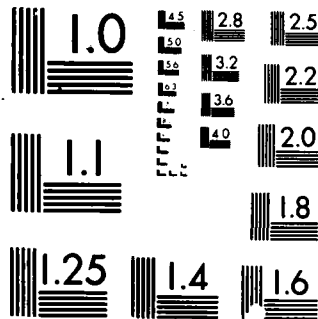


TESTING OF COMPOUNDS FOR EFFICACY AGAINST
SCHISTOSOMIASIS(U) LOWELL UNIV MA CENTER FOR TROPICAL
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Report Number 04-4267/1

"Testing of Compounds For Efficacy Against Schistosomiasis"

Annual Summary Report
John I. Bruce, Ph.D.

July 22, 1986

Supported By

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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Center for Tropical Diseases
University of Lowell
Lowell, Massachusetts 01854

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A solution of absolute ethanol containing varying concentrations of the test compound, Niclosamide, was applied to the abdomen of hamsters at either 2 or 7 days prior to exposure to cercariae of either <u>Schistosoma mansoni</u>, <u>S. haematobium</u> or <u>S. japonicum</u>. Treated animals were examined for presence or absence of schistosomes 49 days after exposure for <u>S. mansoni</u> and <u>S. japonicum</u> and 90 days for <u>S. haematobium</u>. Hamsters treated at 2 days prior to exposure to any of these schistosomes were completely protected against cercarial invasion of their skin. Only partial protection was found in animals treated at 7 days prior to cercarial exposure. Male and female worms were found in nearly all animals not completely protected. Complete protection was not obtained by the animals treated with serial dilutions of the undiluted test compound at either 2 or 7 days prior to cercarial exposure with the exception of Group B treated 2 days prior to <u>S. haematobium</u> cercarial exposure. Mature (Continued)					
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Niclosamide at a concentration of 1.0 gm in 100 ml of absolute ethanol is a candidate for advanced confirmatory animal studies which should be designed to determine its potential for human use.

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SUMMARY

A solution of absolute ethanol containing varying concentrations of the test compound, Niclosamide, was applied to the abdomen of hamsters at either 2 or 7 days prior to exposure to cercariae of either Schistosoma mansoni, S. haematobium or S. japonicum. Treated animals were examined for presence or absence of schistosomes 49 days after exposure for S. mansoni and S. japonicum and 90 days for S. haematobium. Hamsters treated at 2 days prior to exposure to either S. mansoni, S. haematobium or S. japonicum were completely protected against cercarial invasion of their skin. Only partial protection was found in animals treated at 7 days prior to cercarial exposure. Male and female worms were found in nearly all animals not completely protected.

Complete protection was not obtained by the animals treated with serial dilutions of the undiluted test compound at either 2 or 7 days prior to cercarial exposure with the exception of Group B treated 2 days prior to S. haematobium cercariae exposure. Mature male and female worms capable of producing schistosome eggs were found in nearly all animals of the groups treated with serial dilutions.

Niclosamide at a concentration of 1.0 gm in 100 ml of absolute ethanol when applied to hamster skin at 2 days prior to cercarial exposure is a candidate for advanced confirmatory animal studies which should be designed to determine its potential for human use.

FOREWORD

1. Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

2. In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).

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INTRODUCTION

The parasitic disease schistosomiasis is endemic in 74 countries in Africa, Asia, the Middle East, South America and islands of the Caribbean (1). There are approximately 200-300 million infected persons with nearly 600 million constantly at risk in endemic areas (1). The disease has shown remarkable and substantial increases due in part to the creation of new water resources such as dams, lakes and irrigation schemes needed to meet increased energy and food demands. In addition, the introduction of the new water resources has caused dramatic ecological changes to occur in many of the endemic areas for schistosomiasis, due in part to population movements, new irrigation practices and lack of public health funds to adequately address the emerging problems. Another recently occurring problem in the fight against schistosomiasis has been the emergence of drug-resistant strains of the parasite in Brazil (2,3) and more recently in Kenya (4). This emerging problem may compromise the success which has been made against the disease by use of chemotherapy.

An example of the spread of schistosomiasis into an area heretofore free of the disease is the country of Jordan. Up until 1984, Jordan was one of only a few countries in the Middle East free from both susceptible snails and the parasite. But during the past 10 years the threat of the disease becoming established has been growing due to the presence of the vector-snail intermediate host which has defied attempts by the Ministry of Health to prevent its spread by use of chemicals. Indigenous cases have now been discovered nine years after finding the snail intermediate host and eight years after the influx of foreign infected migrant workers (5,6) thus indicating active transmission.

From the military prospectus there are three stages of the schistosomiasis disease process in which medical casualties could be expected to occur: 1) skin penetration which is associated with penetration of the infective larva (cercariae), 2) Katayama fever, associated with the initial stages of egg deposition during the chronic phase of the disease and 3) chronic schistosomiasis, associated with granuloma formation in liver or urinary bladder after the third to fourth month of infection. Currently, there are no infective larva (cercariae) repellents, and neither prophylactic or suppressive drugs nor vaccines are available for use by the military of the United States. Even if it were possible to recognize early stages of the schistosome parasite in exposed personnel, there are no drugs available to treat early infections. Three drugs are currently available to treat mature infections in humans. These are praziquantel,

active against all species of schistosomes infective to man, oxamniquine, active against one species of schistosome infective to man, namely Schistosoma mansoni (most effective against new world S. mansoni), and metrifonate, active against urinary schistosomiasis, namely S. haematobium (7). Strains of S. mansoni resistant to oxamniquine (3,4) have been isolated from patients in Brazil and Kenya. It also appears that strains of S. haematobium resistant to metrifonate have emerged (8). This leaves only one antischistosomal agent, praziquantel, with minimal side effects for use in treatment of schistosomiasis at the present. Studies to determine if praziquantel is capable of causing drug resistant schistosomes to occur are in progress.

The lack of specific preventative measures for use by military personnel poses a significant potential problem for military operations which may occur in areas of strategic interest to the United States. Casualties have occurred during previous operations to British, French and American forces.

During the past several decades many compounds have been tested in an effort to find a formulation which would afford protection against invasion of the skin by Schistosoma sp. infective larva (cercariae). This subject has been amply reviewed by several authors (9,10,11,12).

Evaluation (13,14) of chemical preparations for their topical prophylactic antischistosomal activity is an ongoing program of the United States Medical Research and Development Command and whose overall goal is to develop a substance which will protect personnel unable to avoid water contact when operating in areas where schistosomiasis is endemic.

The objective of the program as supported at the Center for Tropical Disease, University of Lowell is to conduct secondary and definitive test evaluations of compounds showing superior antipenetration prophylactic efficacy against S. mansoni, S. japonicum and S. haematobium in rodents and/or primates. In addition, these evaluations may be made against other schistosome species and/or drug-resistant forms if requested. Curative and/or suppressive evaluation of compounds can also be carried out when requested.

During this funding year, studies were carried out to evaluate the efficacy of the chemical, Niclosamide as a topical antipenetrant against schistosome cercariae invasion of the skin.

A protocol for evaluation of other chemical formulations for their prophylactic efficacy has also been developed.

MATERIALS AND METHODS

I. Techniques for Cultivation and Maintenance of Snail Intermediate Host Species.

The procedures used for cultivating and maintaining the species and strains of schistosomes and their respective snail intermediate hosts are described and presented in Appendix I.

II. Schistosome Species.

The species of schistosomes used in this study were Schistosoma mansoni of Puerto Rican origin, S. haematobium of Egyptian origin and S. japonicum of Philippine origin. The snail intermediate host for S. mansoni is Biomphalaria glabrata from Puerto Rico, for S. haematobium the snail intermediate host is Bulinus truncatus truncatus from Egypt and for S. japonicum the snail intermediate host is Oncomelania quadrasi from the Philippine Islands.

III. Chemicals.

A. Experimental Compound.

The compound tested for its efficacy as an antipenetrant was Niclosamide. This compound is used as an anthelmintic which is active against most tapeworm species including the beef tapeworm (Taenia Saginata), the pork tapeworm (T. solium), the fish tapeworm (Diphylllobothrium latum) and the dwarf tapeworm (Hymenolepis nana) (15,16).

B. Test Compound Solvent or Vehicle.

Pharmaceutical grade absolute ethanol (ETOH) was used to dissolve Niclosamide or serve as the solvent control.

C. Preparation of Test Compound.

Niclosamide was prepared and tested at various concentrations. The various concentrations of Niclosamide used are presented in Table I. The test compound was dissolved into solution A (Table I) by sonication in a bath-style sonicator for 5 minutes to ensure that all of the compound went into solution before making the first dilution.

D. Animals.

The test animals for each experiment were the Charles River strain of male Golden hamsters (Cricetus auratus) ranging

in weight from 64-75 gm (groups treated at two days prior to exposure) and 100-110 gm (groups treated at seven days prior to exposure). The day of exposure, species of schistosomes, and number of animals in experimental and control group are shown in Table II.

TABLE I
SERIAL DILUTION

A	B	C	D	E
1,000 Grams of WRAIR compound for for a final vol of 100 ml with ETOH	25 ml of sol. A into 75 ml of ETOH	25 ml of sol. B into 75 ml of ETOH	25 ml of sol. C into 75 ml of ETOH	25 ml of sol. D into 75 ml of ETOH
DILUTION RATIO				
----	1:4	1:4	1:4	1:4
CONCENTRATION OF COMPOUND (MG/ML)				
10	2.5	0.625	0.15625	0.0390625

TABLE II
NUMBER OF HAMSTERS PER GROUP

DAY OF EXPOSURE	SPECIES OF SCHISTO	SOLUTION DILUTION GROUP (EXPERIMENTAL GROUPS)					ETOH	UNTREATED
		A	B	C	D	E	CONTROL	CONTROL
2	SmC	10	10	10	10	10	10	10
	ShC	10	10	10	10	10	10	10
	SjC	10	10	10	10	10	10	10
7	SmC	10	10	10	10	10	10	10
	ShC	10	10	10	10	10	10	10
	SjC	10	10	10	10	10	10	10

SmC = Schistosoma mansoni cercariae; ShC = S. haematobium
cercariae; SjC = S. japonicum cercariae.

It was shown by experimentation at WRAIR that treatment of animals with ETOH reduces the percentage of adult worm recovery by approximately 20 percent. Therefore, the number of cercariae used for animal exposures was increased to compensate for this reduction. Since the experiment depends on comparing the differences between the number of adult worms recovered from control animals and test compound solution treated animals, the number of cercariae needed to be as high as possible without causing premature mortality of the hamsters. The number of cercariae used was: 240 S. mansoni and S. haematobium cercariae, and 90 S. japonicum cercariae. The number of cercariae used per animal was recorded.

E. Treatment.

On the day prior to treatment, animals were anesthetized and the abdomen of each one was clipped free of hair. On the day of treatment each animal was treated with 7.5 ml of the test compound solution or ETOH (except the infection control group, which received no treatment). The solutions were applied by a 2" X 2" gauze pad and wiped on the abdomen area from anterior to posterior then left to right. This pattern was repeated for 60 seconds, keeping the treated area wet with solution at all times. After the alcohol had evaporated (approximately 5 minutes), the abdomens were washed for one hour with running tap water, 25-27 C, (See Figures 1 and 2). The infection control animals were also washed. The animals were then returned to their cages and allowed to dry and recover from the anesthesia.

On the day of infection, the animals of the appropriate group were anesthetized and their abdomens pre-wetted with aerated "aged" tap water. The hamsters were then placed over a vial with a diameter no more than half the size of the skin area treated by the solutions. The hamsters were then exposed to the appropriate species and number of schistosome cercariae for one hour.

F. Adult Worm Recovery.

After the worms had a chance to mature (49 days for both S. mansoni and S. japonicum, and 90 days for S. haematobium) but before the egg burden caused mortality, the hamsters were sacrificed by injection with 0.5 ml of sodium pentobarbital (65 mg/ml sodium pentobarbital). The animals were necropsied and perfused using a method similar to the Perf-O-Suction method of Radke et al. (17). The number of male, female, and immature worms were counted and recorded for each animal.

The number of worms recovered from the infected control animals was recorded and used to calculate the relative protection of the antipenetrant using the following formula (18):

$$\text{Relative Protection} = \frac{x - y}{x} \times 100$$

Where x = Avg. number of worms recovered from control animals

Where y = Avg. number of worms recovered from protected animals

RESULTS

The number of worms recovered at necropsy for animals treated with the test compound and those of respective control groups is shown in Tables III, IV, and V. Appendix II includes calculations showing mean worm burden calculations used in computing the test compound efficacy for each experimental group of animals.

The efficacy of the test compound against *S. mansoni* cercariae is shown in Table III. Complete protection (100%) was obtained when animals of Group A were treated with the undiluted test compound solution at two days before exposure to cercariae. Efficacies ranging from 96.5% (Group B), 54.9% (Group C), 0.0% (Group D) and 10.2% (Group E) were obtained when serial dilutions (each at 1:4) of the undiluted test compound were used (see Table I). Complete protection was not obtained for any of the groups of animals treated at 7 days before exposure to *S. mansoni* cercariae. The efficacies for these groups ranged from 99.3% (Group A - undiluted test compound), 92.5% (Group B), 67.2% (Group C), 11.2% (Group D), and 17.1% (Group E) respectively, (see Table I).

The efficacy of the test compound against *S. haematobium* cercariae is shown in Table IV. Complete protection of animals in Group A (undiluted test compound solution) and Group B (1:4 dilution of this compound) treated at 2 days before exposure to cercariae was obtained. Efficacies ranging from 98.0% (Group C), 44.5% (Group D), and 0.0% (Group E) were obtained when animals were treated with serial dilutions (each at 1:4) of the undiluted test compound (see Table I). Worms, both male and female, were found in varying numbers from all animals treated at 7 days before exposure to *S. haematobium* cercariae. No complete protection was observed for any single animal of this experiment. The highest efficacy obtained was for Group A (99.2%) treated with the undiluted test compound solution (see Table I). Efficacies for the remaining groups ranged from 64.7% (Group B), 37.7% (Group C), 0.0% (Group D) and 0.0% (Group E) respectively.

The efficacy of the test compound against S. japonicum cercariae is presented in Table V. Complete protection (100%) was obtained when animals of Group A were treated at two days before exposure to cercariae with the undiluted test compound solution. Efficacies ranging from 94.2% (Group B), 42.6% (Group C), 27.1% (Group D) and 27.7% (Group E) were obtained when serial dilutions (each at 1:4) of the undiluted test compound solution were used (see Table I). Complete protection was not obtained for any animal or group of animals treated with either undiluted or diluted test compound solutions at 7 days prior to exposure to S. japonicum cercariae. Worms, male and female, were obtained from animals of each group treated with the test compound solution. Efficacies ranging from 82.9% (Group A), 43.9% (Group B), 48.8% (Group C), 0.0% (Group D) and 26.8% (Group E) were obtained.

TABLE III
ANTIPENETRATION EFFECT OF TEST COMPOUND AGAINST SCHISTOSOMA
MANSONI
IN HAMSTERS EXPOSED EXPERIMENTALLY TO 240 CERCARIAE

DAYS PRIOR TO EXPOSURE	CONTROL DILUTION GROUP	WORM BURDENS AFTER PERFUSION										TOTAL	EFFICACY (%)
		COLLECTION FILTER *					TISSUE EXAMINATION **						
		F	M	SF ¹	SM ²		F	M	SF	SM			
2	Untreated	388	424	24	5		13	6	-	-		860	-
	ETOH (Veh)	220	299	-	7		7	8	-	-		541	-
	E	160	279	1	9		18	19	-	-		486	10.2
	D	390	457	11	6		39	41	-	-		944	0.0
	C	100	132	3	1		4	4	-	-		244	54.9
	B	6	11	-	-		-	-	-	-		17	96.5
	A	-	-	-	-		-	-	-	-		-	100.0
7	Untreated	143	194	7	5		6	4	-	-		359	-
	ETOH (Veh)	274	429	1	4		28	29	-	-		765	-
	E	252	371	2	8		1	-	-	-		634	17.1
	D	261	401	7	4		3	3	-	-		679	11.2
	C	104	141	1	1		2	2	-	-		251	67.2
	B	25	29	-	-		2	1	-	-		57	92.5
	A	3	2	-	-		1	-	-	-	6	99.3	

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

TABLE IV
ANTIPENETRATION EFFECT OF TEST COMPOUND AGAINST SCHISTOSOMA HAEMATOBIIUM
IN HAMSTERS EXPOSED EXPERIMENTALLY TO 240 CERCARIAE

DAYS PRIOR TO EXPOSURE	CONTROL DILUTION GROUP	WORM BURDENS AFTER PERFUSION				TISSUE EXAMINATION**				TOTAL	EFFICACY (%)
		COLLECTION FILTER*				F	M	SF	SM		
		F	M	SF ¹	SM ²						
2	Untreated	28	56	-	2	7	8	-	-	101	-
	ETOH (Veh)	34	58	3	-	3	3	-	-	101	-
	E	47	83	1	2	15	16	-	-	164	0.0
	D	20	26	9	1	-	-	-	-	56	44.5
	C	-	-	2	-	-	-	-	-	2	98.0
	B	-	-	-	-	-	-	-	-	-	100.0
7	A	-	-	-	-	-	-	-	-	-	100.0
	Untreated	48	97	-	1	8	8	-	-	162	-
	ETOH (Veh)	32	82	1	-	3	4	-	-	122	-
	E	42	124	-	1	3	5	-	-	175	0.0
	D	50	70	-	-	17	21	-	-	158	0.0
	C	24	42	2	-	4	4	-	-	76	37.7
	B	9	19	1	-	5	5	-	-	39	64.7
	A	-	1	-	-	-	-	-	-	1	99.2

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesenteric veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

TABLE V
ANTIPENETRATION EFFECT OF TEST COMPOUND AGAINST SCHISTOSOMA JAPONICUM
IN HAMSTERS EXPOSED EXPERIMENTALLY TO 90 CERCAIRIAE

DAYS PRIOR TO EXPOSURE	CONTROL DILUTION GROUP	WORM BURDENS AFTER PERFUSION						TOTAL	EFFICACY (%)		
		COLLECTION FILTER*				TISSUE EXAMINATION**					
		F	M	SF ¹	SM ²	F	M			SF	SM
2	Untreated	36	57	37	1	10	10	-	-	171	-
	ETOH (Veh)	46	46	51	1	6	5	-	-	155	-
	E	33	33	34	2	5	5	-	-	112	27.7
	D	32	34	33	3	6	5	-	-	113	27.1
	C	30	31	24	-	2	2	-	-	89	42.6
	B	3	3	3	-	-	-	-	-	9	94.2
	A	-	-	-	-	-	-	-	-	-	100.0
7	Untreated	10	19	2	1	3	3	-	-	38	-
	ETOH (Veh)	11	20	3	1	3	3	-	-	41	-
	E	6	13	2	1	1	1	-	-	24	26.8
	D	21	30	-	-	3	3	-	-	57	0.0
	C	3	11	1	-	3	3	-	-	21	48.8
	B	5	11	3	-	2	2	-	-	23	43.9
	A	3	4	-	-	-	-	-	-	7	82.9

* Collection Filter: Filter used to trap worms perfused from the liver and mesenteric veins.

** Tissue Examination: Examination of liver, mesentary veins and adipose tissue containing veins for lodged worms after perfusion.

1: Denotes stunted female worms. 2: Denotes stunted male worms.

DISCUSSION

Complete protection of hamsters was obtained when Niclosamide dissolved in absolute ethanol was applied to the skin in a single treatment at 2 days prior to exposure to either S. mansoni, S. haematobium, or S. japonicum cercariae. These results indicate that this preparation is highly active as an antipenetrant against the three major species of schistosomes infective to man.

Application of the dissolved Niclosamide in absolute ethanol at 7 days prior to exposure to cercariae of either of the three schistosome species did not completely protect the hamsters. In most instances, male and female worms were found indicating that the possibility of tissue damage from schistosome ova may occur, thus indicating that the viability of the preparation at 7 days prior to exposure to cercariae is not sustained for this length of time.

The use of serial dilutions of the undiluted test compound preparation (Group B, C, D, and E) did not (except for Group B exposed to S. haematobium 2 days after treatment with the undiluted preparation) completely protect hamsters at either 2 or 7 days prior to exposure to cercariae of either of the three species of schistosomes. The presence of male and female worms capable of depositing eggs which would cause pathological manifestation (despite the presence of low worm burdens in some of the groups) indicates that the concentrations of the serial dilutions used should not be a candidate for further testing as an antipenetrant against schistosome cercariae invasion.

These studies suggest that the undiluted preparation of Niclosamide in absolute ethanol is a candidate for advanced studies (in primates) which should be designed to determine its potential for human use.

CONCLUSIONS

Niclosamide has been used very effectively in the treatment of persons with tapeworm infections. No toxic effects are known to occur in man. The results obtained with the undiluted preparation suggests that this compound has promise as a successful anti-schistosome prophylactic agent (15,16).

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APPENDIX I *

Techniques for Cultivation and Maintenance
of Snail and Schistosome Species and
Safety Practices

* This Appendix I includes Figures 1-3 and Forms 1-3

A number of papers have been published which describe the requirements for laboratory cultivation of snail intermediate hosts (Biomphalaria sp, Bulinus sp, Oncomelania sp, and Tricula sp) for schistosomes (Schistosoma mansoni, S. haematobium, S. japonicum, and S. mekongi) which infect man in sufficient numbers to support simultaneous programs in chemotherapy, immunology, physiology, pathology vector control and other areas of biomedical research (1, 2, 3, 4, 5, 6, 7, 9). Certain optimal physical conditions are necessary for the successful cultivation of snails. These are pH, oxygen content, water free of chlorine, copper, zinc and other harmful protozoans and metazoans, and regular removal of decayed food and other debris from the culture aquaria. These physical environmental conditions are given high priority by persons involved in the maintenance of snails in the laboratory.

The procedures used in the cultivation of snails in the University of Lowell's laboratory represent an adaptation and/or modification of those techniques published as cited above. During the past five years through an ongoing National Institutes of Health contract, several new snail cultivation and maintenance units as well as some cost efficient techniques designed for large scale operation have been developed (8).

I. TECHNIQUES FOR CULTIVATION OF BIOMPHALARIA SPECIES

A. Units for Snail Maintenance

Three types of units are used.

1. The prototype of the "cabinet drawer" arrangement as described (10) and modified (1) is used for holding large numbers of exposed snails. The overall dimensions of this unit with 12 (3-pan) slide drawers and a shelf for accommodating 5 (40-liter) aquaria are shown in Figure 1. The unit consists of an air manifold system for aerating each individual pan, a water-oil extractor and air pressure gauge for removing harmful oil-droplets from the air and measuring of the air pressure, respectively. This unit is sufficient for maintaining approximately 1000 exposed snails.
2. A mobile unit similar to that described (2) is used for holding large numbers of breeder and stock snails (Figure 2). The overall dimensions of this unit are 52 X 175 X 243 cm. This unit is capable of holding 32 glass aquaria (20-liter), 64 plastic trays or 288 petri dishes. There are two types of set-ups for this unit. One is equipped with air lines and

fluorescent lightes and is used to accommodate glass aquaria for stock snail cultures. Its air manifold system is also connected to an oil extractor and air pressure gauge for removing harmful oil droplets from the air and measuring the air pressure, respectively. There are 2 of these units available in this laboratory, which is more than sufficient to meet Biomphalaria culture requirements. The second type of set-up is one in which only fluorescent lights are used for accommodating the type of aquaria (plastic trays and petri dishes) which do not require aeration. This mobile unit is also used for cultivating algae needed as food for snails.

3. A mobile unit, constructed of heavy-duty steel (Figure 3) is used for holding exposed snails and algal cultures. The overall dimensions of this unit are 61 X 122 X 188 cm. It is capable of holding 50 plastic trays or 180 petri dishes. This unit is set up in two types, neither of which is equipped with air lines. The first type of set-up employs fluorescent lights. Three of these are available for accommodating plastic trays for exposed snails and petri dishes for algal cultures. The second type of set-up does not employ fluorescent lights. Two of these are available for accommodating plastic trays containing exposed snails.

Four kinds of aquaria are used: 1) twenty-liter and forty-liter glass aquaria are used for rearing and maintaining stock snails; 2) stainless steel pans with a holding capacity of 2 liters of water are used as holding aquaria for maintaining exposed snails; 3) plastic trays with a holding capacity of 1.5 liters of water are used as aquaria for breeder snails as well as for both pre-exposed and exposed snails; 4) petri dishes with a holding capacity of 40 ml of water are used as aquaria for newborn snails.

B. Environmental Parameters

1. Light. In the first type of snail maintenance unit described above, 40-liter glass aquaria are maintained under constant light provided by a 15-w "cool" white fluorescent tube suspended 4 inches over the top of each aquarium. Stainless steel pans (holding aquaria for exposed snails) are covered at all times. In the second and third type of snail maintenance units described above, 40-w "cool" white fluorescent tubes are suspended 12 inches and 18 inches respectively above each shelf. The 20-liter

glass aquaria used for maintaining stock snails are held under 12-hour light and 12-hour darkness. The plastic trays (when used for breeding snails and for maintaining pre-exposed snails) are placed under constant light.

2. Water. Tap water is conditioned by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. This water is then aerated for one day prior to use. The pH of the water is initially 7.1.
3. Aeration. The water of all glass aquaria and holding pans is continuously aerated. The air is supplied by a centrally located air compressor and passes through a water-oil extractor into the culture units. The water-oil extractor filters out the minute droplets of water and oil carried by the air which might be harmful to the snails. The water contained in plastic trays and petri dishes is not aerated.
4. Temperature. Temperature is monitored daily. A central air conditioning unit maintains the temperature in the snail cultivation rooms between 25-27 C.
5. pH. The pH of the water within glass aquaria, holding pans and plastic trays averages 7.1 and is monitored weekly.
6. Food. Romaine lettuce is used as a basic food source. In addition, one or two dishfuls of specially prepared mud, on which blue-green algae (*Nostoc muscorum*) has been grown (5) is placed in all glass aquaria, holding pans and plastic trays to serve as an additional food source. Powdered commercial fish food is added daily. Powdered oyster shell is added to glass aquaria to ensure adequate supply of calcium. Snails in petri dishes are given only blue-green algae with mud.

C. Breeding

Snails measuring 12 - 15 mm are removed from glass aquaria and placed in plastic trays (10 per tray) containing 1.5 liters of aerated tap water. Trays are placed under continuous light and supplied with food. After one week the trays are changed and the egg masses are removed and placed in petri dishes with aerated tap water and incubated under continuous light for about one

week until hatching occurs. Newborn snails (0.6 - 0.8 mm in shell diameter) are transferred with a pipette to petri dishes containing blue-green algae, mud and water and kept at a density of 50 snails per dish. Dishes are maintained under continuous light. In 14 days, young snails 3-5 mm in size are available for use in initiating new cultures or for eventual exposure to pathogens.

D. Rearing and Maintenance

For initiating new cultures, snails are removed from petri dishes in groups of 400 and placed in 40-liter glass aquaria (or in groups of 250 in 20-liter glass aquaria) with aerated tap water. After exposure to pathogens, the snails are maintained in groups of 50 in plastic trays containing 1.5 liters of water.

E. Source and Strain of Snail

The albino Biomphalaria glabrata snail strain is from Puerto Rico and was originally obtained from the University of Michigan.

II. TECHNIQUES FOR CULTIVATION OF BULINUS SPECIES

A. Unit for Snail Maintenance

Two types of units are used.

1. A mobile unit similar to that designed by Davis (1971) is used for accommodating large numbers of snails (Figure 2). The overall dimensions of this unit are 52 X 175 X 243 cm. It is equipped with air lines and fluorescent lights and is used to accommodate glass aquaria holding stock. This unit is capable of holding 32 glass aquaria (20-liter size), 64 plastic trays or 288 petri dishes.
2. A mobile unit constructed of heavy-duty steel (Figure 3). The overall dimensions of this unit are 61 X 122 X 188 cm. It is equipped with or without fluorescent lights. The unit equipped with fluorescent lights is used for accommodating the type of aquaria (plastic trays and petri dishes) which do not require aeration. This type of mobile unit is also used for cultivating algae needed as food for snails. The unit without fluorescent lights is used for accommodating snails exposed to pathogens. Each of these units is capable of

holding 50 plastic trays of 180 petri dish cultures. Three kinds of aquaria are used: 1) twenty-liter glass aquaria are used for maintaining stock snail cultures; 2) plastic trays with a holding capacity of 1.5 liters of water are used as breeding aquaria for collecting eggs and for maintaining pre-exposed and exposed snails; 3) petri dishes with a holding capacity of 40 ml of water are used for incubating and hatching eggs. Newborn snails are also kept in the petri dishes until they reach 3 - 4 mm in size.

B. Environmental Parameters

1. Light. The plastic trays (when used for breeding and for maintaining pre-exposed snails) are placed in continuous artificial light provided by a 40-w "cool" white fluorescent tube suspended 12 inches (or 18 inches in Type 2 unit) above each shelf. Likewise, the petri dishes containing egg masses and newborn snails are placed under similar light. The 20-liter glass aquaria used for maintaining stock snails are held under 12-hour light and 12-hour darkness. Plastic trays with exposed snails are maintained with or without illumination depending on experimental designs.
2. Water. Tap water is conditioned before use by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. The water is then aerated for at least one day prior to use. The pH of the water is approximately 7.1.
3. Aeration. Air supplied to glass aquaria is passed from a centrally located air compressor through a water-oil extractor in culture units. The extractor filters out the minute droplets of oil which can be harmful to the snails. The water contained in plastic trays and petri dishes is not aerated.
4. Temperature. Temperature is monitored daily. It is maintained 25-27 C.
5. pH. The pH of the water within stock aquaria and trays is monitored weekly.
6. Food. Romaine lettuce supplemented with blue-green algae (*Nostoc muscorum*) and mud forms the diet of stock snails in glass aquaria as well as those kept in plastic trays. Commercial fish food is added

daily. Powdered oyster shell is added to glass aquaria. Snails in petri dishes are given only blue-green algae with mud.

C. Breeding

After populations of snails have been established in glass aquaria, they are transferred to plastic trays containing 1.5 liters of aerated tap water and maintained at a density of ten snails per tray. Trays are placed under continuous light and supplied with food as described above. Trays are changed weekly, at which time egg masses are scraped from the walls of the trays as well as from the surface of lettuce.

D. Hatching of Eggs and Maintenance of Newborn

Egg masses are placed in petri dishes with aerated tap water and incubated under continuous light for one week until hatching is apparent. Approximately 50 newborn (0.6 - 0.8 mm in shell length) are transferred with a pipette to petri dishes with blue-green algae and mud, and are placed under continuous illumination. Within 14 days young snails will reach 2 - 4 mm in size which is suitable for exposure to pathogens.

E. Source and Strain of Snail and Parasite

The Egyptian strain of Bulinus truncatus truncatus snail originated from the University of Michigan stock. The Ghanian and Voltan strains of Bulinus truncatus rohlfsi also originated from the University of Michigan stock.

III. TECHNIQUES FOR CULTIVATION OF THE 4 SUBSPECIES OF ONCOMELANIA HUPENSIS AND TRICULA SPECIES

A. Unit for Snail Maintenance

A mobile unit, constructed of heavy-duty steel (Figure 3) is used to accommodate large numbers of snails. The overall dimensions of the unit are 61 X 122 X 188 cm. Two types of units are used, neither of which is equipped with air lines. The unit with lighting is used for accommodating petri dish cultures containing exposed, pre-exposed, breeder snails, and algal cultures. The unit with lighting is used for incubating snail eggs.

Three kinds of aquaria are used: 1) a petri dish (2 X

10 cm) containing peripherally placed mud, blue-green algae (Nostoc muscorum) and water is used for breeding and obtaining eggs (3, 4); 2) a petri dish containing a centrally placed mud mound, blue-green algae (Nostoc muscorum) and water is used for rearing young snails and maintaining both pre-exposed and exposed snails (5); 3) a petri dish containing small amounts of blue-green algae (Nostoc muscorum), mud and water is used for newborn snails (6).

B. Environmental Parameters

1. Light. Breeder and pre-exposed snails are maintained under a 40-w "cool" white fluorescent light with 12-hour light and 12-hour darkness. Exposed snails are maintained with or without illumination depending on experimental designs. Eggs collected from breeding aquaria are placed in clean petri dishes with aerated tap water and placed on shelves without direct illumination.
2. Water. Tap water is conditioned before use by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. The water is then aerated for at least one day prior to use. The pH of the water is approximately 7.1.
3. Temperature. Temperature is monitored daily and maintained at 25-27 C.
4. Food. Blue-green algae (Nostoc muscorum) grown in petri dishes with mud (5) is used exclusively as the food source.

C. Breeding

A modification of the culture method described previously (3, 4) is used for cultivating all subspecies of Oncomelania snails. Adult snails (5 males and 5 females per aquarium) are introduced into petri dish aquaria containing peripherally placed mud. After the surface of the mud is washed several times, a small amount of blue-green algae is added. The aquaria are checked weekly for eggs which are then removed into a petri dish containing aerated tap water. At the same time, snail feces and soiled water are removed and fresh water and algae (if necessary) are added. Cultivation of Tricula species follows previously described methods (6, 7).

D. Hatching of Eggs and Maintenance of Newborn

Eggs previously placed in petri dishes with aerated tap water are placed on shelves without direct illumination for incubation and hatching. Eggs usually hatch within 18 days for Oncomelania species and 35 days for Tricula species. Newborn snails are then placed in newly established aquaria containing a small amount of blue-green algae and allowed to grow for 2-3 weeks. The snails are maintained under 12-hour light and 12-hour darkness. After reaching 2 - 3 mm in shell length, the snails are used for exposure to pathogens or for establishing new rearing cultures by placing snails in petri dishes containing centrally placed mud with blue-green algae.

E. Source and Strain of Snail

Oncomelania hupensis hupensis (Vogel Chinese strain) is from the University of Michigan stock. O. h. hupensis (Shanghai strain) was obtained through Dr. Mao direct from People's Republic of China approximately 3 years ago.

O. h. nosophora (Kofu strain) were supplied from Hamamatsu University direct to the University of Lowell.

O. h. formosana (Pu-yen strain) were obtained through Dr. Cross (NAMRU-2) direct from Taiwan and from this laboratory.

O. h. quadrasi (Leyte strain) originated from Leyte, Philippines through Dr. Sano. Hamamatsu University, Japan.

O. h. chiui was from the University of Michigan stock and from this laboratory.

Tricula paerta originated from Mahidol University, Thailand.

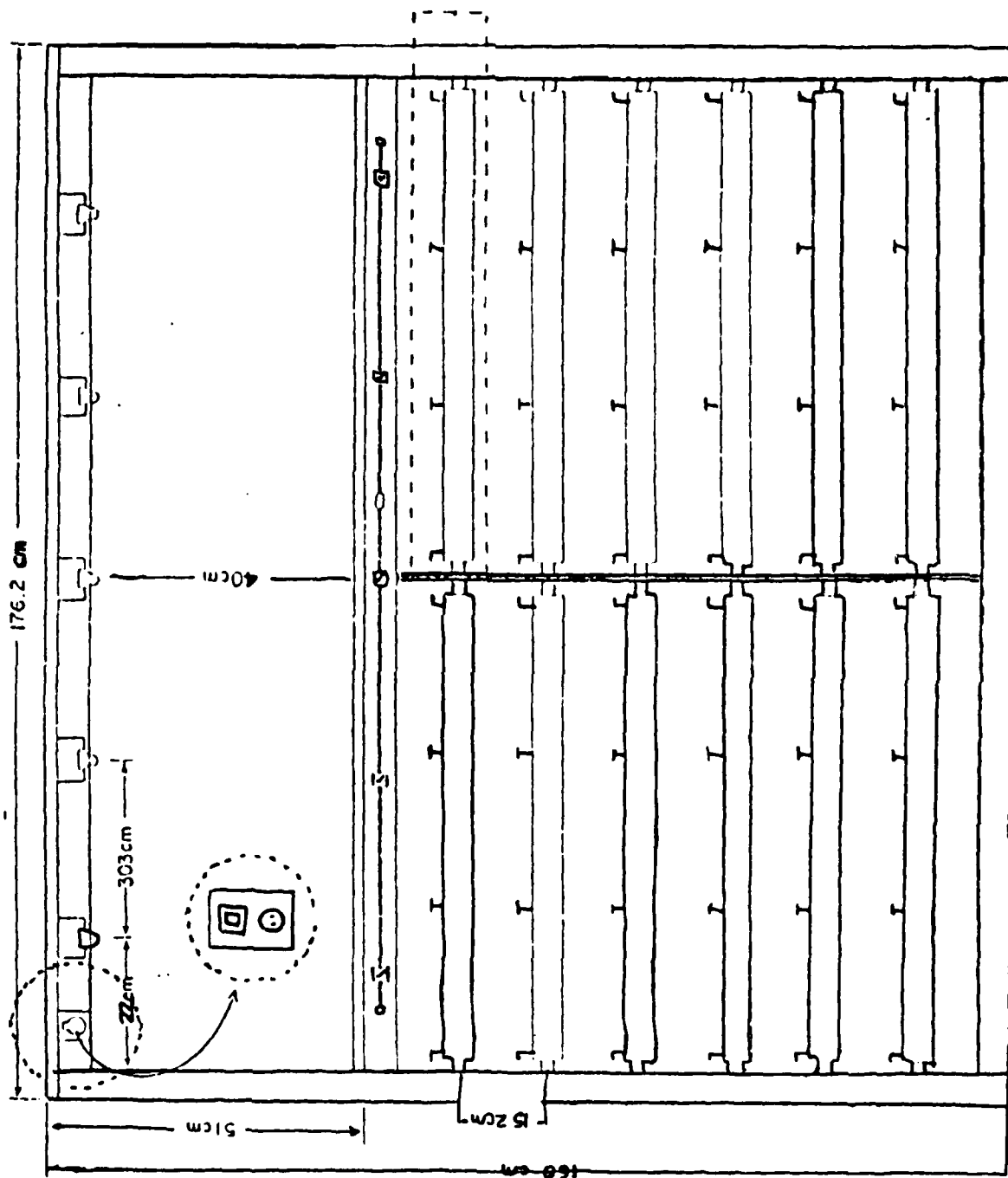


Figure 1: Snail Maintenance Unit (Cabinet Drawer Type)

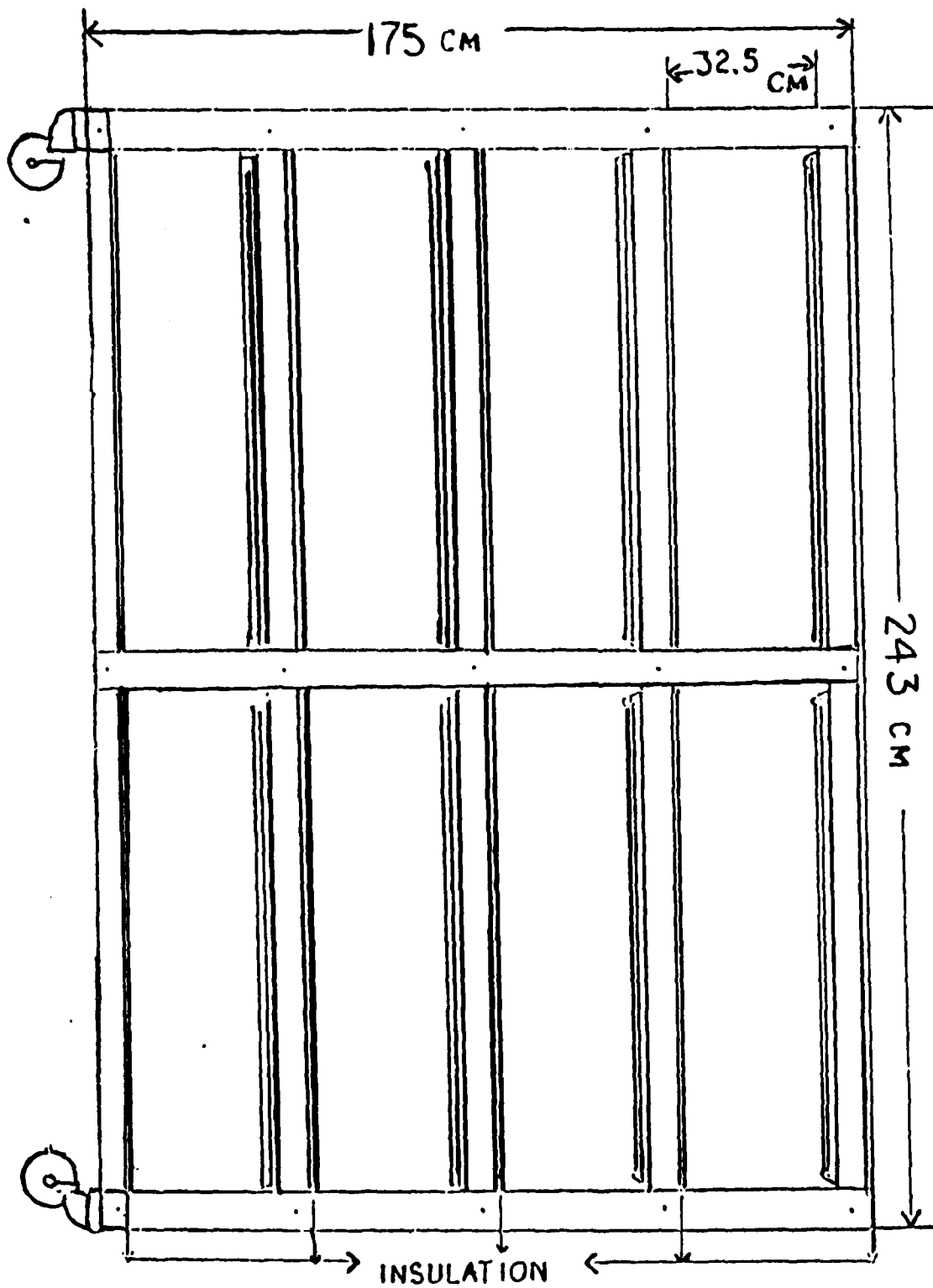


Figure 2: Snail Maintenance Unit (Wooden, Mobile Type)

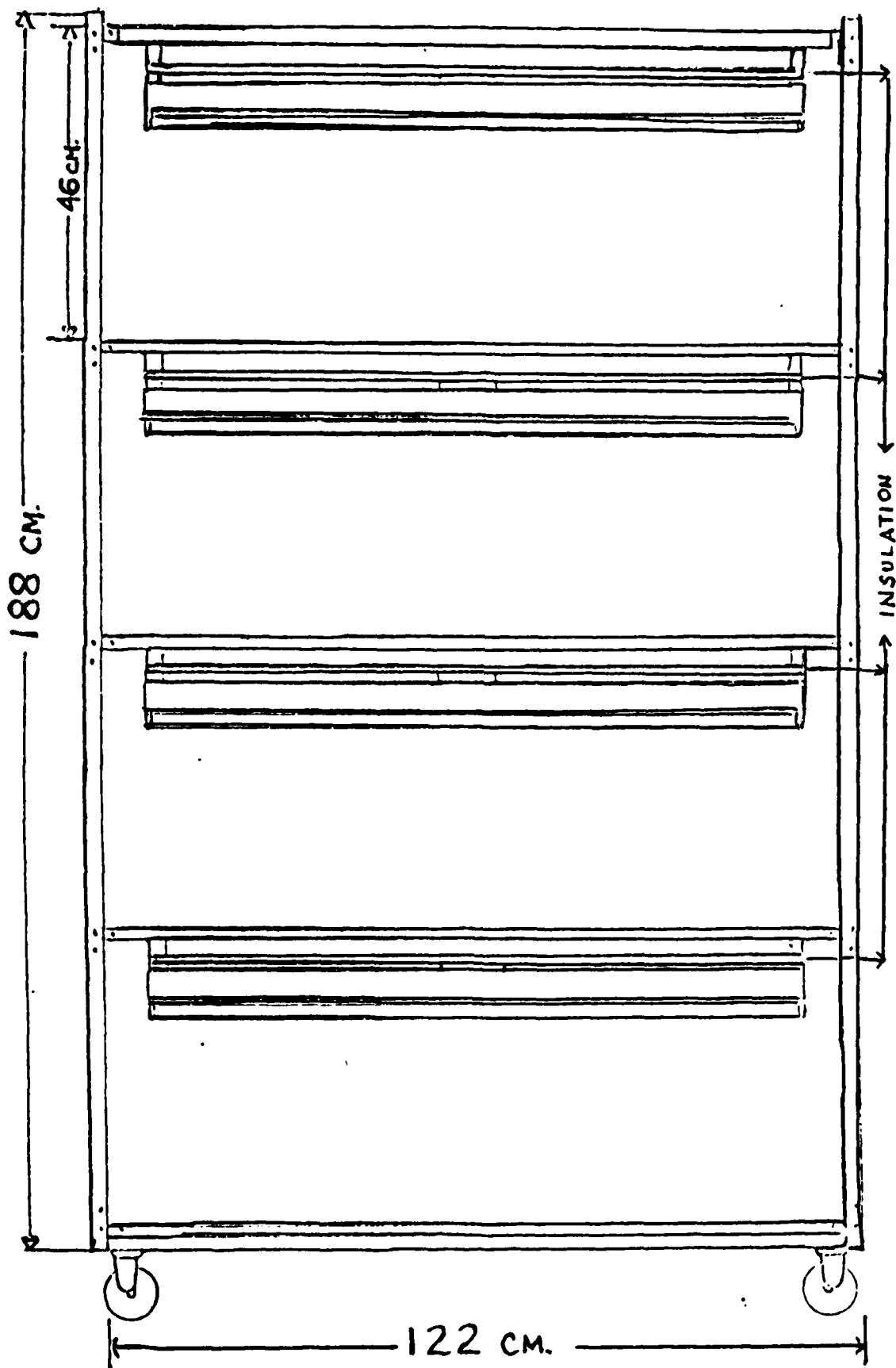


Figure 3: Snail Maintenance Unit (Steel, Mobile Type)

Laboratory Safety Practice

The following routine procedures are carried out to ensure good laboratory practice and safety of laboratory personnel as well as protection of the environment.

1. Responsibility for the life cycle of a single species of schistosome is delegated to a single technician. This permits full accountability in case of questions regarding shipments to investigators arise.
2. Patent snails are housed in properly labeled pans, trays or petri dishes instead of large glass vessels. This allows easy and safe access and handling.
3. Separate sinks are provided for washing of lettuce, glassware and animal cages to prevent accidental exposure of snails to schistosome eggs and miracidia.
4. Infected tissue remains, miracidial and cercarial suspensions are discarded into a bleach solution. Contaminated glassware and instruments are disinfected with 5% solution of bleach for 5 minutes prior to washing.
5. The drainage systems of all laboratory rooms are connected to a decontamination tank which has been especially designed for environmental safety. After decontamination, the contents of the tank are released into the general sewage system. This treatment is in excess of requirements for complete killing of all schistosome stages and ensures protection of the environment.

Routine Maintenance of Snail Laboratory (Applies for all snail species)

Daily checks are made of water levels and the temperature of glass aquaria, holding pans, plastic trays, and petri dishes. Snails which have climbed above the water level are returned to the water. Dead snails and decaying food matter are removed, and mortality among pre-patent and patent snail populations is recorded.

Aquaria containing cloudy water, protozoa, metazoa, etc., are changed immediately. The aquaria are thoroughly soaked with a 10% bleach solution, then thoroughly washed with hot running tap water followed by a final prolonged rinse with aerated tap water.

Routine Laboratory Tasks

Tasks	Daily	Weekly
Feed snails	X	
Check temperature	X	
Check level and condition of water in aquaria	X	
Check aeration and filtration system	X	
Check snail mortality; remove dead snails	X	
Record vital statistics	X	
Check pH of water		X
Collect egg masses		X
Set up breeding cultures		X
Set up rearing cultures		X
Change water of cultures holding pre-patent and patent snails		X
Screen snails for infection		X
Expose snails to miracidia		X

Sources of Snails, Strains and Species

The University of Lowell currently has established interactions with several foreign universities and research institutions where fresh field isolates of snail and parasites can be obtained. Oncomelania hupensis nosophora (infected and uninfected) can be obtained from Dr. Yukio Hosake, NIH, Japan. O. h. quadrasi (infected and uninfected) are obtainable from NAMRU-2 (Philippines). Oncomelania hupensis formosana (infected and uninfected) and Oncomelania hupensis chiui (uninfected) from Dr. P.C. Fan (Yang-ming College, Taiwan) and can be obtained at any time from this source. Infected and uninfected Bulinus truncatus truncatus (Egyptian strain) and Biomphalaria alexandrina can be obtained from Drs. El Banhawy and Saoud of Ain Sham University, Cairo, Egypt. Bulinus truncatus rohlfschi (Ghanian strain) both infected and uninfected can be obtained from Dr. Lo from specific areas in Africa including Ghana. Biomphalaria glabrata (Puerto Rican strain) both infected and uninfected can be obtained from Lt. Col. Willis R. Reid, Walter Reed Army Institute of Research, and B. glabrata (Brazilian strain) can be obtained from Dr. N. Katz, Belo Horizonte, Brazil.

It should be pointed out that all strains of schistosomes and corresponding snail hosts currently in the laboratory and those which have not originated recently from the field can be eventually replaced with fresh field isolates.

Maintenance of Records
(Applies for all species)

Three types of data recording forms are used to provide continuous monitoring of the production of stock snails and for maintenance of infected snails and mammals. They are designated as: Form 1 Snail Rearing; Form 2 Snail Infection; and Form 3 Mammal Infection.

Form 1 is used for recording data pertinent to snail rearing such as date of set-up, numbers of breeders, survivals, eggs laid, and number and date of young snails produced as well as other pertinent data.

Form 2 is used to record conditions of exposure of snails to miracidia, sources of miracidia, survival of snails and number of snails determined to be positive by shedding and crushing.

Form 3 is used to record exposure of mammals to cercariae, source of cercariae, survival of mammals, and status of infection at the time the mammals are sacrificed.

With these forms one may monitor the infection parameters of schistosome species from the snail host to the experimental mammals. These records are bound periodically and maintained in a separate record room at this facility.

Information pertaining to purchase of mammals from animal breeders is also kept in a bound ledger book.

SNAIL REARING

Species:

No.

Source:

No. Snail: (♂: ♀:)

Age: Size:

Date Set-up:

Date	No. Alive	No. of				Remarks	
		Mass	Egg	E/M	Young		
-						-	-
-						-	-
-						-	-
-						-	-
- - - - -							
-						-	-
-						-	-
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SNAIL INFECTION

Tray No.:
Date Inf.:
No. Exp.:
Method: en masse _____ ind. _____
No. Mir.:
Definitive Host:
 Date Inf.
 Dose:

Parasite Strain:
Snail:
 Age:
 Size:
Exposure Time:

[illegible]

Date _____

Remarks

MAMMAL INFECTION

Cage No.	Mammal Strain
	Age Weight
	Date Exposed
	Number Exposed
	Exposure Method
	Time Start Shed
	Time Last Mammal Exp.
	Age of Cercariae
	Number of Cerc. Counts
	Aver. Number Cerc.

<u>Parasitic Strain</u>	<u>Snail Species</u>	<u>Date Snail Exp.</u>	<u>Number of Snails</u>	<u>Number of Times Previously Shed</u>
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[illegible]

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APPENDIX II

Calculations for Mean Worm Burdens and Their Use in
Computing Test Compound Efficacies

Calculations for Mean Worm Burdens and Their Use in
Computing Test Compound Efficacies

I. Efficacy against Schistosoma mansoni (Puerto Rican)

A. Day 2 Group

Mean = 54.1

1. E	$100 \times \frac{54.1 - 48.6}{54.1}$	= 10.17	
2. D	$100 \times \frac{54.1 - 94.4}{54.1}$	= 0.0	
3. C	$100 \times \frac{54.1 - 24.4}{54.1}$	= 54.90	
4. B	$100 \times \frac{54.1 - 1.9}{54.1}$	= 96.49	(1 animal died during exp.)
5. A		100.00	

B. Day 7 Group

Mean = 76.5

1. E	$100 \times \frac{76.5 - 63.4}{76.5}$	= 17.12	
2. D	$100 \times \frac{76.5 - 67.9}{76.5}$	= 11.24	
3. C	$100 \times \frac{76.5 - 25.1}{76.5}$	= 67.19	
4. B	$100 \times \frac{76.5 - 5.7}{76.5}$	= 92.55	
5. A	$100 \times \frac{76.5 - 0.6}{76.5}$	= 99.26	

II. Efficacy against Schistosoma haematobium (Egyptian)

A. Day 2 Group

Mean = 10.1

1.	E	$100 \times \frac{10.1 - 16.4}{10.1}$	=	0.0
2.	D	$100 \times \frac{10.1 - 5.6}{10.1}$	=	44.55
3.	C	$100 \times \frac{10.1 - 0.2}{10.1}$	=	98.02
4.	B			100.00
5.	A			100.00

B. Day 7 Group

Mean = 12.2

1.	E	$100 \times \frac{12.2 - 17.4}{12.2}$	=	0.0
2.	D	$100 \times \frac{12.2 - 15.8}{12.2}$	=	0.0
3.	C	$100 \times \frac{12.2 - 7.6}{12.2}$	=	37.70
4.	B	$100 \times \frac{12.2 - 4.3}{12.2}$	=	64.75 (1 animal died during exp.)
5.	A	$100 \times \frac{12.2 - 0.1}{12.2}$	=	99.18

III. Efficacy against Schistosoma japonicum (Philippine)

A. Day 2 Group Mean = 15.5

1. E	$100 \times \frac{15.5 - 11.2}{15.5}$	= 27.74
2. D	$100 \times \frac{15.5 - 11.3}{15.5}$	= 27.10
3. C	$100 \times \frac{15.5 - 8.9}{15.5}$	= 42.58
4. B	$100 \times \frac{15.5 - 0.9}{15.5}$	= 94.19
5. A		100.00

B. Day 7 Group Mean = 4.1

1. E	$100 \times \frac{4.1 - 3.0}{4.1}$	= 26.83 (2 animals died during exp.)
2. D	$100 \times \frac{4.1 - 5.7}{4.1}$	= 0.0
3. C	$100 \times \frac{4.1 - 2.1}{4.1}$	= 48.78
4. B	$100 \times \frac{4.1 - 2.3}{4.1}$	= 43.90
5. A	$100 \times \frac{4.1 - 0.7}{4.1}$	= 82.93

APPENDIX III *

Protocol for Secondary Prophylactic Testing

* This Appendix III includes Tables I, II, and III

Introduction

Two compounds (ZM65703 and BC78878) have been received for evaluation as possible prophylactic against the major three species of human schistosomes. A third compound (AG63908), Niridazole, will be used as a positive drug control. The compounds were selected from the test results of the Brazil schistosomiasis primary prophylactic test (PMT) and Walter Reed Army Institute of Research, Division of Experimental Therapeutics in-house testing program. The following protocol describes, in general terms, secondary rodent testing for the experimental and the drug control compounds.

Compounds

The following compounds have been received in quantities indicated in Table I. There are no known adverse effects in handling these compounds, however, good laboratory practice will be used. All compounds will be stored according to the labeling instructions: Red label = hygroscopic, Blue label = refrigerate, and Green label = compound in liquid form. No label indicates that no special storage conditions are required.

TABLE I
TEST COMPOUNDS RECEIVED

BOTTLE NUMBER	WALTER REED NUMBER	QUANTITY
ZM65703	007930	13 Grams
BC78878	102796	20 Grams
AG63908 (Niridazole)	005950	20 Grams

Animals

The mice will be male ICR (out bred) weighing between 17-25 grams. The golden hamsters will be males weighing between 75-90 grams.

Parasites

Three species of schistosomes will be used. The Philippine strain of Schistosoma japonicum, Egyptian strain of S. haematobium, Nigerian strain of S. haematobium, and Kenyan strain of S. mansoni.

Vehicles

Two vehicles will be used. Pharmaceutical grade peanut oil for subcutaneous injections and Tween 80-methyl cellulose-saline (TMCS) for per os. TMCS is made as indicated in Table II.

TABLE II

MAKE-UP OF TWEEN 80-METHYL CELLULOSE-SALINE

Ingredient	Amount
Tween	0.40 ml
Methyl cellulose	0.20 gr
Sodium chloride	0.85 gr
Distilled water	98.55 ml

The sodium chloride is dissolved into the distilled water and warmed to 70 C. 5 ml of the warm saline solution is added to the Tween 80 and stirred until dissolved. The rest of the saline solution is then added. The solution is allowed to come to room temperature and the methyl cellulose added. The mixture is placed in the refrigerator overnight to facilitate the dissolution of the methyl cellulose.

Drug Solutions

All animals will be given 100 mg/kg of body weight for subcutaneous and per os treatments. To obtain the proper concentrations of drug in a reasonable volume of carrier the following formula is used.

- 1) 10 mg of compound per ml of carrier (weight per volume)
- 2) Animal weight (in grams) X 0.01 = ml of solution per animal per day.

To calculate the volume of drug solution needed for each day of treatment the formula below will be used.

Number of		Avg. Weight		0.01 ml per		Tot. vol. used
Animals	X	plus 10%	X	gr body wt.	=	for treatment

Ex:1) 10 mice X (25 gr + 2.5 gr) X 0.01 ml = 2.75 ml of solution

2) 10 hamsters X (90 gr + 9 gr) X 0.01 ml = 9.9 ml of solution

To example 1 above, 27.5 mg of compound is added to 2.75 ml of carrier solution (Weight/Volume) and to example 2, 99.0 mg of compound is added to 9.9 ml of carrier solution (Weight/Volume).

Treatment

Subcutaneous: Each animal will be injected under the skin in the anterior - dorsal area just posterior to the neck for five days.

Per os: Each animal will be administered the appropriate volume by gavage needle for five days.

The following experiments will be conducted using 10 animals for each compound, 10 animals as vehicle controls, and 10 animals for infection controls (no treatment). Each experiment will use 50 animals per parasite species using the two experimental compounds and Niridazole. The animals will be treated on Monday, Tuesday, Wednesday, Thursday, and Friday. All animals (including the infection controls) will be infected Wednesday, but prior to treatment.

TABLE III
EXPERIMENT SCHEDULE

Exp. No.	Animal Host	Parasite Species	Cercariae per Animal	Route	Perfusion Day After Infec.
1	Mouse	<u>S. mansoni</u>	150	SQ	49
2	Mouse	<u>S. japonicum</u>	50	SQ	35
3	Mouse	<u>S. mansoni</u>	150	ORAL	49
4	Mouse	<u>S. japonicum</u>	50	ORAL	35
5	Hamster	<u>S. mansoni</u>	200	SQ	49
6	Hamster	<u>S. japonicum</u>	75	SQ	35
7	Hamster	<u>S. haematobium</u>	250	SQ	95
8	Hamster	<u>S. mansoni</u>	200	ORAL	49
9	Hamster	<u>S. japonicum</u>	75	ORAL	35
10	Hamster	<u>S. haematobium</u>	250	ORAL	95

Perfusion

Worm burdens will be determined by hepatoportal perfusion of the animals. All animals will be killed by intraperitoneal injection of 0.02 ml/gr/bw of heparinized (100 units per ml) of pentobarbital (65 mg/ml) solution. Worms will be collected by whole body perfusion according to a modified method of Radke et al. (Journal of Parasitology, 1961, 47:366-368) using heparinized (10 units per ml) 0.9 percent saline solution following the protocol which is currently being used at Lowell University. The worms from each perfused animal will be counted as to male or female.

Forms

The information collected will be entered on the Walter Reed Army Institute of Research Anti-Schistosomiasis Drug Screen data form which has been received along with a description of the codes to be used. A photocopy of the forms will be sent to WRAIR when each experiment is completed. The information will also be transcribed onto a data disk and electronically transferred to WRAIR using Kermit or a copy of the disk will be sent to the COTR at WRAIR:

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