced in 2 hours by miracidia of		
		S. japonicum	*S. mansoni*	*S. haematobium*
B. glabrata M-line	Foot muscle	100(100)†	16(0-26)	23(22 - 32)
	Digestive gland	100(100)	100(100)	90(79-100)
	Ovotestis	98(96-100)	9(3-10)	17(14 - 19)
B. pfeifferi	Foot muscle	100(100)	21(0-30)	16(0 - 33)
	Digestive gland	100(100)	100(100)	100(100)
	Ovotestis	100(100)	8(7 - 9)	37(33 - 40)
Bulinus guernei	Foot muscle	73(69 - 76)	38(33-43)	14(14 - 16)
(Gambia strain)	Digestive gland	100(100)	100(100)	100(100)
	Ovotestis	100(100)	53(52-54)	19(10 - 28)
Bulinus. sp. Ethiopia	Foot muscle	100(100)	33(30-34)	—
	Digestive gland	100(100)	100(100)	—
	Ovotestis	100(100)	9(7-10)	—
Helisoma trivolvis	Foot muscle	100(100)	73(70-75)	41(33 - 50)
	Digestive gland	100(100)	44(36-50)	82(78 - 85)
	Ovotestis	100(100)	4(0 - 7)	30(27 - 33)
Lymnaea stagnalis	Foot muscle	69(64 - 73)	—	—
	Digestive gland	12(10 - 13)	—	—
	Ovotestis	13(11 - 15)	—	—
Oncomelania nosophora	Foot muscle	28(23 - 32)	15(7-21)	—
	Digestive gland	16(8 - 25)	14(7-21)	—
Distilled water control		14(11 - 18)	15(11-25)	18(12 - 27)

* 5 %original extracts were diluted 1 : 2.

† Figures in parentheses indicate the range in percent of immobilization observed.

1. Immobilizing activity using extracts from certain tissues from various species of snails to three species of schistosome miracidia.

In order to determine whether certain tissues were specific in causing miracidial immobilization, extracts were prepared from three tissues which were dissected from the animal. In this experiment, distilled water was used as an extraction medium.

(1) Miracidia of *S. japonicum* : The extracts were prepared from three tissues ; foot muscle, digestive gland (liver) and ovotestis (includes some part of seminal vesicle and hermaphroditic duct) of 7 species of snails. In the case of *O. nosophora*, the gonad extract could not be used because of the minute size of this organ. Miracidial immobilization tests were run with two trials using three or more extracts from each species of snail to *S. japonicum* miracidia. As shown in Table 5, two exceptions (*L. stagnalis* and *O. nosophora*), the extract from three tissues of all species tested showed complete or a high percentage of immobilization.

(2) Miracidia of *S. mansoni* : Tissue extracts, as mentioned above, were prepared from 6 species of snails and the tests were repeated with two trials in 3 extracts from each species to *S. mansoni* miracidia. Complete miracidial immobilizations were observed only from the digestive gland extract of 4 species of snails, *B. glabrata*,

Table 6 Immobilizing activity of tissue extracts prepared from various tissue of various species of snails to *S. japonicum* miracidia

Species of snails used	Extracts prepared from	Mean score of miracidia immobilization in 2 hours							
		Dilution of extracts*							
		1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
B. glabrata (M-line)	Foot muscle	++++	++++	++++	++++	++++	++++	+++	−
	Digestive gland	++++	++++	+++	+++	±	−	−	
	Ovotestis	++++	+++	±	±	−	−	−	
B. pfeifferi	Foot muscle	++++	++++	++++	++++	++++	+++	−	−
	Digestive gland	++++	++++	++++	±	−	−	−	
	Ovotestis	++++	++++	±	−	−	−		
Bulinus sp. (Ethiopia)	Foot muscle	++++	++++	++++	++++	++++	+++	−	−
	Digestive gland	++++	++++	++++	++++	±	−	−	
	Ovotestis	++++	++++	±	−	−	−		
NaCl 0.035 M control		− (3—13%)							

* 5 % original extracts were diluted with 0.035 M NaCl solution.

B. pfeifferi, *Bulinus guernei* and *Bulinus* sp. However, the extract from the digestive gland of *O. nosophora* and *Helisoma trivolvis* showed only a rather low percent of immobilization. In other tissue extracts, the degree of immobilization varied from 4 to 73 %.

(3) Miracidia of *S. haematobium:* Extracts from three tissues of 4 species of anails were used in the test with *S. haematobium* miracidia. A higher percent of miracidial immobilization was produced by the extract from the digestive gland of all species of snails to the other tissue extracts.

2. Titration of tissue extracts on the immobilizing activity of *S. japonicum* miracidia.

The extracts were prepared from three tissues of *B. glabrata*, *B. pfeifferi* and *Bulinus* sp. and tested with a series of twofold dilution from each extract to *S. japonicum* miracidia. The results of four replicate trials in 2 extracts from each tissue were scored on the following scale for the convenience in comparing the data. The mean percent of immobilization which showed more than 25 % to 50 % was scored as "±" and was regarded as doubtful in causing miracidial immobilizing activity. A score of "++" was assigned when the mean percentage of immobilization observed was more than 50 % as possibly a positive reaction, because more than 50 % immobilization was significantly high as compared with that of concurrent control in all cases. When more than 75 % occurred as complete immobilization a score of "+++" was given. The results (Table 6) indicated that the extract from foot muscle showed the highest activity in immobilizing the miracidia in all cases. The extract from the digestive gland produced rather low titer of immobilization in comparison with that of foot muscle. The lowest titer was obtained from the extract of the ovotestis from all of the species of snails used.

D. *Some characteristics of immobilizing activity.*

1. Effect of high temperature:

(1) On the activity of whole tissue extracts: These were prepared from 4 species of snails and divided into three series. One was stored at 5°C until tested, while one of the other series was subjected to a water-bath temperature of 46°C for 30 minutes and the third was held at 56°C for 30 minutes. Miracidial immobilizing activity to *S. japonicum* miracidia was tested simultaneously with the extract from all series. The

Table 7 Effects of high temperature on the immobilizing activity of whole tissue extracts from various snails to *S. japonicum* miracida

Extracts* prepared from	No. of test	Mean percent miracidia immobilized in 2 hours		
		Extracts heated at		Non-heated control
		46°C for 30min.	56°C for 30 min.	
B. glabrata M-line	4	91.3(86-93)†	6.4(3-21)	100 (100)
B. pfeifferi	3	100 (100)	23.1(14-36)	100 (100)
Bulinus sp. Ethiopia	3	95.7(93-97)	16.7(8-31)	98.9(97-100)
H. trivolvis	4	88.5(66-100)	12.0(9-50)	100 (100)
NaCl 0.035 M control	5			12.1(6-36)

* 5 % original extracts were diluted 1 : 2.

† Figures in parentheses indicate the range in percent of immobilization observed.

Table 8 Effect of high temperature on the immobilizing activity of extracts from various tissue parts of *B. glabrata* to miracidia of *S. japonicum*, *S. mansoni* and *S. haematobium*

Extracts* prepared from	Treatment	Mean percent of miracidia immobilization produced in 2 hours by miracidia of		
		S. japonicum	*S. mansoni*	*S. haematobium*
Foot muscle	Heated at 56°C for 30m.	13.1(7-19)†	13.5(8-19)	19.2(16-22)
	Non-heated control	100 (100)	15.6(10-21)	22.2(18-32)
Digestive gland	Heated at 56°C for 30m.	100 (100)	100 (100)	100 (100)
	Non-heated control	100 (100)	100 (100)	93.1(89-100)
Ovotestis	Heated at 56°C for 30m.	9.9(3-17)	3.6(3- 4)	16.5(7-22)
	Non-heated control	98.4(97-100)	9.0(7-10)	18.5(14-33)
Whole tissue	Heated at 56°C for 30m.	7.7(5-14)	11.1(9-21)	21.4(15-28)
	Non-heated control	100 (100)	15.9(6-25)	21.3(13-36)
Distilled water control		9.5(7-12)	12.1(10-15)	15.0(10-27)

* 5 % original extracts were diluted 1 : 2.

† Figures in parentheses indicate the range in percent of immobilization obserued.

results of 3 or 4 replicate experiments indicated that no significant diminution in the activity of the extract subjected to a temperature of 46°C was observed, whereas the immobilizing activity of the extract was destroyed remarkably by heating to 56°C for 30 minutes in all cases (Table 7).

(2) On the activity of tissue extracts: Two series of an appropriate amount of extract from whole tissue and three isolated tissues (foot muscle, digestive gland and ovotestis) of *B. glabrata* M-line snails were prepared. One series was held for 30 minutes at 56°C and the other was kept in a refrigerator at 5°C until tested. Immobilizing activity of these series of extracts was observed on the miracidia of *S. japonicum*, *S. mansoni* and *S. haematobium*. Repeated tests with the three extracts of each series showed that heated extracts from all sources, except from the digestive gland, lost their immobilizing activity to *S. japonicum* miracidia, while heating at 56°C for 30 minutes did not alter the activity of the extract from digestive gland to any species of the miracidia used in the test (Table 8).

2. Effect of low temperature.

The extract from whole tissue of *B. glabrata*, *B. pfeifferi* and *Bulinus* sp. were stored at 5°C for two weeks, and immobilizing activity of the extract to *S. japonicum* miracidia was then tested. The results of this test showed no significant diminution in the activity of the extracts.

In another experiment, the extract prepared from foot muscle of *B. glabrata* and *B. pfeifferi* were stored at 5°C for 7 days and −20°C for 10 days, respectively. The results of immobilization tests with three extracts to *S. japonicum* miracidia indicated no significant difference in the activity of the extracts as between the before and after storage.

3. Effect of dialysis on the immobilizing activity of foot muscle extract.

The extract from foot muscle of *B. glabrata* was dialyzed through a Visking tube against distilled water at 5°C for about 17 hours. This sample and non-dialyzed extract were tested to determine the immobilizing activity of *S. japonicum* miracidia. No loss in titer of miracidial immobilizing activity by dialysis was noted.

Discussion

In these screening experiments, it appears that the response of miracidia of *S. mansoni* and *S. haematobium* to snail tissue extracts is substantially different from that of the miracidia of *S. japonicum* and *S. duthitti*. The miracidia of *S. japonicum* and *S. douthitti* are highly responsive to the immobilizing activity of snail tissues, whereas the miracidia of *S. mansoni* and *S. haematobium* are not. Furthermore, even among the highly responsive miracidia, the degree of response to the same exract is different, depending upon the species of miracidia. It can be said that the miracidia immobilization in snail tissue extract appears principally dependent on physiologic characteristics of the species of miracidia. Benex and Lamy (1959) suggested that snail species found refractory to *S. mansoni* infection may possess immobilizing substances. But Michelson (1964) mentioned that his results did not support the hypothesis proposed by Benex and Lamy of a universally distributed immobilizing substance, which may account for the resistance of many snail species to *S. mansoni* infection. The results obtained in the present work concerning *S. mansoni* miracidia support Michelson's view.

The presence of miracidial immobilizing activity in snail tissue extracts is suggested in 29 different combinations with certain miracidia and many species or strains of snails. As shown in Tables 3 and 4, 22 extracts from 22 different species or strains of snails were used to test immobilization of *S. japonicum* miracidia. *S. douthitti* miracidia were exposed to 20 different extracts, whereas 18 extracts were employed using *G. huronensis* miracidia. Of these 60 combinations, 29 were found to have some degree of miracidial immobilizing. Of the 29 groups, 14 groups produced complete immobilization of certain miracidia, while the others ranged from 50 to 98 per cent immobilization. Although occurrence of miracidial immobilizing activity in snail tissues vary according to the species of miracidia, the activity is distributed in a wide range depending on the species of snail used. In general, certain related species of snails belonging to the Planorbinae and Bulininae exhibit a high degree of immobilizing activity. Lesser degree of the activity might be produced by some species belonging to the families Lymnaeidae and Physidae, while those belonging to the Amnicolidae are not capable of immobilizing any species of miracidia to a high extent.

It is of interest that the extract from *L. emarginata* (also called *L. catascopium*) immobilizes miracidia of *S. japonicum* and *G. huronensis* to a great extent, however, the extract does not immobilize *S. douthitti* miracidia which uses *L. emarginata* as its intermediate host. Another similar example is that the extract from *Physa gyrina* (the intermediate host of *G. huronensis*) is capable of immobilizing the miracidia of *S. japonicum* and *S. douthitti* to a high degree, whereas the extract fails to immobilize *G. huronensis*

miracidia. In all cases, the combinations with certain miracidia and susceptible snails to its trematode infection show a rather low immobilization. It is suggested that the immobilizing activity present in some snail tissues may act, in part, because of the incompatibility between miracidia and snails.

The immobilizing activity of the extract from three separate tissues, i.e., foot, digestive gland and gonad, of various snails to *S. japonicum* miracidia show similar results to those using the extract prepared from the entire soft parts of the snail. In contrast, whole tissue extracts from various snails showed no activity in immobilizing the miracidia of *S. mansoni* and *S. haematobium*, while the extract from the digestive gland of various snails immobilized these species of miracidia to a greater degree.

It is of considerable interest that the immobilizing activity in the extract from foot muscle, ovotestis and whole soft tissue are destroyed by heating at 56°C for 30 minutes, whereas the activity in the digestive gland is not altered by heating. This may suggest that at least two different kinds of immobilizing substances are present in the tissue extracts from snails. One has "heat labile activity" which is found in whole soft tissue, and especially the activity which is high in foot muscle, and the substance which remains inside the Visking tube in dialysis. The other has "heat stable activity" which is present in the digestive gland of some snails. Michelson (1963, 1964) found that miracidia immobilizing substances which immobilized *S. mansoni* miracidia appeared in the tissue extract from *B. glabrata* infected with this trematode and the substances were heat stable. He also observed similar immobilizing substances in the extract from *B. glabrata* either infected with *Daubaylia potomaca* or inoculated with *S. mansoni* eggs. It is not known whether the immobilizing activity is produced by the heat stable substance observed in the present study is similar to Michelson's substances or completely different from them. It should be noted, however, that the snails used in the present experiment were non-infected with any trematode or nematode and the immobilizing activity is found in the extract from the digestive glatnd of both *Biomphalaria* and *Bulinus* snails. The possibility, therefore, cannot be excluded that two different kinds of heat stable substances immobilizing *S. mansoni* miracidia may be found in the tissue extract from *B. glabrata* either infected with some helminths or specimens which are free of infection.

It is of additional interest to note that when whole tissue extracts from snails are heated to 56°C for 30 minutes both the heat labile and heat stable substances which immobilize miracidia of schistosome species are destroyed. This fact suggests that heat stable activity from the digestive gland is blocked or neutralized by other tissues of the snails. If so, the immobilizing activity produced from whole tissue extract of many snail species might show mainly the characteristics of heat labile activity.

In any event it is not contingent that the miracidia are never immobilized by heat labile activity of the extract from compatible snails (specific host or susceptible snail). Further as to compatibility, heat stable immobilizing activity in the digestive gland of snails may be capable of immobilizing miracidia of compatible and incompatible species; however, heat labile activity may act on miracidia of incompatible species. Sudds (1960) observed that when *Trichobilharzia elvae* miracidia penetrate an abnormal host, the parasite died and began to degenerate within a few days without any indication of a host tissue reaction. The results indicate that there is some degree of host defense mechanism operating as a humoral factor in such incompatible hosts. Schistosome miracidial penetration to snail tissue could be found in the foot-head region or the foot muscle to a greater extent. It is of interest that the heat labile immobilizing activity of snail tissue is found in the foot muscle in incompatible snails to a great degree. It can be said that the miracidial immobilizing activity in snail tissues, especially heat labile

activity, may play some role in the host's defense mechanism belonging to the same category as an innate humoral reaction as mentioned by Cheng (1968). Further studies on an analysis of the miracidial immobilizing substances have been initiated.

Summary

Experiments were undertaken to ascertain whether miracidial immobilization of 5 schistosome species: *Schistosoma japonicum*, *Schistosoma mansoni*, *Schistosoma haematobium*, *Schistosomatium douthitti* and *Gigantobilharzia huronensis*, would occur in tissue extracts from various species or strains of snails. Some characteristics of immobilizing substances were also observed. The results obtained are summarized as follows: (1) The miracidial immobilization in snail tissue extracts appears principally dependent on physiological characteristics of the species of miracidia. (2) The presence of miracidial immobilizing activity in snail tissue extracts is suggested in 29 different combinations with certain miracidia and many species or strains of snails. (3) The activity is distributed in a wide range depending on the species of snails used. In general, certain related species of snails belonging to the Planorbinae and Bulininae exhibit a high degree of immobilizing activity. A lesser degree of activity is produced by some species belonging to the families Lymnaeidae and Physidae, while those belonging to the Amnicolidae are not capable of immobilizing any species of miracidia to a high degree. (4) In all cases, the combinations with certain miracidia and the whole tissue extract fron susceptible snails to its trematode infection show a rather low immobilization. It is suggested that the immobilizing activity present in some snail tistues may act, in part, because of the incompatibility between miracidia and snails. (5) The extract from three separate tissues, i.e. foot, digestive gland and goand, of various snails to *S. japonicum* miracidia show similar results in appearnce to the extract prepared from entire soft parts of the snails. Whole tissue extracts from various snails show no activity in immobili zing the miracidia of *S. mansoni* and *S. haematobium*, while the extract from the digestive gland of certain snails immobilize these species of miracidia to a greater degree. (6) At least two different kinds of immobilizing substances are suggested in the tissue extracts from certain snails. One has "heat labile activity" which is found in whole soft tissus, and especially the high activity occurring in foot muscle. The other has "heat stable activity" which is present in the digestive gland of some snails. (7) The heat stable immobilizing activity in the digestive gland of snails may be capable of immobilizing miracidia of both compatible and incompatible species, however, heat labile activity may act on miracidia of incompatible species. (8) The storage of two weeks at 5°C does not alter the activity of the whole tissue extract from some of the *Biomphalaria* and *Bulinus* snails studied. The foot muscle extract stored at 5°C for 7 days or −20°C for 10 days indicates no significant difference in the activity as between before and after storage. (9) No loss in the activity of the foot muscle extract from *B. glabrata* by dialysis with the Visking tube was noted.

Acknowledgments

The work reported here was mostly done in the Mollusk Division in the Museum of Zoology, the University of Michigan. The authors wish to express their thanks to the staff of the Mollusk Division for their cordial help. Thanks are also due to Dr. Tatsushi Ishizaki, former Director of the Department of Parasitology, National Institute of Health, Tokyo, for his valuable advice and cordial help.

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各種貝類組織抽出液中でおこる住血吸虫類ミラシジウムの運動阻止について

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各種貝類組織抽出液中でおこる5種住血吸虫（日本住血吸虫，マンソン住血吸虫，ビルハルツ住血吸虫，Schistosomatium douthitti および Gigantobilharzia huronensis）のミラシジウムの運動阻止について試験した．また運動阻止物質の特性の一部についても観察し，以下のような結果を得た．

1) 貝類組織抽出液中でおこるミラシジウムの運動阻止は，第1には吸虫の種に特有な生理的特性に支配され，それぞれの種により異ることがわかつた．

2) 貝類組織抽出液中のミラシジウム運動阻止活性は，貝の種又は株と各種ミラシジウムとの間の29組にもおよぶ組合せにおいて証明された．

3) 貝類組織中に存在する運動阻止活性を貝の分類学上よりみると，その存在分布範囲は非常に広いことがわかつた．

4) 或種のミラシジウムに対し感受性を示す貝の組織抽出液中では，そのミラシジウムの運動阻止はおこらない．このことより貝組織中に存在するミラシジウム運動阻止活性が，貝とミラシジウムとの適合性に関係するらしいことを示唆される．

5) 貝の軟体の部分別（足部，中腸腺および生殖腺）の組織抽出液は，全軟体部のそれと同様に，日本住血吸虫ミラシジウムに対しては運動阻止をおこさせる．ところが足部および生殖腺の組織抽出液は，マンソン住血吸虫およびビルハルツ住血吸虫ミラシジウムに運動阻止をおこさせないが，中腸腺の抽出液は，これらいずれのミラシジウムにも高率の運動阻止をおこさせることがわかつた．

6) 少くとも2種の異つた運動阻止物質が存在し，1つは易熱性であり，全軟体部に分布するが，とくに足部において活性が高い．他の1つは耐熱性で，とくに貝の中腸腺にのみその存在が示唆された．

7) 中腸腺に存在し耐熱性である物質は，貝とミラシジウムの適合性に関係なく，易熱性である物質は，適合性を示さない貝とミラシジウムとの組合せにおいて活性を示すようである．

8) *Biomphalaria* と *Bulinus* の全軟体組織抽出液および同貝の足部抽出液は，5°C 以下の低温で保存すれば，比較的長時間にわたり運動阻止活性に変化がおこらない．

9) *B. glabrata* の足部組織抽出液は，透析してもその運動阻止活性に変化がないことがわかつた．