Infection of *Australorbis glabratus* with *Schistosoma mansoni* under Bacteriologically Sterile Conditions.* (26088)

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Australorbis glabratus, major snail vector of human schistosomiasis in the Western Hemisphere, can be secured free from demonstrable bacteriological contaminants(1) and grown axenically for protracted periods *in vitro*(2). Although axenic snails appear normal grossly and histologically, it seemed important to determine their susceptibility to infection with *Schistosoma mansoni* and their capacity to sustain complete larval development of this parasite.

Materials and methods. 1. *Strains of A. glabratus.* Four Puerto Rican (PR) strains provided eggs: PR-2, maintained in our laboratory since 1957(2); PR-2A and PR-2B, derived from the same Puerto Rican colony as PR-2 but kept in this laboratory since Aug. 1959; PR-3, established in Aug., 1959, from wild caught snails from Padin's Pond, San Juan. Stock colonies of PR-2 and PR-2A snails were maintained as described(2); colonies of PR-2B and PR-3 were maintained in a similar manner except that aquaria contained commercial potting soil (150 g/10 liters) in addition to marble chips, and tap water passed through a charcoal column instead of distilled water. 2. *Technics for axenic cultivation.* Mature snail eggs were dissected from egg-masses, disinfected, placed in individual screw-cap tubes and permitted 3-4 days to hatch in a balanced salt solution containing penicillin G., streptomycin sulfate, $NaHCO_3$, and phenol red(1). This fluid was then replaced with 5 ml/tube of filtered, autoclaved (15 lb/30 min) aquarium water to which all of the foregoing were added except streptomycin(2,3). This is referred to as maintenance fluid. All snails were fed a diet of autoclaved brewer's yeast and formalin-killed *Escherichia coli*(2). Snails which reached or exceeded 2 mm in diameter after the first feeding were refed after transfer (usually at 14-25 days) to individual 160 ml screwcap bottles containing 25 ml of fresh maintenance fluid. At weekly intervals thereafter fluid was replaced, and snails refed. When snails reached 5-6 mm in diameter, allotments of maintenance fluid and food per snail per week were doubled for the balance of experiments. All snails were kept in incubators at 27-28°C and were measured weekly by ocular micrometer. Sterility tests were done prior to each feeding by inoculating aliquots of fluid and fecal debris from each snail into tubes of liquid thioglycollate medium which were incubated at 28-32°C for one week and at room temperature for another week. These tests were supplemented periodically by parallel use of nutrient broth and blood agar media. 3. *Securing bacteriologically sterile miracidia of S. mansoni.* Webster strain mice, each infected intraperitoneally with 100 cercariae of a Puerto Rican strain of *S. mansoni,* were supplied by Dr. Henry van der Schalie, Univ. of Michigan. Livers were removed aseptically from 3-6 mice infected 8-10 weeks previously, pooled, and processed as described by Lee and Lewert(4) with the following modifications: a) instruments, glassware, and solutions were sterile; b) miracidia were hatched under light in volumetric flasks containing sterile aquarium water and 100 units/ml penicillin. Miracidia were transferred *en masse,* within 30 minutes of hatch, to a small Petri dish and picked out by Pasteur pipette under low magnification. Aliquots of miracidial suspension in each experiment were inoculated into tubes of liquid thioglycollate medium and of nutrient broth; incubation for 2 weeks disclosed no contaminants. 4. *Exposure of axenic A. glabratus to miracidia.* Most snails were exposed to infection at 2-3 weeks of age (mean diameter 2.1-2.4 mm),

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just before transfer from tubes to bottles. Prior to exposure, maintenance fluid was removed from tubes and replaced by 3 ml of sterile aquarium water containing 100 units/ml penicillin (pH = 7-8). Counted miracidia were introduced in 1-2 drops into each tube by Pasteur pipette, caps were tightened, and tubes placed in an incubator at 27-28°C. The inner glass door of the incubator was closed, but the outer door was left open during exposure period to allow a constant low level of illumination from artificial lights of the room. Snails were removed from tubes after 18-20 hours, transferred to individual bottles, and maintained as described earlier. For exposure to infection of older snails (60-66 days, mean diameter 6.7-6.9 mm), maintenance fluid was discarded, 10 ml per bottle of sterile aquarium water containing 100 units/ml penicillin was substituted, and counted miracidia were added. Other exposure conditions were as described above. After exposure, maintenance fluid and food were substituted and all snails were maintained in dark incubators (27-28°C). Bottles were removed from incubators only for feeding, measuring, or examining snails. As indirect controls in each experiment, aquarium-reared snails (all strain PR-2), comparable in size to axenic snails, were individually exposed to infection in tubes or bottles under conditions identical to those outlined for axenic snails, including batch and number of miracidia. After exposure, control snails were kept in groups in aerated battery jars (26°C) supplied with watercress; ceiling-level fluorescent lights were kept on at all times since natural light was excluded from the room. 5. *Examination of snails for schistosome infection.* Snails *in vitro* were examined 8-10 days after exposure for presence of sporocysts using transmitted light, 45× magnification, and viewing each snail from its ventral aspect. Snails were examined for cercarial emergence every 1-2 days starting about 20 days post-exposure. Bottles were placed under a desk lamp for about 2 hours, then examined individually for cercariae using a 7× lens and right-angle, narrow beam, transillumination. Snails shedding cercariae were usually reexamined every 3-4 days thereafter to determine duration of cercarial production. In one experiment snails exposed to infection were fixed in Newcomer's solution in groups of 2-3 starting 24 hours after exposure, and at weekly intervals thereafter for 8 weeks, and stained 8 μ serial sections of each specimen were examined for parasite-development. 6. *Exposure of mice to cercariae from bacteriologically sterile A. glabratus.* Although cercariae were shed regularly and apparently survived well in the bicarbonate-containing maintenance fluid in stoppered bottles, newly-emerged cercariae became inactive or moribund within 2-3 hours after being decanted into open Petri dishes. This phenomenon appeared to be related to a rapid alkaline shift in pH. Accordingly, when infectiousness of cercariae for mice was to be tested, maintenance fluid was removed and replaced with 10 ml sterile aquarium water containing 100 units/ml penicillin, and the bottles then exposed to light. When a suitable density of cercariae had accumulated, the fluid from each bottle was decanted into a Petri dish from which counted cercariae were transferred by Pasteur pipette to 15 x 75 mm tubes. This procedure was done without sterility precautions since anaesthetized white mice (20 g) were immediately exposed to the cercariae for 1-2 hours by the tail immersion method(4). Mice were sacrificed and examined for adult worms and the presence of viable eggs about 8 weeks after exposure.

Experimental results. Data summarized in Table I show that infections in partly-grown *A. glabratus* were readily established under bacteriologically sterile conditions. Of 66 axenically-reared snails exposed to miracidia, sporocysts were visible in 53 individuals 8-10 days later, and 61/63 survivors (97%) shed cercariae by 33 days. Mortality during this period among infected, bacteriologically sterile, snails was low and comparable to that among uninfected axenic controls. On the other hand, mortality among schistosome-exposed aquarium-reared snails was much higher (67%) than among the bacteriologically sterile snails (<5%). Losses among the former generally occurred before infections reached maturity (Table I), and ranged

TABLE I. *Schistosoma mansoni* Infections in Bacteriologically Sterile and in Aquarium-Reared *Australorbis glabratus*.

		Axenic snails exposed				Post-exposure			
				Per snail			Day 25-27		Day 30-33	
Exp. No.	Strain	Age (days)	Mean diam. & range (mm)	No. miracidia	Vol (ml)	No. snails	Snails alive	Cum. No. shedding	Snails alive	Cum. No. shedding
HP-2	PR-2A	13	2.3 (2 -2.5)	5-10	3	13	13	10	13	13
HP-3a	PR-2B	14	2.1 (1.8-2.5)	10	3	20	20	19	18	18
-3b	PR-2	25	2.4 (1.8-2.6)	10	3	6	6	1	6	6
-3c	PR-2A	66	6.9 (6.6-7.2)	15-20	10	5	5	2	4	3
-3d	PR-2A	60	6.7 (6.2-7.0)	15-20	10	9	9	6	9	8
HP-4	PR-3	17	2.2 (2 -2.6)	10	3	13	13	4	13	13
					Totals	66†	66	42	63‡	61
					%	—	100	64	95	97
	Aquarium-reared snails exposed*				Totals	48	25	9	16	13
					%	—	52	30	33	81

* All strain PR-2.
† 53/66 had 1-3 visible sporocysts per snail 8-10 days after exposure.
‡ Loss of 3 snails accounted for by 1 death and 2 contaminated.

from 37% to 93% in the several groups exposed.

Comparison of growth rates for infected snails and uninfected controls, maintained under identical bacteriologically sterile conditions, was made in experiments HP-2, HP-3a, and HP-4. These data disclosed that infected snails consistently grew at a more rapid rate than controls, and that these differences regularly became apparent within the first week after exposure. Representative data from experiment HP-3a are shown in Table II.

The course of development of *S. mansoni* in bacteriologically sterile *A. glabratus* appeared normal as judged by tissue sections (Fig. 1), and the cercariae produced *in vitro* were indistinguishable in appearance and behavior from those shed by aquarium-reared snails. Direct counts of cercariae done on the entire contents of a few bottles disclosed that as many as 500-1000 cercariae emerged from individual snails during 2 hour periods at peak of cercarial production.

Bacteriologically sterile cercariae proved to be infectious. Seven mice, each exposed to 100-150 cercariae derived from several snails in Exp. HP-2, were found to have mature schistosomes 8 weeks later. The presence of adult worms was confirmed by direct observation of portal and mesenteric blood vessels or by perfusion of the liver. Gross mottling and enlargement of the liver was evident in all mice, large numbers of eggs were seen in press preparations examined microscopically, and viability of these eggs was confirmed by hatching miracidia from ground livers. The entire cycle was then repeated by recovering these miracidia under sterile conditions and successfully infecting 7 8 axenically-reared *A. glabratus*.

The first emergence of cercariae from the bacteriologically sterile snails in HP-2, 3, and 4 usually occurred 25-30 days after exposure, although in a few instances cercariae were first seen as early as the 22nd day or as late as the 33rd day. However, in one experiment in which newly-hatched, previously unfed, axenic *A. glabratus* were exposed to infection with *S. mansoni* somewhat different results were obtained. Fourteen such snails (1-4 days old, .65-.85 mm diam., strain PR-3) were each exposed to 10 miracidia in 3 ml under described conditions of exposure and subsequent sterile maintenance. Only 6/14 (43%) became infected; 3/6 first shed cercariae 24-31 days after exposure, but cercarial emergence from the remaining 3 did not occur until 42-49 days after exposure.

TABLE II. Mean Shell Diameter (mm) of Schistosome-Infected and Uninfected *A. glabratus* Maintained under Sterile Conditions (Exp. HP-3a).

	Days post-exposure						
Snails	0	9	16	22	29	36	43
Infected*	2.1	4.2	5.1	5.5	5.8	6.0	6.2
Uninfected†	2.1	3.3	4.4	5.0	5.2	5.6	5.8

* N = 20 initially; 18 after day 29. † N = 14.

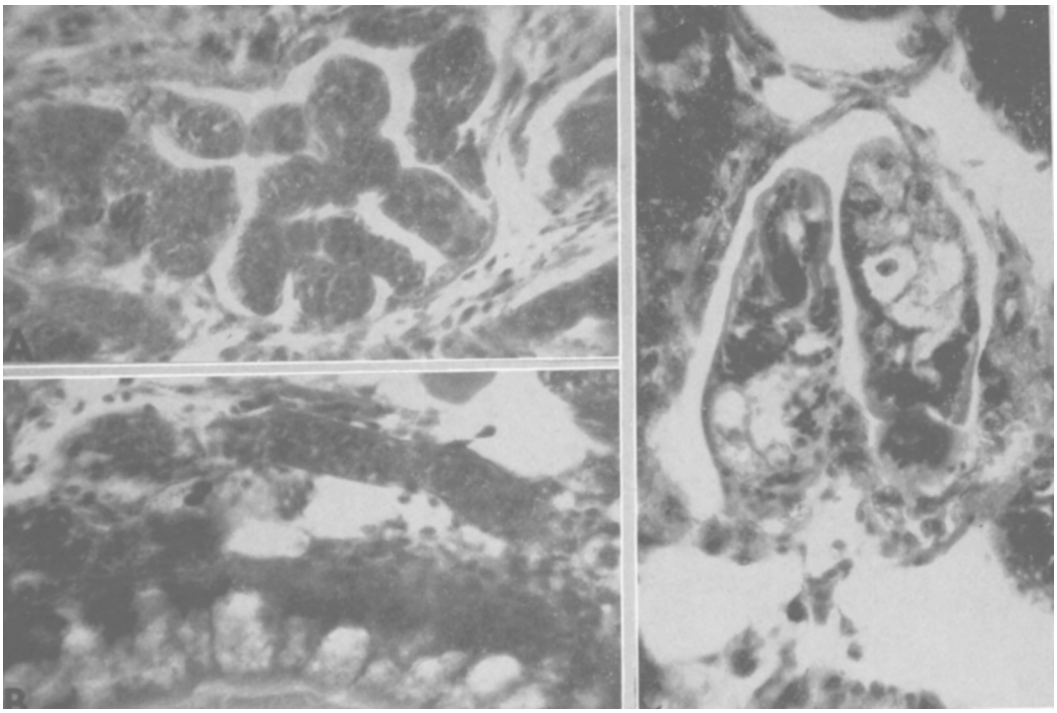

FIG. 1. Development of *S. mansoni* in bacteriologically sterile *A. glabratus* (Exp. HP-4). A. Mother sporocyst in foot 8 days post-infection. B. Daughter sporocyst in liver 15 days post-infection. C. Mature cercariae in liver 27 days post-infection. A and B stained with haematoxylin-eosin; C with haematoxylin-azure II-eosin. All sections ×425.

While a small proportion of bacteriologically sterile snails infected with *S. mansoni* shed cercariae for only 3-10 days, most produced cercariae for several weeks. About 15% produced cercariae for 50 days or more, and 2 individuals regularly liberated cercariae for 87 and 98 days. Some snails produced cercariae until they died, but in most instances several days or several weeks elapsed between last emergence and death of the snail host. When maintained under sterile conditions for extended periods following onset of cercarial shed, infected snails tended not to survive as long as uninfected controls.

Discussion. *A. glabratus* grown in the absence of demonstrable contaminants were readily infected with *S. mansoni* and served as suitable hosts for normal development of the extra-mammalian phase of the parasite life-cycle. The sequence of events from miracidial exposure to cercarial emergence was consistent with that reported in the literature for snails reared under conventional conditions. The unusually high mortality noted among infected aquarium-reared snails is unexplained but may have resulted from a) secondary microbial infections, b) differences in strain, nutritional status, or handling methods, or c) constant exposure to light. The latter possibility was explored experimentally, but no correlation was observed in mortality among infected snails kept in the light or in the dark.

The experimental demonstration that *S. mansoni* develops normally in snails kept in the dark may have epidemiological significance since the absence of light in covered irrigation conduits or similar situations would probably not inhibit parasite development in infected snails. It may also be noted in this context that, in the absence of information as to the significance of light in terms of successful miracidial penetration, low illumination was provided when snails were actually being exposed to miracidia. A preliminary

experiment with aquarium-reared snails has since shown, however, that penetration of miracidia into *A. glabratus* can occur in total darkness, conjectures to the contrary(5) notwithstanding.

It is of interest that schistosome-infected snails grew somewhat more rapidly than uninfected ones under sterile conditions. Parasitization by certain larval trematodes is said to cause excessive growth of some snails (6, for review), and it has been suggested that such snails apparently overcompensate by voracious feeding for nutrients supposedly withdrawn by the parasite(7). However, under the bacteriologically sterile conditions of the present study, parasitized and control snails were fed the same amount of food per week and little or no food remained unconsumed in either case. If the parasites were "withdrawing" food, parasitized snails might have been expected to be smaller than control snails, while in fact the converse was observed. It seems doubtful that "parasitic castration" could have been significant in this since most of the snails were small and sexually immature, and since the differences in growth become apparent within a week of infection.

Successful cultivation *in vitro* of schistosome-infected *A. glabratus* opens new areas for investigation, particularly insofar as the bacteriologically sterile nature of the system provides a source of uncontaminated larval stages, including cercariae, for possible use as antigens or for studies on cultivation *in vitro*.

Summary. *Australorbis glabratus,* reared axenically to 2-7 mm before exposure, were found to be highly susceptible to infection with *Schistosoma mansoni* in the absence of demonstrable bacteriological contamination. Larval development was normal in all observable respects, and cercariae infectious for mice were produced after normal periods of incubation. Maintenance of these snails in the dark for extended periods did not inhibit larval development. Mortality was very low among schistosome-infected snails maintained in the sterile state. Infected snails tended to grow more rapidly than uninfected snails when both were fed identical rations under controlled, bacteriologically sterile conditions.

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1. Chernin, E., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 204.
2. Chernin, E., Schork, A. R., *Am. J. Hyg.*, 1959, v69, 146.
3. Chernin, E., *ibid.*, 1957, v66, 321.
4. Lee, C. L., Lewert, R. M., *J. Inf. Dis.*, 1956, v99, 15.
5. Wright, C. A., *Bull. Soc. Path. Exot.*, 1956, v49, 1211.
6. Boettger, C. R., in *Verhandlungen der Deutschen Zoologischen Gesellschaft in Freiburg* 1952, 468, Geest and Portig, Leipzig.
7. von Brand, T., Files, V. S., *J. Parasit.*, 1947, v33, 476.

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Effects of Amethopterin on Cell Growth and Viral Synthesis.* (26089)

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Mammalian cells have been found not to require any purine or pyrimidine for growth in Eagle's medium(1,2), but the presence of some form of folic acid is obligatory(3). A variety of cultivated mammalian cells have been found unable to proliferate in growth media containing amethopterin (4-amino-N^{10}-methylpteroyl-glutamic acid) an anti-

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