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STUDY ON THE ETHIOPIAN FRESHWATER MOLLUSCS, ESPECIALLY ON IDENTIFICATION, DISTRIBUTION AND ECOLOGY OF VECTOR SNAILS OF HUMAN SCHISTOSOMIASIS

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Abstract: Many surveys were carried out in Ethiopia from January 1969 to January 1971 to study freshwater molluscs, especially the intermediate and potential host snails of *Schistosoma mansoni* and *S. haematobium,* to collect their ecological data, and to clarify the distribution of the snails in the country. The gastropods collected consisted of two orders, the Prosobranchia and Pulmonata. The former order contained three families (Thiaridae, Viviparidae and Valvatidae) and the latter four families (Planorbidae, Physidae, Lymnaeidae and Ancylidae). The pelecypods contained four families: the Unionidae, Mutelidae, Corbiculidae and Sphaeriidae. *Biomphalaria pfeifferi rueppellii* and *Bulinus (Physopsis) abyssinicus* are the most important hosts of *S. mansoni* and *S. haematobium* respectively. The freshwater snail species could be grouped into two distibution patterns, one of which is ubiquitous and the other sporadic. *B. pfeifferi rueppellii* and *Bulinus sericinus* belong to the former pattern and *Biomphalaria sudanica* and the members of the subgenus *Physopsis* to the latter. Pictorial keys were prepared for field workers of schistosomiasis to identify freshwater molluscs in Ethiopia. Habitats of bulinid and biomphalarian snails were ecologically surveyed in connection with the epidemiology of human schistosomiasis. Rain falls and nutritional conditions of habitat appear to influence the abundance and distribution of freshwater snails more seriously than do temperature and pH, but water current affects the distribution frequently. Slight pollution of water brought about by immigration of people with their domestic animals under reclamation plans of land often results in abundance of snails in the waters and subsequently new endemic foci of schistosomiasis.

Two species of human schistosomes are endemic in Ethiopia: *Schistosoma mansoni* and *S. haematobium.* Some species of freshwater snails of the genera *Biomphalaria* and *Bulinus* are known as the intermediate hosts of these blood flukes in various parts of Africa. But, it has been insufficiently examined in Ethiopia what species of *Bulinus* and *Biomphalaria* act as the intermediate hosts and how wide they are

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distributed in the country. Ayad (1956), Brown (1964, 1965), Burch (1965) and others conducted surveys to collect the molluscan intermediate hosts and to know their distribution in Ethiopia, which, however, did not sufficiently cover broad range enough to elucidate the state of the case.

During the stay in Ethiopia surveys were many times undertaken to know the distribution and habitat conditions of the intermediate and potential snail hosts of the human schistosomes in this area. In surveys and experiments on medical and veterinary important flukes such as the schistosome, common liver fluke and paramphistome, it is frequently a troublesome task for parasitologists, physicians and veterinarians to identify intermediate host snails, because they have few characteristics available to discriminate each other. Meskal (1967) prepared an identification key of Ethiopian freshwater gastropods which had been known in the country, but the key was not pictorial. Hence an attempt was made to prepare the pictorial keys of Ethiopian freshwater molluscs for field work.

Materials and Methods

The surveys were carried out through the country from January 1969 to January 1971.

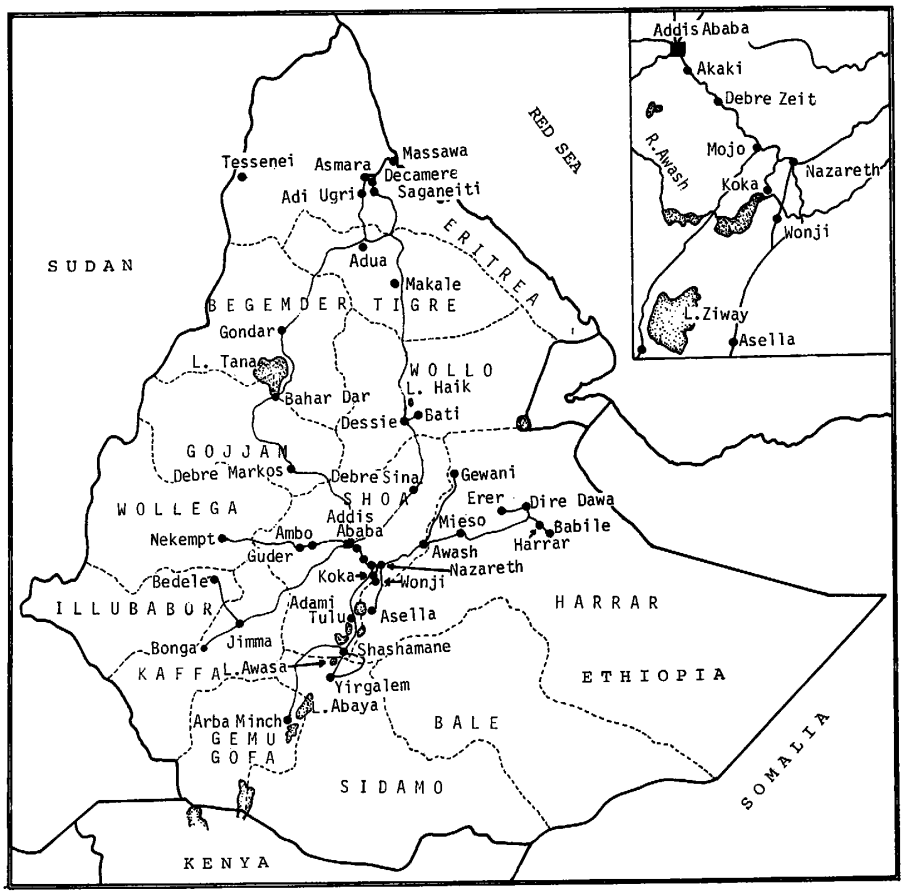

Fig. 1 Ethiopia. Boundaries of provinces indicated by broken lines; routes followed in making the present collection indicated by solid lines.

The collecting loci of molluscs amounted to 336 in number, which are distributed principally along the main all-weather roads (Fig. 1). All the molluscan specimens collected were identified and ecological data of the habitats were recorded in regard to pH and water temperature, associated plants and animals, pollution of water, and so on. pH was principally obtained by measurement with pH indicator papers.

The snails collected were identified by shell characteristics alone, and Brown (1965) was principally followed in the present study to identify and classify Ethiopian gastropods.

Results

1. Freshwater molluscs collected

The present survey revealed the occurrence of the following freshwater molluscs in Ethiopia including gastropods and pelecypods. The gastropoda consisted of two orders of the Prosobranchia and Pulmonata. The former order contained three families: Thiaridae, Viviparidae and Valvatidae and the latter did the four families of Planorbidae, Physidae, Lymnaeidae and Ancylidae. The family Planorbidae contained at least six genera of *Bulinus, Biomphalaria, Anisus, Gyraulus, Segmentorbis* and *Armiger,* and the genera *Bulinus* and *Biomphalaria* include the hosts of the human schistosomes. The Pelecypoda consisted of the following genera of *Pisidium, Sphaerium* and *Etheria,* together with at least one unnamed species of the family Unionidae and two species of Corbiculidae (Table 1).

Table 1 Ethiopian freshwater molluscs collected in the present survey

Class Gastropoda
Subclass Prosobranchia
Order Mesogastropoda
Family Viviparidae
Genus *Bellamya*
B. unicolor
Family Valvatidae
Genus *Valvata*
V. sp.
Family Thiaridae
Genus *Melanoides*
M. tuberculata
Genus *Cleopatra*
C. sp.
Subclass Pulmonata
Order Basommatophora
Family Lymnaeidae
Genus *Lymnaea*
L. natalensis
L. truncatula

Family Planorbidae
Genus *Bulinus*
B. (*Bulinus*) *sericinus*
B. (*B.*) *forskalii*
B. (*B.*) sp.
B. (*Physopsis*) *africanus ovoideus*
B. (*P.*) *ugandae*
B. (*P.*) *abyssinicus*
Genus *Biomphalaria*
B. pfeifferi rueppellii
B. sudanica
Genus *Anisus*
A. natalensis
A. coretus
Genus *Gyraulus*
G. castulatus
Genus *Segmentorbis*
S. angustus
Genus *Armiger*
A. crista
Family Physidae
Genus *Physa*
P. sp.
Family Ancylidae
Genus *Burnupia*
B. caffra
Genus *Ferrissia*
F. clessineana
Genus *Ancylus*
A. sp.
Class Pelecypoda
Family Unionidae
An unnamed species
Family Mutelidae
Genus *Etheria*
E. elliptica
Family Corbiculidae
Two unnamed species
Family Sphaeriidae
Genus *Sphaerium*
S. sp.
Genus *Pisidium*
P. sp.

TABLE 2 Classification of Ethiopian freshwater mollusca

Phylum	Class	Subclass	Family	Subfamily	Genus
Mollusca	Pelecypoda (Bivalves)		Unionidae		
			Mutelidae		*Etheria*
			Corbiculidae		*Corbicula* (?)
			Sphaeriidae		*Sphaerium, Pisidium*
	Gastropoda	Prosobranchia	Thiaridae		*Melanoides, Cleopatra*
			Viviparidae		*Bellamya, Lanistes*
			Pilidae (Ampullaridae)		*Ampullaria*[1)]
			Valvatidae		*Valvata*
			Amnicolidae	Buliminae (Bythininae)	*Gabia*
		Pulmonata	Ancylidae		*Ancylus, Burnupia, Ferrissia*
			Planorbidae	Bulininae	*Bulinus*
				Planorbinae	*Anisus, Gyraulus, Lentorbis, Segmentorbis, Biomphalaria, Armiger*
			Lymnaeidae		*Lymnaea*
			Physidae		*Physa*

1 Empty shells of *Ampullaria* were collected by Brown (1965)

2. The intermediate and suspected snail hosts of the human schistosomes and their distribution in Ethiopia

The genera of *Bulinus* and *Biomphalaria* contain the species incriminated as the intermediary hosts of *Schistosoma haematobium* and *S. mansoni* respectively.

Two species of *Biomphalaria* were collected: *B. pfeifferi rueppellii* and *B. sudanica.* The former species is the most important snail host of *S. mansoni* in Ethiopia, while the latter is of very little importance as the host. *S. haematobium,* on the other hand, has been reported to be transmitted by *Bulinus* snails in various parts of the African continent. The genus *Bulinus* is usually composed of two subgenera, *Bulinus* and *Physopsis.* At least six species of the subgenus *Bulinus* have been reported from Ethiopia: *B.* (*B.*) *sericinus, B.* (*B.*) *forskalii, B.* (*B.*) *scalaris, B.* (*B.*) *hexaploidus, B.* (*B.*) *octoploidus* and *B.* (*B.*) sp., and the subgenus *Physopsis* includes three species in Ethiopia: *B.* (*P.*) *abyssinicus, B.* (*P.*) *africanus ovoideus* and *B.* (*P.*) *ugandae.* Of these species, *B.* (*P.*) *abyssinicus* is the only confirmed snail host of *S. haematobium* in the country.

The freshwater snails have different distribution patterns in Ethiopia according to the species. Speaking roughly, some species have a ubiquitous distribution throughout the country and some are sporadic in distribution. *Bulinus sericinus* and *Biomphalaria pfeifferi rueppellii,* for example, belong to the former pattern of distribution,

whereas the members of the subgenus *Physopsis,* the *forskalii* group complex of the subgenus *Bulinus* and *Biomphalaria sudanica* to the latter pattern. *Bulinus sericinus* was mainly distributed in the areas around Addis Ababa, along the Asmara road from Addis Ababa to Lake Haik, and around Lake Tana and Harer (Fig. 2), while *Biom-*

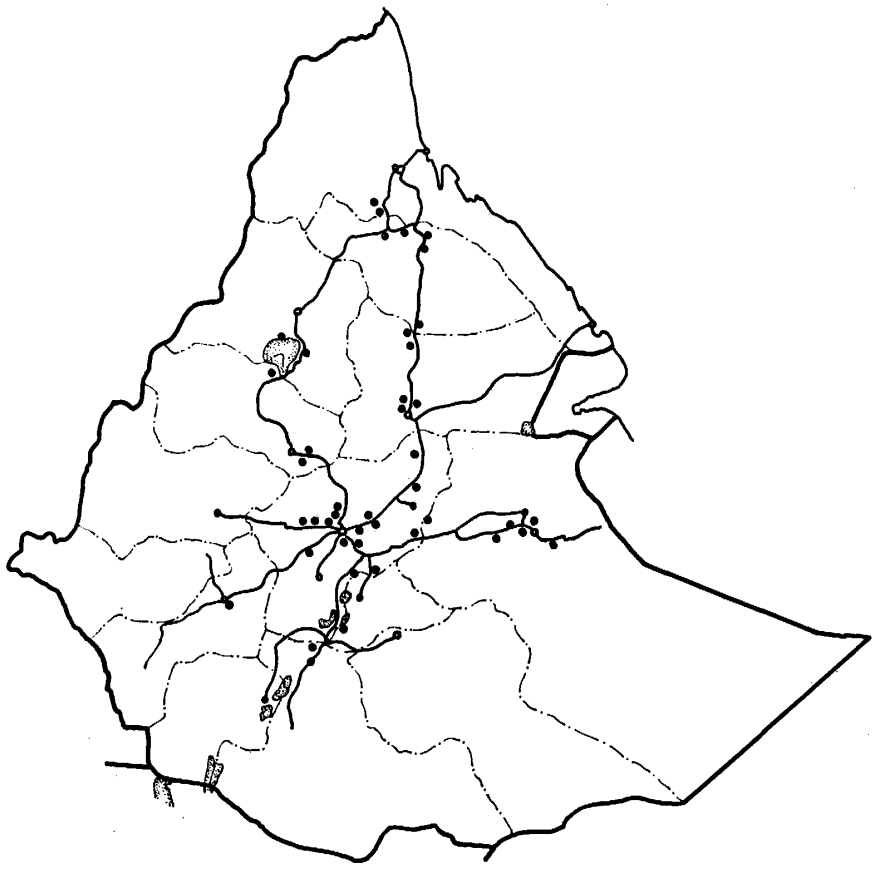

Fig. 2 Distribution of *Bulinus* (*Bulinus*) *sericinus* in Ethiopia.

phalaria pfeifferi rueppellii in the areas along the all-weather roads except the areas between Lake Haik and Makale, from Gondar to the border between the Tigre and Begemder provinces, between Debre Markos and Lake Tana, and Nazareth to Mieso (Fig. 3). The areas without any species of freshwater snails have no bodies of water suitable for the snails to inhabit. *Biomphalaria sudanica* was limited in distribution to some of the Great Rift Valley lakes area (the lakes of Ziway, Awasa and Abaya) and to a separated focus at the Artuma Bilu river (a tributary of the Awash river) about the midst between Debre Sina and Dessie (Fig. 4). *Bulinus* (*P.*) *africanus ovoideus* was collected at some foci in the north-east part of the country, principally along the main road from Bahar Dar to Asmara and in the Little Gibe river on the road from Addis Ababa to Jimma (Fig. 5). *Bulinus* (*P.*) *ugandae* and *B.* (*P.*) *abyssinicus* have a sporadic distribution; the former species was found in Beda Buna (a swamp near Jimma) and Lake Abaya and the latter at Gewani and Ofonofei in the Lower Awash Valley (Fig. 5). *Bulinus* (*B.*) sp. described by Brown (1965) was also found restrictedly in the Great Rift Valley lakes area (Fig. 6) and *B.* (*B.*) *forskalii*

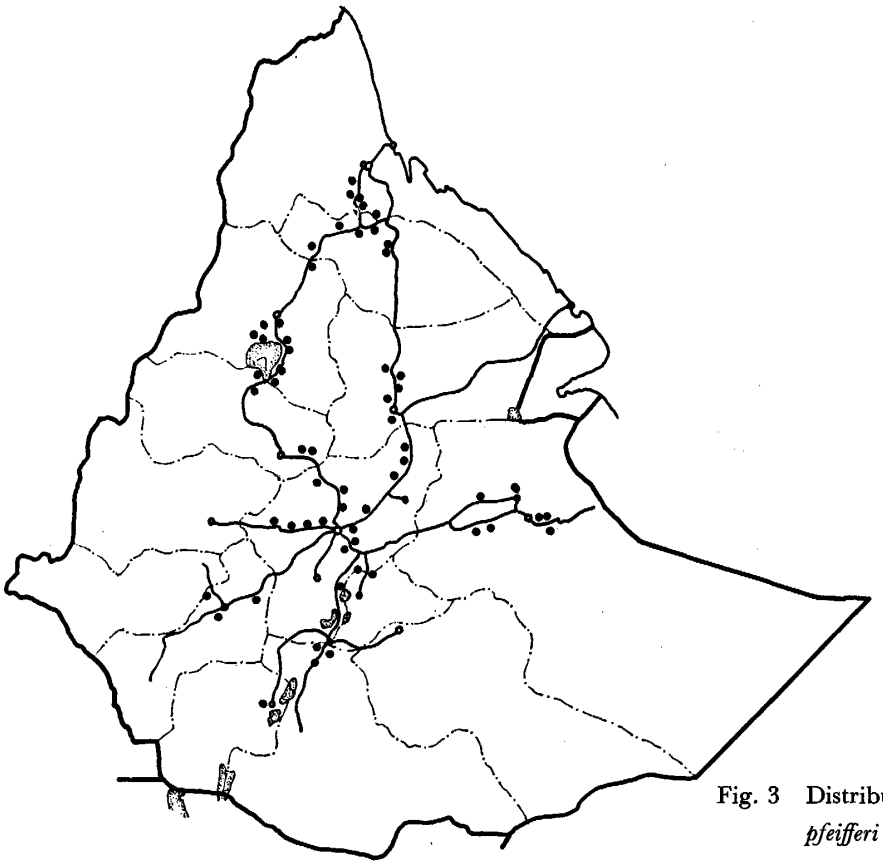

Fig. 3 Distribution of *Biomphalaria pfeifferi rueppellii* in Ethiopia.

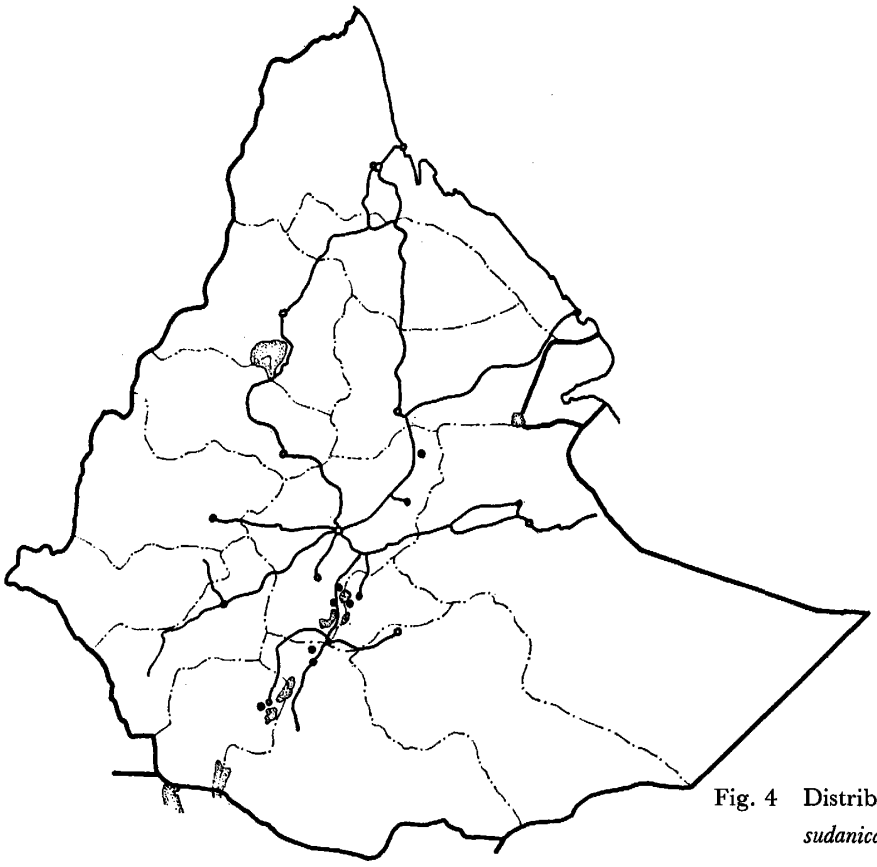

Fig. 4 Distribution of *Biomphalaria sudanica* in Ethiopia.

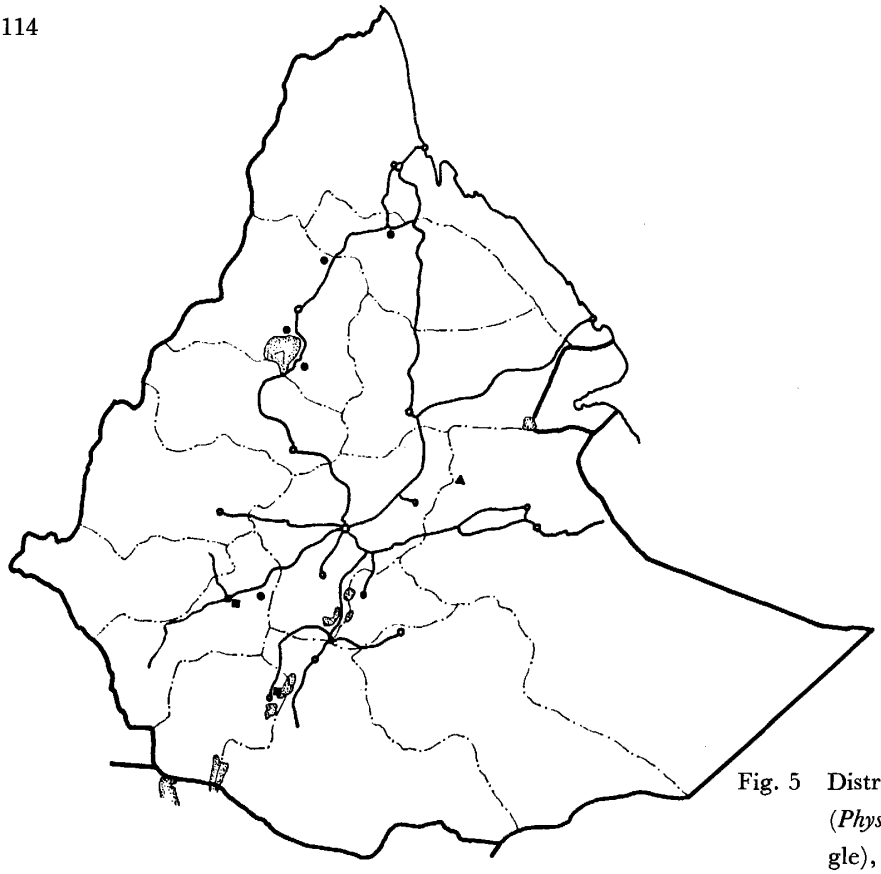

Fig. 5 Distribution of *Bulinus* (*Physopsis*) *abyssinicus* (triangle), *B.* (*P.*) *ugandae* (square) and *B.* (*P.*) *africanus ovoideus* (circle).

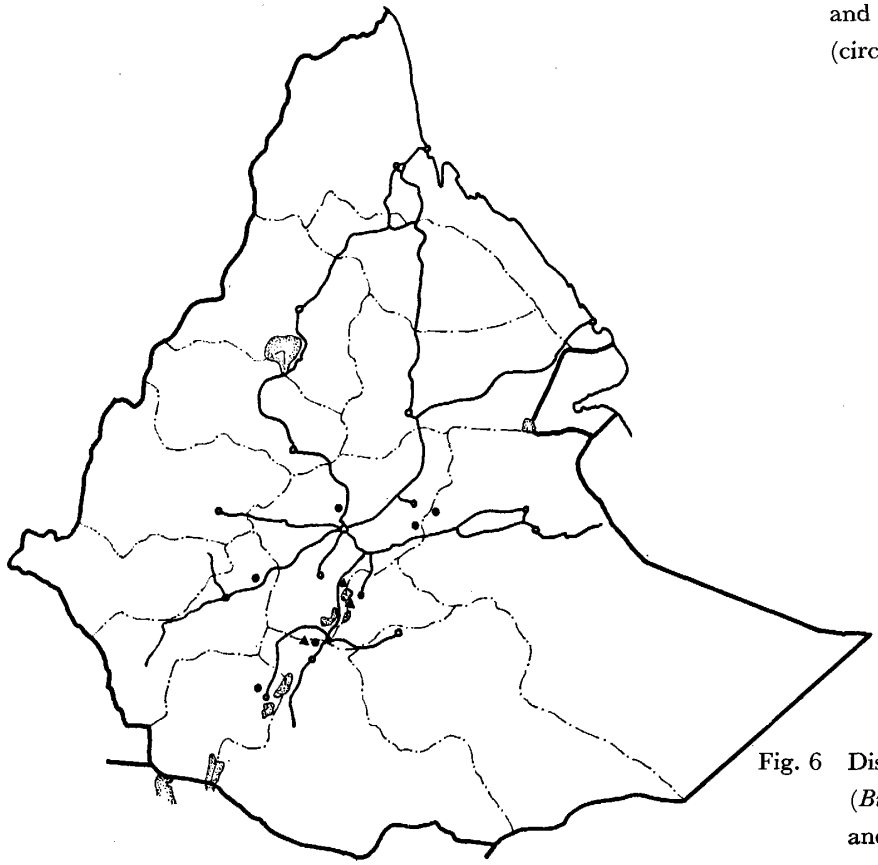

Fig. 6 Distribution of *Bulinus* (*Bulinus*) *forskalii* (circle) and *B.* (*B.*) sp. (triangle).

was also sporadic in distribution (Fig. 6). No snails were found in the area between Nazareth and Awash where no permanent bodies of water exist except for a few alkaline lakes.

3. Environmental factors in snail habitats and their correlation with prevalence of schistosomiasis

Water temperature of habitat ranged from 9.5 to 31.5 C with the maximum frequency near 15 and 22 C in both of the cases of *Bulinus* and *Biomphalaria,* and higher temperatures may be more suitable for *Biomphalaria* than for *Bulinus* (Figs. 7, 8). *Bulinus,* in general, has a tendency to tolerate the wider range of pH than *Biomphalaria,*

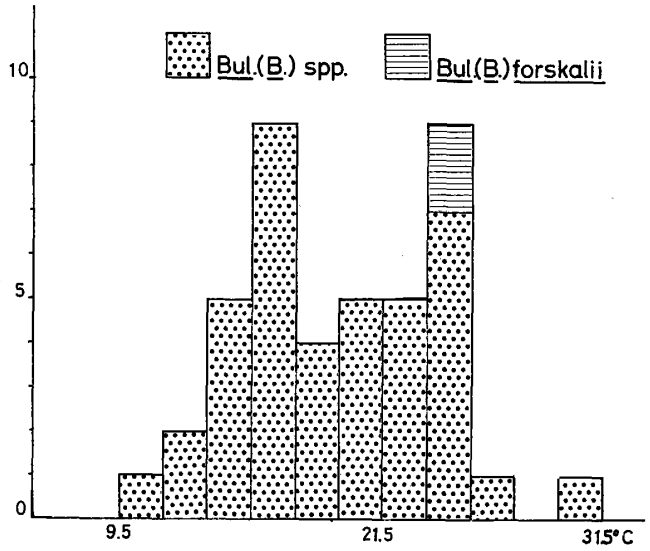

Fig. 7 Histogram showing range of water temperatures of habitats of *Bulinus* snails and frequency (number of localities).

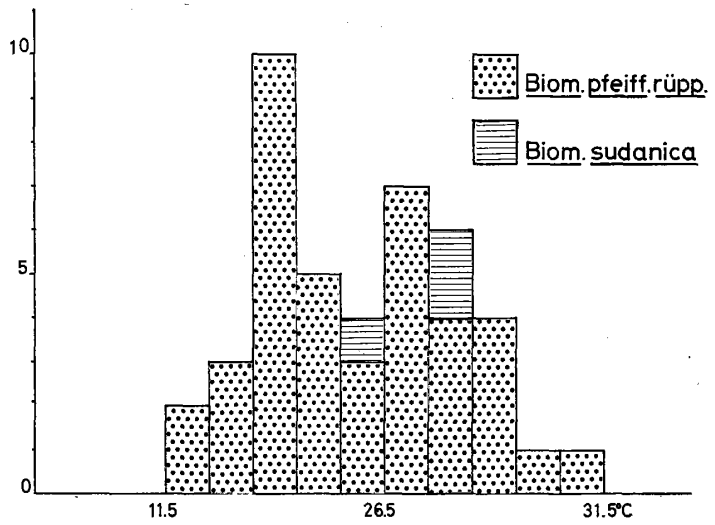

Fig. 8 Histogram showing temperature range of habitats of *Biomphalaria* snails and frequency.

that is, pH range was from 5.3 to 9.5 in the case of *Bulinus* and from 5.4 to 7.8 in *Biomphalaria,* with the maximum frequency in pH ranges of 5.0 to 6.2 and of 6.6 to 7.8 respectively. Consequently hydrogen ion concentration appears rarely to be a limiting factor of the distribution (Figs. 9, 10).

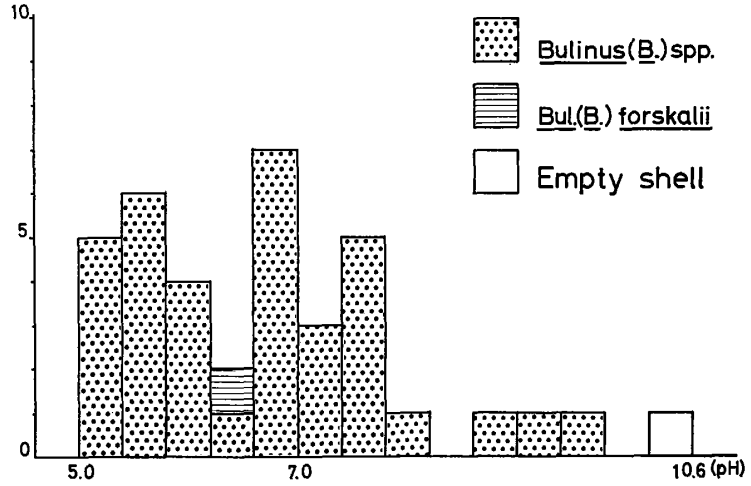

Fig. 9 Showing pH range of habitats of *Bulinus* spp. and frequency.

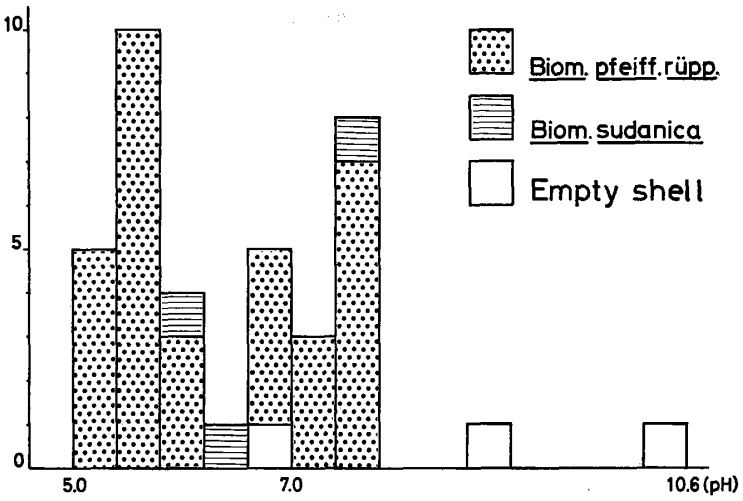

Fig. 10 Histogram showing pH range of habitats of *Biomphalaria* spp.

Two main factors responsible for marked change in abundance of snails will be temperature and rainfall. The rainfall of Ethiopia is seasonal and varies in different parts of the country. The year can be divided into rainy and dry seasons. The rainy season usually consists of the 'little rains' from April to June and big rains from July to September. Freshwater pulmonate snails can breed in temporary waters too and snail populations rapidly increase after the start of rainy season.

Another factor for snails to breed is nutritional. The ability and willingness of the snail hosts to eat almost any organic material offered to them are largely res-

ponsible for the correlation between human pollution and number of freshwater snails; in other words, mild pollution by the human has an enriching effect on the snails. The sites inhabited by a lot of *Bulinus* or *Biomphalaria* snails were often polluted slightly with domestic wastes, excreta of domestic animals and man, and so on; and waters containing much organic materials were frequently accompanied with a large quantity of floating, emersed or submersed vegetation. For example, into a farm along the Kella river near Shashamane many people have immigrated with their domestic animals to result in pollution of the streams where *Biomphalaria* snails crowded on muddy silt. Another example was observed in the Wonji Sugar Plantation where irrigation ditches had been constructed and many people, some of whom were infected with schistosomes, had migrated, so schistosomiasis masoni had come endemic and *Biomphalaria* snails were abundantly found in the ditches.

Another factor influencing snail inhabitation is water current. *Bulinus, Biomphalaria, Lymnaea* and *Physa* species were unusually collected from large and rapid streams such as the Hedie river near Guder, 143 km from Addis Ababa on the road to Ambo, the Gibe river and the Gojeb river on the Jimma road and the Awash river in the Awash National Game Reservoir, in most of which substratum was sandy or rocky and had no or scarce vegetation. The reason for this appears that the snails can not tolerate a high rate of flow and fast stream may wash away muddy silt which contains much of organic materials important as the food of bulinid and biomphalarian snails. Consequently, stream gradient seems to have an important influence on the distribution of those intermediate host snails.

Aquatic animals found with the snails do not always have direct ecological correlation with them, but sometimes they may be fair indicators of habitats suitable for the snails. Some species of molluscs were frequently collected with bulinines or biomphalarians. A high degree of association between *Bulinus* species and such small planorbids as *Anisus* and *Gyraulus* and a very low degree of association between *Bulinus* and *Biomphalaria pfeifferi rueppellii* make it possible to suggest that *Bulinus* has ecological requirements similar to the small planorbids and different from *B. pfeifferi rueppellii. Biomphalaria sudanica,* on the contrary, was frequently collected with *Bulinus* in habitats where luxriant aquatic vegetation was present. *B. pfeifferi rueppellii* appears to inhabit sites with much muddy silt containing organic materials and was often found associated with a small bivalve, *Sphaerium* sp.

Aquatic plants provide good surface for freshwater snails to feed on and lay eggs. Some species of the plants were observed to have a high degree of association with certain species of freshwater snail; the submersed vegetation such as *Potamogeton crispus,* the water weed *Elodea* sp., *Chara* sp., *Hydrilla*-like sp. and filamentous algae, the floating plants such as *Potamogeton* sp., *Nymphaea* and *Lemna*-like sp., *Pygonum* sp., *Cradamine*-like sp., and *Commelina*-like sp. were seen associated with *Bulinus, Biomphalaria* and *Lymnaea. Bulinus forskalii* was frequently collected on the undersides of floating leaves of *Nymphaea* in Lake Awasa. A few sites with luxuriant submersed vegetation and clean water were observed inhabited by many species of freshwater molluscs, for example, the Chacha river about 110 km from Addis Ababa on the road to Asmara and a small body of water 100 km from the capital on the road.

4. Keys of Ethiopian freshwater molluscs

Freshwater molluscs, in general, are difficult to identify, because they have generally not so discriminative features as sea shells. For this reason it will be a necessary but troublesome job for parasitologists, physicians and veterinarians to identify the intermediary and potential host molluscs of human and veterinary important parasites, hence the keys of the host snails, especially the pictorial are useful for the purpose. Pictorial keys were prepared for the identification of important

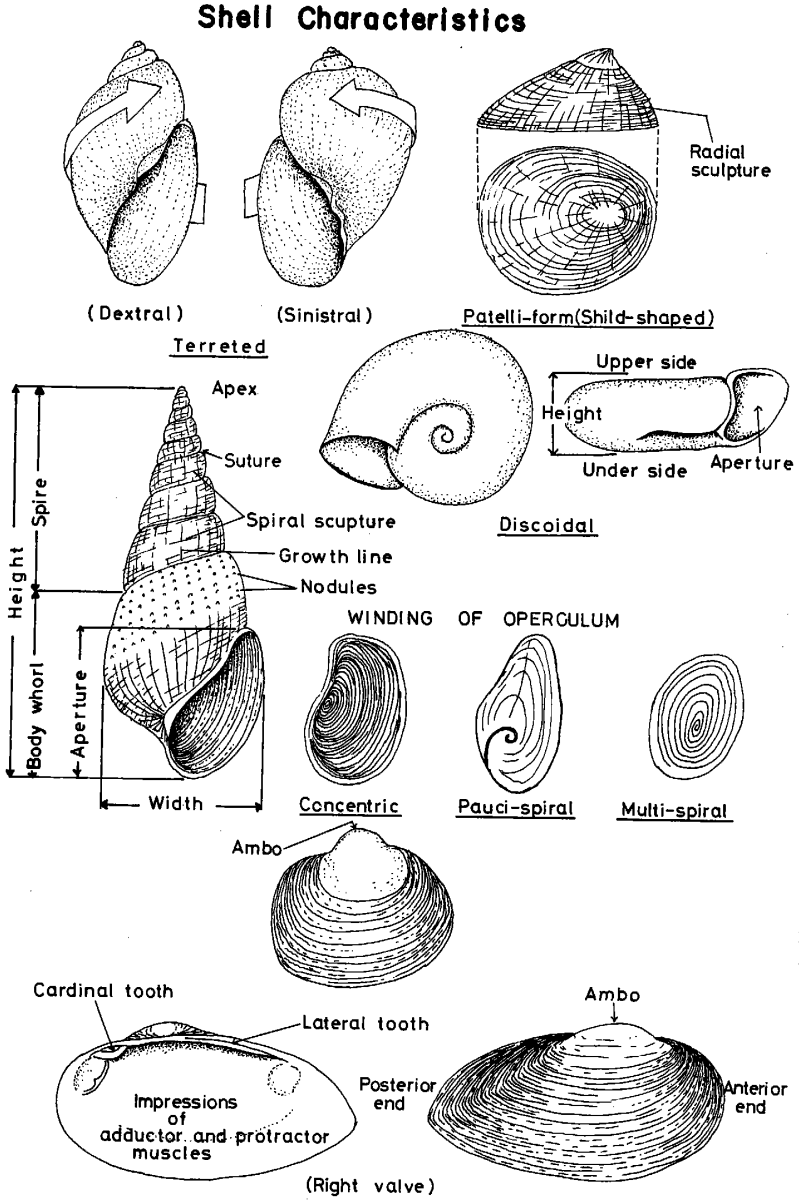

Fig. 11 Showing shell characteristics available for identification of molluscan species.

Ethiopian freshwater molluscs. The identification keys were principally based on shell characteristics for the purpose of field work and the freshwater molluscs reported from Ethiopia were delt with in the present keys (Figs. 11–15, Tables 3–6).

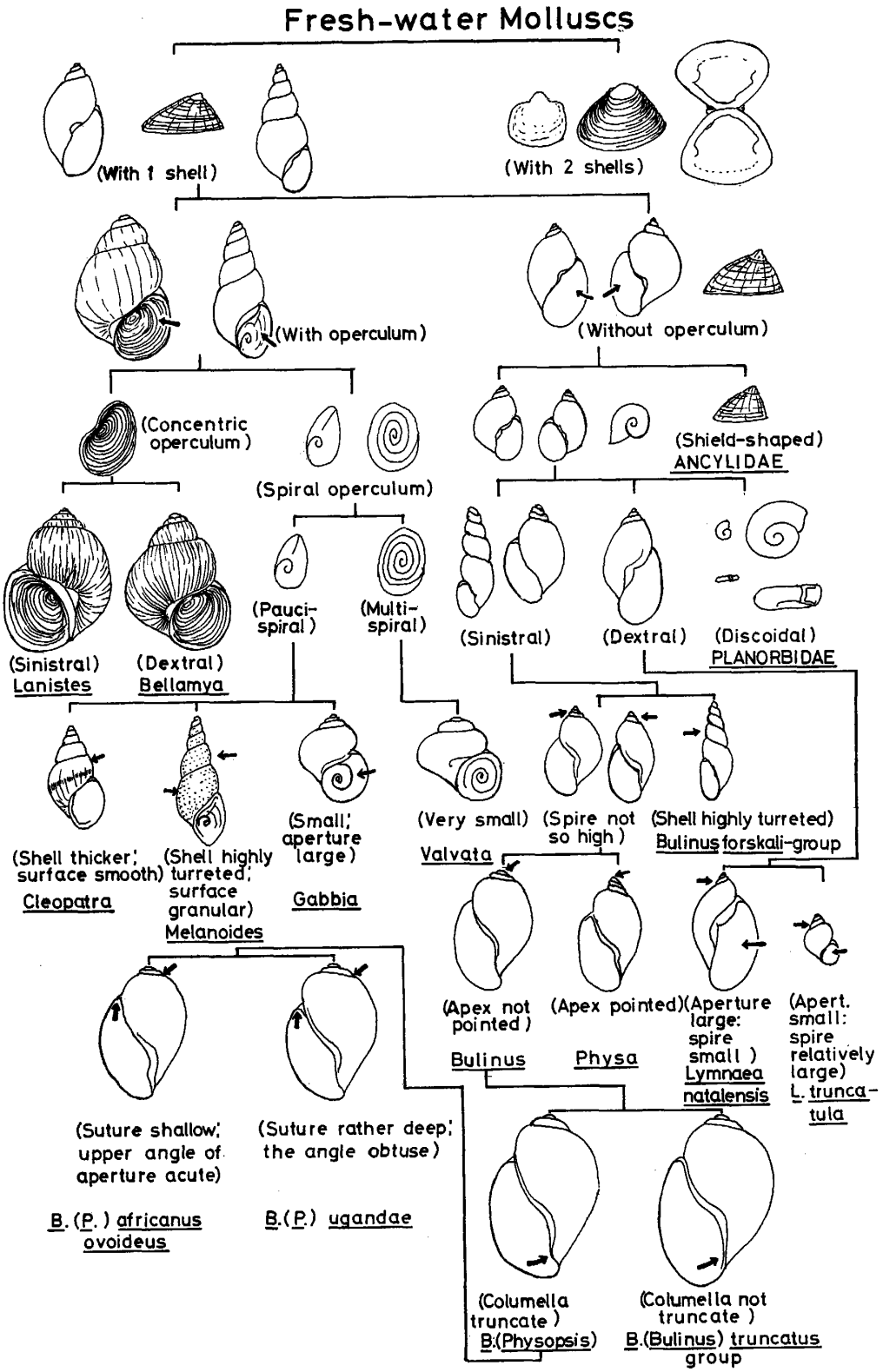

Fig. 12 Pictorial key to identification of Ethiopian freshwater molluscs.

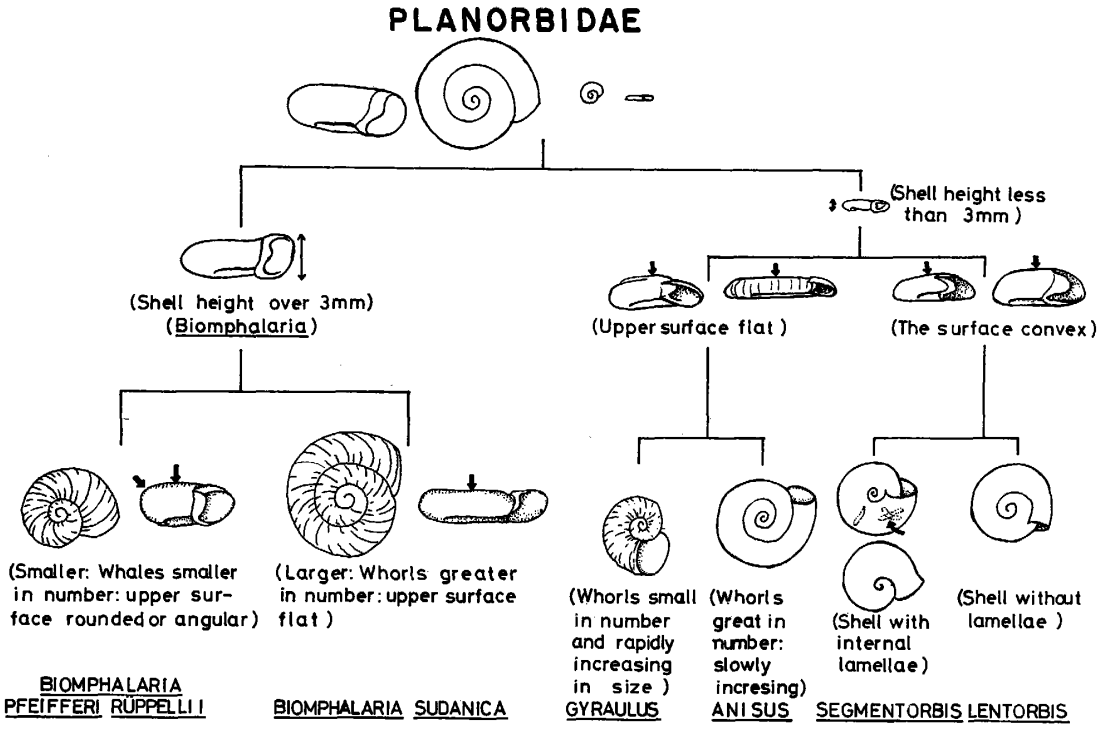

Fig. 13 Pictorial key to Ethiopian Planorbidae.

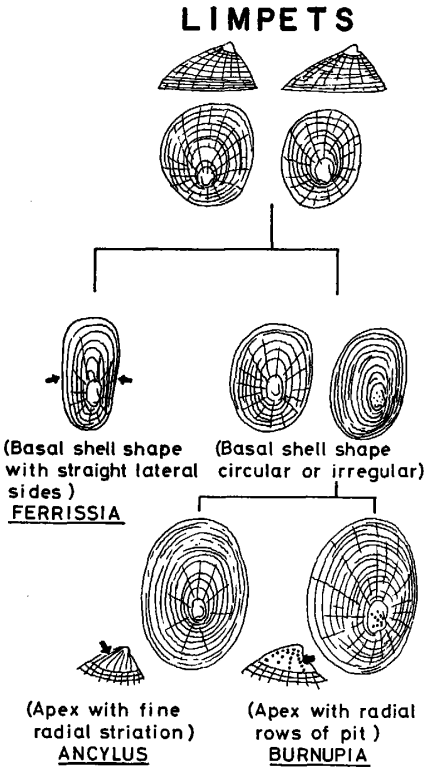

Fig. 14 Pictorial key to Ethiopian Ancylidae (limpets).

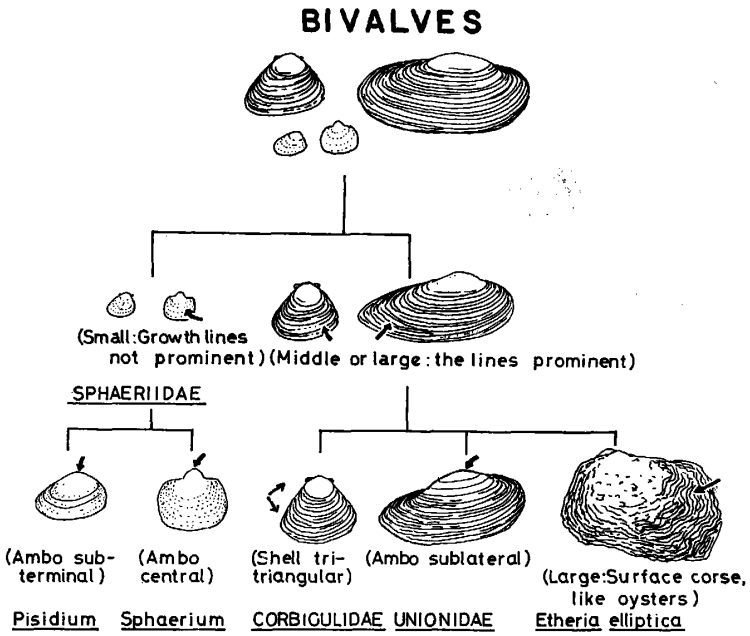

Fig. 15 Pictorial key to Ethiopian Pelecypoda (bivalves).

TABLE 3 Key to the genera of Ethiopian freshwater mollusca

1	Shell one in number and spiral or discoidal or shield-shaped	2 (Gastropoda) (Fig. 12)
	Shell two in number and dish-like	14 (Pelecypoda) (Fig. 15)
2	Shell usually thick and has a lid (=operculum); (radula has 7 teeth in a transverse row; dioecious)[1]	3 (Prosobranchia)
	Shell usually thin and has no lid; (radula more than 7 teeth in the row; hermaphrodite)	9 (Pulmonata)
3	Operculum concentric; shell large, dextral or sinistral	4
	Operculum spiral; shell middle or small, dextral	6
4	Shell very large (up to 10 cm in height), often nearly globose, smooth in surface; dextral or sinistral	5 (Pilidae)
	Shell smaller (less than 4 cm), broad conical; dextral; (right tentacle of male acts as copulatory organ)	*Bellamya* (Viviparidae)
5	Shell dextral	*Pila*
	Shell sinistral	*Lanistes*
6	Operculum multispiral; shell small in size	*Valvata* (Valvatidae)
	Operculum paucispiral; shell small or middle (less than 2 cm in height)	7
7	Shell turreted, surface tuberculate or not; whorls more than 4 in number	8 (Thiaridae)
	Shell turreted, surface smooth; whorls less than 4	*Gabia* (Bithyniidae)
8	Shell tuberculate on surface; (mantle edge fringed)	*Melanoides*
	Shell surface often smooth or with few spiral ribs; (mantle edge smooth)	*Cleopatra*
9	Shell cap- or shield-shaped	Ancylidae (see the key) (Fig. 14)
	Shell spiral	10
10	Shell turreted	11
	Shell discoidal	Planorbidae (see the key) (Fig. 13)
11	Shell dextral	*Lymnaea*
	Shell sinistral	13

12	Shell rather small (7–12 mm) with more slowly increasing whorls; spire as high as aperture; columella not obliquely folded	*L. tuncatula*
	Shell large (up to 25 mm), with rapidly increasing whorls; aperture large, more than two times as high as spire; columella obliquely folded	*L. natalensis*
13	Apex pointed; sutures shallow; columella twisted; (mantle with fringed edge; proboscis with pointed side-corners; no pseudobranch)	*Physa* (Physidae)
	Apex blunt, sutures deep; columella not twisted; (mantle with smooth edge; proboscis with rounded side-corners; with pseudobranch)	*Bulinus* (see the key) (Fig. 13)
14	Shell large or middle in size and thick; concentric growth lines prominent	15 (Fig. 15)
	Shell small and thin; concentric growth lines not prominent	16 (Sphaeriidae) (Fig. 15)
15	Shell sub-triangular in outline; middle in size	Corbiculidae
	Shell blade-shaped or oval in contour; large.....	Unionidae
16	Beak (=ambo) central or subcentral and protruded; shell oval, 2 cardinal teeth in the left valve and none in the right	*Sphaerium*
	Beak sub-terminal and not protruded; shell inequilateral, anterior end of valves longer; 2 cardinal teeth in each valve	*Pisidium*

1 Features in parentheses are those of others than the shell, or supplementary

TABLE 4 Key to the genera of common Ethiopian Ancylidae[1] (Limpet snails) (Fig. 14)

1	Shell almost oval or broadly oval, or rather angular in basal shape	2
	Shell relatively narrow, wider in front than behind in basal shape; lateral sides of shell more or less straight	*Ferrissia*
2	Shell almost oval or broadly oval in basal shape; apex depressed and provided with fine radial striations[2]	*Ancylus*
	Shell variable in basal shape, but frequently with rather angular outline; apex with radial rows of pits[2]	*Burnupia*

1 Some authors have divided the lipet snails into 2 families, Ancylidae and Ferrissiidae; then the genus *Ancylus* belongs to the former and *Ferrissia* and *Burnupia* to the latter

2 Radial striations and rows of pits can be easily seen by removing the periostracum (epidermal horny layer of shell) by submerging shells in a sodium hypochloride solution

TABLE 5 Key to the genera of Ethiopian Planorbinae (Fig. 13)

1	Larger species with a shell more than 3 mm in height (in thickness) (except in very young specimens)	2 (*Biomphalaria*)
	Small species, less than 2 mm high	3
2	Shell size, more than 20 mm in full grown specimens; whorls 5 1/2–6 1/2 in number, more closely coiled; upper side of whorls flat	*B. sudanica*
	Shell size, less than 20 mm in full grown specimens; whorls 5–5 1/2 in number, not so closely coiled as *sudanica*; upper side rounded or angular, or partly flat	*B. pfeifferi rueppellii*
3	Shell serrated in contour and minute in size (2–3 mm)	*Armiger crista*[1]
	Shell without serrated contour	4
4	Shell flat or concave on both sides with only slightly embracing whorls	5
	Shell lens-shaped or convex above and flat below; whorls deeply embracing	6
5	Shell consists of 4–5 slowly increasing whorls	*Anisus*
	Shell consists of 3–4.5 more rapidly increasing whorls; (verge with stylet, pear-shaped at base)	*Gyraulus*
6	Shell without internal lamellae	*Lentorbis*
	Shell has 4–9 sets of internal lamellae	*Segmentorbis*

1 This species is very small and of rare occurrence in Ethiopia, which is not shown in Fig. 13

TABLE 6 Key to the species of Ethiopian *Bulinus* (Fig. 13)

1	Shell highly turreted; spire high; (radular teeth small)	3 (*forskalii* group)
	Shell not highly turreted; spire not so high	2
2	Columella truncate in lower portion	4 (*africanus* group) (Subgenus *Physopsis*)
	Columella not truncate	6 (*truncatus* & *tropicus* groups)
3	Shell greater; spire thicker; aperture larger; lower whorls not shouldered	*B.* (*B.*) *scalaris*[1]
	Shell smaller; spire narrower; aperture smaller; the whorls shouldered	*B.* (*B.*) *forskalii*
4	Whorls flattened near suture to form no or blunt shoulder; spiral microsculpture well developed especially on apex; inner edge of aperture folded well over umbilicus, which completely open	*B.* (*P.*) *africanus ovoideus*
	Whorls shouldered; upper angle of aperture obtuse	5
5	Whorls shouldered near suture; especially the upper angle of aperture obtuse and frequently of a right-angle; microsculpture fine, never consisting of well defined nodules; inner edge of aperture folded narrowly over umbilicus which is open	*B.* (*P.*) *ugandae*

5	Whorls convex and shouldered; upper angle of aperture is obtuse, microsculpture in upper part of shell is finely corrugate rather than spirally sculptured; inner edge well folded over umbilicus; colour of shell varying from pure white to a light yellowish grey.....	*B.* (*P.*) *abyssinicus*[2]
6	Ultimate (or body) whorl relatively large and spire larger than in the following species; spiral sculpture occasionally present; colour of shell horny brown	*B.* (*B.*) *sericinus*
	Ultimate whorls large; spiral sculpture well developed on apex in many cases and occasionally present in ultimate whorl also; shell colour of living snails pale straw-coloured or almost white	*B.* (*B.*) sp.

1 Intermediate form of these two species has been found in Ethiopia and another small species belonging to *forskalii* group, *B. reticulatus reticulatus*, is restrictedly distributed in the Begemeder province (37 km south of Gondar) and is like those of *truncatus* group in shape (Brown, 1967)

2 The distribution of this species is restricted in the Lower Awash Valley (Assaita, Gewani, etc.)

3 *Bulinus* sp. from Lake Awasa was proposed by Burch (1964) to belong to the *tropicus* group

Discussion

Surveys had insufficiently conducted on the distribution of human schistosomiasis in Ethiopia. Broadly speaking, *Schistosoma mansoni* infection was found mainly in the highland areas, but has also been reported to be endemic in the Lower and Upper Awash Valley (Lemma, 1969). Namely, this infection was recorded from Asmara, Decamere, Maaraba, Saganeiti and Adi Ugri in Eritrea, Bahar Dar in the Gojjam province (Ayad, 1956), Harrar in the Harrar province (Ayad, 1956), Tessenei in Eritrea (Nutrition Survey, 1959), Gorgora north of Lake Tana in the Begemder province, Adua in the Tigre province (Duncan, 1970), the regions along the Gojeb river near Bonga (Oliver and Buzo, 1964) and around the lakes of Awasa, Langano and Ziway (Oliver and Buzo, 1964; Ito *et al.*, 1973), and the Akaki and Adami Tulu districts (Ito *et al.*, 1973). On the other hand, urinary schistosomiasis is mainly restricted to the warm and arid lowlands such as the Middle and Lower Awash Valley and the endemic foci were reported in the Gewani and Awash districts (Russell, 1958; Lo, 1970a, b), though immigrated patients have been detected in Addis Ababa, Massawa, Tessenei and other regions.

Bulinines act as the intermediary hosts of *Schistosoma haematobium* and *Biomphalaria* snails are those of *S. mansoni*. Experimental infections of *Biomphalaria* and *Bulinus* snails with *S. mansoni* and *S. haematobium* respectively have yielded divergent results. This appears to be caused by the difference in susceptibility of the different species,

subspecies or local populations of the snails to the different strains of the schistosomes.

The genus *Bulinus* is usually divided into two subgenera *Bulinus* and *Physopsis*, though another subgenus *Pyrgophysa* may be added to the above two. By the way, four species groups of *Bulinus* were proposed by Mandahl-Barth (1958) instead of the subgenus system and he considered the 4-group system was the better classification method in view of parasite-host relationship. The four groups are the *africanus*, *tropicus*, *truncatus* and *forskalii*; and the first group belongs to the subgenus *Physopsis* and the rest to the subgenus *Bulinus*. If the subgenus *Pyrgophysa* is considered to be valid, the fourth group belongs to it. The *africanus* group, confined in distribution to the Ethiopian geographical region (south of the Sahara), contains important hosts of the schistosomes with terminalspined eggs including *S. haematobium* in the African continent. The *truncatus* group involves the host species of human and cattle schistosomes. On the other hand, the *tropics* group has not been known to be the hosts for the schistosomes and the *forskalii* group contains only two species implicated as the snail host of the urinary schistosome in the African continent (Mandahl-Barth, 1958; Wright, 1966), hence these two groups are generally of little importance as the vector snails of the human schistosomes. All the four groups will occur in Ethiopia, if the species "*sericinus*" is considered to belong to the *truncatus* group by means of the features in the radula, mantle pigmentation, reproductive maturity and proportion of euphallic specimens as stated by Brown (1965) and Wu and Burch (1973), and a *Bulinus* species from Lake Awasa, tentatively considered by Brown (1965) a member of the *truncatus* group, rather belongs to the *tropicus* group by the fact that it has haploid chromosome number ($n=18$) (Burch, 1964). Of the four groups the *africanus* and *truncatus* groups will be most suspected for transmission of human urinary schistosomiasis. The sole incriminated host of *S. haematobium* in Ethiopia is *B. abyssinicus* (Lo, 1970a, b), which is also responsible for the infection in Somalia. In addition to this species *B. africanus ovoideus* and *B. ugandae*, both belonging to the *africanus* group, occur in Ethiopia. The former species is responsible for transmitting *S. haematobium* in Kenya (Teesdale, 1962), while the latter is not the host of the blood fluke in Uganda, the Lake Region of Tanzania (Cridland, 1955) and Kenya (Teesdale and Nelson, 1958). Hence *B. africanus ovoideus* may act as the host in Ethiopia too.

Bulinus sericinus had been considered to be a suspected vector of the urinary schistosome which has been recorded from a broad range of the Ethiopian plateau up to 2,900 m elevation (Brown, 1964a, b, 1965), but the incrimination of this species will require further verification. Lo (1972) failed to infect *Bulinus* sp. ($n=18$) from Lake Bishoftu near Debre Zeit and from Asmara with the Egyptian strain of *S. haematobium*, but recently *B. sericinus* from its type locality (the Toquor river at Kekerka, Ethiopia) was successfully infected with the Egyptian and Iranian strains by Wu and Burch (1973). The so-called "*Bulinus sericinus*" from the Ethiopian highlands, furthermore, has been known to include a few polyploids (Burch, 1967a, b, 1972; Brown and Burch, 1967), of which an octoploid bulinine *B. octoploidus* was infected with the endemic strain of the blood fluke at a rate of 7% (Lo, 1972).

One of the considerable reasons for the different results of infection trials is that *S. haematobium* has local strains or might wrongly contain other related species. LeRoux (1958) stated that the schistosome infecting the *truncatus* group was *S.*

haematobium and that from the *africanus* group was *S. capense* (Harley, 1964), and the local strains of *S. haematobium* and *S. capense* were reported by Wright (1962). Another reason is present in complexity of identifying host snails. *Bulinus (P.) africanus,* for example, is discriminated with difficulty from *B. (P.) globosus* by means of shell characters alone, but reliably done by the characters of the copulatory organ (Wright, 1973). Identification used by Brown (1965) of the Ethiopian freshwater snails was principally followed in the present paper and only shell characters were used for identification. But, other characteristics must be utilized in further study, for differences in immuno-cytological patterns are present between different *Bulinus* populations (Burch and Lindsay, 1970).

Biomphalaria snails containing the vectors of *S. mansoni* are divided into four species groups: the *pfeifferi, sudanica, alexandrina* and *choanomphala,* of which the first two have been reported from Ethiopia (Ayad, 1956; Wright and Brown, 1962; Brown, 1965). *B. pfeifferi* and its subspecies have been confirmed as the hosts of *S. mansoni* (Teesdale and Nelson, 1958; LeRoux, 1961; Webbe, 1962a, 1965; Ito *et al.,* 1973b). But, field and experimental infections of *B. sudanica* and its subspecies with *S. mansoni* revealed that this species varied in susceptibility to the local strains of the parasite (McClelland, 1956; Webbe, 1962a, 1965; Wright, 1962) and, at least in Ethiopia, is not so much responsible for transmitting the parasite (Ito *et al.,* 1973).

Considering the distributions of the blood fluke diseases and of the vector snails and the susceptibility of the snails to the parasites, the most predominant host snails of *S. mansoni* in Ethiopia will be *Biomphalaria pfeifferi rueppellii* and the only host of *S. haematobium* incriminated by field and experimental examinations is *Bulinus abyssinicus* which is principally distributed in the Gewani district of the Lower Awash Valley. In addition to this species of snail, *B. africanus ovoideus* might be a responsible host in Ethiopia. Consequently it will be highly possible that schistosomiasis haematobium becomes endemic in the highlands, when the northern African or Near Eastern urinary schistosome strain would be introduced into the highlands, as surmized by Wu and Burch (1973).

Identification keys have been prepared by previous authors of the intermediate snail hosts of blood flukes and the related species of snail for physicians and veterinarians' use, and Mandahl-Barth (1962a) dealt with the East and Central African freshwater snails and Meskal (1967) with the Ethiopian freshwater gastropods. Meskal's key is not pictorial, with insufficient explanation of terms and with a few mistakes.

Main ecological factors responsible for marked changes in abundance of freshwater snails will be temperature and rainfall in sub-tropical and temperate regions, but in the tropical arid regions such as Sudan and the coastral plain of Tanzania it is only the high temperature of summer that appears to reduce the reproduction (Malek, 1962). The maximum temperature for *Biomphalaria pfeifferi* appears to be about 32 C, but the colonies might not survive temperatures much above 28 C under field conditions (Sturrock, 1966). The optimum temperature for rapid population expansion of *B. pfeifferi* and *Bulinus globosus* is close to 25 C, and at other temperatures the snail expands population much more slowly, surviving favourably at 19 C and not at 30 C (Shiff 1964; Harrison and Shiff, 1966; Sturrock, 1966). *Biomphalaria*

and *Bulinus* snails will be able to tolerate the climatic condition in Ethiopia except very high altitudes of the plateau, but the low temperature might inhibit the development of the intramolluscan stages of blood flukes to limit the distribution of schistosomiasis in the country. The data on prepatent periods obtained from experimental infection with *S. haematobium* conducted in outdoor aquaria in South Africa showed that transmission in natural habitats in this area was curtained during the four coldest months of the year when the mean maximum temperature is about 20 C or less (Pitchford and Visser, 1965). Monthly mean temperatures in Addis Ababa during 1959 and 1967 ranged from 13.6 to 16.4 C with an average of 13.0 C; and monthly means of maximum temperature were 18.9 to 23.3 C with an annual average of 21.0 C and those of mean minimums 6.2 to 10.2 C with an average of 5.8 C (The Geographical Observatory, Haile Sellasie I Univ.).

As to the alkalinity of habitats Watson (1958) mentioned that establishment and multiplication of *Bulinus truncatus* was favoured by neutral or slightly alkaline water, and hard water was better than soft one. In the present survey *Bulinus* was found more abundantly in slightly acid waters, whereas *Biomphalaria* was more abundant in slightly alkaline waters. Bilharziasis vectors appear to tolerate a wide range of pH, generally speaking, from 6.0 to 9.0 (Malek, 1958).

In Tanzania *Bulinus nasutus* rapidly increases in population after the start of rainy season (Webbe, 1962a) and *B. globosus* responds to rainfall by very active breeding in Rhodesia (Shiff, 1964). The same phenomenon was observed in the case of *B. truncatus rohlfsi* in Ghana. *Biomphalaria sudanica* is flushed out of its stream habitats by rains and reaches high densities only during the dry season (Webbe, 1962a). *Bulinus* breeds, in general, more rapidly than *Biomphalaria* under favourable conditions and bulinines were frequently found in Ethiopia at temporary waters such as road-side pits from which clay had been taken for making houses.

Another important factor affecting snail distribution may be water current. *Bulinus truncatus* does not occur in rapidly flowing waters; a rate of 20 to 30 cm/sec is approximately the maximum in which this species is found (Watson, 1950, 1958) and the distribution of *B. glabrata* (=*Australorbis glabrata*) and *B. alexandrina* was also observed to be seriously influenced by stream gradient (Harry and Cumbie, 1956; Pimentel and White, 1959a). Most of large and permanent rivers in the Ethiopian plateau such as the Gibe river on the Jimma road and the Awash river near Awash and Mojo were hardly populated by *Bulinus* and *Biomphalaria* snails, probably owing to rapid current and absence of deposited organic materials.

Changes in the distribution and abundance of vector snails have been reported to be produced by reclamation and rehabilitation of lands which bring about migrations of people and domestic animals, construction of small reservoirs and irrigation channels, damming of streams and mild pollution of waters. In the Aswan province of Egypt the amount of *S. haematobium* infection increased from four- to 40-fold in three years following the substitution of the perennial type of basin irrigation (Wright, 1973). Watson (1958) reported *Bulinus truncatus* was abundant in waters polluted to some extent by human excrement, but was conspicuously absent from areas heavily polluted with animal excrement. In Ethiopia the largest dam is situated at Koka on the Awash river near Nazareth, which was constructed to supply water to power

mills and for irrigation, and the Koka reservoir (Lake Koka) was inhabited by a great number of *Bulinus, Biomphalaria, Lymnaea* and the related pulmonate snails. On executing developing plans the endemic foci of schistosomiasis will possibly spread over the country, as suggested by Ayad (1956).

Some kinds of plants have been frequently observed associated with a peculiar species of freshwater snail. A large part of the association of snails and plants is said to be brought about by the common cause of generally favourable aquatic habitats, rather than to cause and effect (Watson, 1958); but it may be sure that vegetation provides good surface for snails to feed on and to spawn. The food of freshwater snails is algae, especially diatomes, or a combination of this and decaying vascular plants (Watson, 1958; Dazo and Moreno, 1962). A blue green alga, *Oscillatoria formosa,* was proved to be a very satisfactory diet for rearing *Bulinus* (Xavier *et al.,* 1968).

The following species of plants have been reported to be associated with *Bulinus* and *Biomphalaria*: *Nymphaea,* the water hyacinth, *Potamogeton crispus, Potamogeton* spp., *Callitriche, Polygonum,* the rush, *Ranunculus, Carex,* etc. The snails are not always associated with a specific species of aquatic animals, but a certain species of mayfly was observed as an indicator of the habitats of *Biomphalaria pfeifferi* and *Bulinus africanus* in South Africa (de Meillon *et al.,* 1958).

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Appendix

Collecting loci of freshwater molluscs where ecological observations were made. Distance in km shows that from Addis Ababa, when without any notice, and temperatures show those of water. Freshwater snails collected are given in parentheses with abbreviations (A=*Ancylus* sp., An=*Anisus* sp., Ar=*Armiger* sp., B=*Bulinus* (*Bulinus*) sp., Ba=*Bulinus* (*Physopsis*) *abyssinicus*, Bf=*Bulinus* (*B.*) *forskalii*, Bis=*Biomphalaria sudanica*, Bpr=*Biomphalaria pfeifferi rueppellii*, Bs=*Bulinus* (*B.*) *sericinus*, Bu=*Burnupia* sp., E=*Etheria elliptica*, F=*Ferrissia* sp., G=*Gyraulus* sp., Ln=*Lymnaea natalensis*, Lt=*Lymnaea truncatula*, M=*Melanoides tuberculata*, P=*Pisidium* sp., Ph=*Physa* sp., S=*Sphaerium* sp., Se=*Segmentorbis* sp., V=*Valvata* sp.). "*Bulinus sericinus*" (Bs) may contain the related polyploid species.

1. Along the road from Addis Ababa to Asmara via Dessie

River Ordida near Koba, 100 km, 13 C, pH 6.8, (Ln, An, G, Bpr, Bs, V, Bu); River Chacha near Chacha, 110 km, 18.0 C, pH 6.8, (Bpr, Bs, An, V, A); A pool by River Baresa near Debre Berhan, 21.5 C, pH 6.8, (Bs, An); River Baresa near Debre Berhan, about 130 km, 21.0 C, pH 7.4, (G, An); Lake Gorbo (=L. Gobara) in Gorbo Village north of Dessie, 24.0 C, pH 7.6, (Bpr, Bs, Ln); A stream near the slaughter house in Kembolcha, (Bpr); Lake Haik, 24.0 C, pH 8.2, (Bs, G); River Tita in Tita Village near Dessie, 665–670 km, 16 C, pH 6.8, (Bs, An); River Haik in Haik Village, (Ln, Bpr); River Keralu, 19.0 C, pH 7.6, (Bpr, Bs, Ln); River Ajowa in Wuchale Village, 23.5 C, pH 7.2, (Bpr); River Silinga in Silinga Village, 23.0 C, pH 7.0, (Bpr); River Borchenna in Kembolcha, 365 km, 21.0 C, (Ln, Bpr); River Wolke in Kamise Village, 315 km, 22.5 C, pH 7.4, (Bs, Bpr); River Artuma Bilu near Mita, about 303 km, 25.0 C, pH 7.6, (Bis, Ln, An); River Senbete near Senbete, about 256 km, 25.5 C, pH 7.6, (Bpr); River Sibilu (a tributary of R. Mugher) in Chancho Village, about 50 km, (A); River Damotu in Chancho, about 55 km, (Bpr, Bs, A, G, S, V, Bu); River Alellu in Chancho, about 60 km, (Bpr, Bs, An, G, S); A stream, 35–40 km, 20.0 C, pH 5.6, (Lt, A); River Ubebarha, 11 km, 15.0 C, pH 5.6, (A); A stream between Chacha and Debre Berhan, 120–125 km, 9.5 C, pH 5.4, (A); River Kabigiza, 115–120 km in Chacha Village, 11.0 C, pH 5.4, (S, Bs, An, Bs); River Chacha in Chacha Village, 110 km, 16.0 C, pH 5.6, (Bpr, Bs, An, G, V, S); River Ordida in Chacha Village, 100 km, 14.5 C, pH 5.4, (An, G, Ln, Bu, Bs); A pool, 75 km, 18.0 C, pH 5.6, (Bs); River Jisa, 70–75 km, in Sheno Village (Amsgaba Saraishu), 18.0 C, pH 5.6, (An, Bpr); A stream near Yegudo, 65–70 km, in Yegudo Village, 16.0 C, pH 5.8, (Bs, Bpr, Lt); A stream, 65 km, 16.0 C, pH 5.6, (Bpr, Bs, An, S); A stream in Alelfu Village, 60–65 km, 17 C, pH 5.8, (G); River Kamte in Alelfu Village, 55–60 km, 20.0 C, pH 5.4, (Bpr); New Reservoir of Addis Ababa, 30 km, 20.5 C, pH 5.6, (Bs, Bpr, An); A stream 5 km from Kekere, (Bpr); River Borkena, (Bpr, Ln, Bu, P); A stream between Makale and Enda-Sellasie, (Bpr, Ln, Bs, An); River Atai near Atai Town, (G)

2. Along the road from Addis Ababa to Dire Dawa and Erer via Nazareth

Awash Fall in Awash Natl. Pk., (Bu, M, Unionidae); Hot Spring in Awash Natl. Pk., (M, An); River Hiruna in Hiruna Village, 365–370 km, 27.0 C, pH 7.6, (Bpr);

A pool (Hubeta), 390–395 km, 19.5 C, pH 6.8, (small planorbids); River Erer in Erer, 400–405 km, 30.0 C, pH 7.8, (Lt); River Harawe, 535 km, 23.0 C, pH 7.8, (Bpr); A pool near Babile, 555 km, 21.0 C, pH 7.8, (Bs); Lake Alem Maya in Agriculture College, 20.0 C, pH 7.2, (Ln, Bs); Lake Alem Maya near Agriculture College, 515 km, 29.5 C, pH 7.8, (Ln, Bs, An, Bpr); Lake Alem Maya in Alem Maya Village, 505 km, 26.5 C, pH 7.8, (Bs); A small deep pool by Lake Adele, 500 km, 21.5 C, pH 7.6, (Ln, Bs); River Chalenko, about 445 km, 17.0 C, pH 7.6, (Bpr, Lt, Ln, small planorbids); River Akaki in Akaki Village near Addis Ababa, 20.0 C, pH 6.8, (Bs, Unionidae); Lake Chalalaka, 45 km, 22.0 C, pH 7.1, (Bs); Lake Bishoft in Debre Zeit, 24.0 C, pH 9.3, (Bs); Lake Hora Orsendi in Debre Zeit, 24.5 C, pH 9.5, (Bs); Lake Babogaie near Debre Zeit, 26.0 C, pH 8.9, (Bpr, Bs, An, Ln, G, V; all the shells empty, except for 2 *Bulinus* snails); A small stream flowing into Lake Chilotes near Debre Zeit, 28.0 C, pH 6.8, (Bpr, Ln); Lake Chilotes, pH 6.6, (small planorbids); River Mojo crossing the road near Mojo, 24.0 C, pH 6.8, (G, F, P); A small pool in Koka Hotel in Koka, (Ph); Gewani in Harrar province, (Ba)

3. Along the road from Addis Ababa to Adigrat via Bahar Dar and Axum

River Deneba, 5–10 km, 14.0 C, pH 6.0, (F); River Dima in Sululta (?) Village, 20–25 km, 15.0 C, pH 6.0, (Bs, An, Bu, F); River Ragateruma in Gabra Gracha Village, 135–140 km, 14.0 C, pH 5.4, (F); River Dadausarubi near Dagamu, 125–130 km, 13.5 C, pH 5.4, (Bs, An, P); A stream, 125–130 km, 14.0 C, pH 5.4, (An, P); A stream, 115–120 km, 18.0 C, pH 5.6, (Lt); A stream, 110–115 km, 18.0 C, pH 5.6, (Bs, Bpr); River Guru near Debre Libanos, 21.0 C, pH 6.8, (Bs, G, Bpr); River Wasarbu, 18.0 C, pH 5.4, (Bpr, Bu, S, V); River Nagaljele, 45–50 km, 13.5 C, pH 5.4, (Bpr, Bs, G, V); River Adillo, 20–25 km, 14.0 C, pH 6.0, (Bs); River Sululta, (Bs, Bpr, Bu, An, G, Lt, Ln, V, S, P); River Chagel near Debre Libanos, (Bpr); River Ijere Ragagora, (Bpr, G, Bs); River Duber, (Bpr, G, An, Lt); River Chancho near Chancho, (P); A stream between Enda-Sellasie and Gondar, (Bpr)

4. Along the road from Mojo to Awasa via Shashamane

Lake Koka (Koka Reservoir Lake, Lake Galila), 25.0 C, pH 7.0, (Ln, Bs); River Redjebo in Goldjota Village, 24.0 C, pH 5.8, (Lt); River Redjebo, 16.0 C, pH 5.8, (Bs); River Buccanisa in Goldjota Village, 15.5 C, (F, Bu); Lake Shalla, 23.0 C, pH 10.4, (M, Bs, Bpr, V; all the shells empty); River Dadabbu, 18 km from Negelle to Shashamane in Negelle Village, (P); River Garbite, 18.0 C, pH 5.6, (Bu); River Laftu between Adami Tulu and Shashamane, 18.0 C, pH 5.6, (A, Ar); Lake Langano, (M, Corbiculidae; all the shells empty); River Markaoda, 22.0 C, pH 5.6, (Bs, S); Lake Abiata, (M; all empty shells); River Wosha at Hydrogical Station No. 4 (MNCD Awasa Farm), 19.5 C, pH 5.8, (An, Lt, P); River Wolka, 20.0 C, pH 6.0, (P); River Karo, 18.0 C, pH 5.6, (An); River Kella near Philadelphia Mission Clinic, 19.0 C, pH 5.4, (Bpr, Lt, P); River Gomoso at Hydrogical St. No. 8, 17.0 C, pH 5.6, (Bpr, An, P, Lt); River Wodessa, 18.5 C, pH 5.4, (P, A); River Abowassa, (Sphaeriidae); A small stream in the swamp (Marge Wando), 24.0 C, (An, Se); A pool near Black Water River, 21.0 C, pH 6.0, (Bis); A pool (Oa) in Sciella Swamp, 21.5 C, pH 6.6, (An); Lake Awasa (Daleti), north shore, 25.0 C, pH 6.6, (Ln, Bis, B, Bf, An); Lake Awasa, east shore, 1 km from a petrol station, 23.0 C, pH 6.2, (Bpr, Ln, B); Lake Awasa, east shore, 25 C, (Bf, B, An)

5. Along the road from Addis Ababa to Jimma and Bonga

River Fuleha (R. Sabata), 20–25 km, 22.0 C, pH 5.6, (F); River Rebiur between Ghion and Jimma, (A); River Awash crossing the road, (Bs, An); Tadji, (Bs); A stream near Bonga, (Ln); River Ejiasar, (F); River Gibe, (M, Corbiculidae)

6. Along the road from Addis Ababa to Ambo and Nekempt

A stream from Water Reservoir of Addis Ababa, 20–25 km, 16 C, (Bs, small planorbids); River Hobi, 25–30 km, 16 C, (Lt, Bs, Bu); River Wolmera, 35–40 km, 17 C, (Lt, P); River Urunie, 35–40 km, 17 C, (Bpr, G, Bu, P); River Gale, 40–45 km, 21 C, (Bs, Bpr, An); River Birgi, 40–45 km, 18.5 C, (Bu); River Worofe, 65–70 km, 24 C, (Bpr); River Wuluko, 65–70 km, 22.5 C, (Bpr, small planorbids); River Abadebela, 65–70 km, 20.0 C, (Bpr); River Chenktu, 70–75 km, 23 C, (Ln, Bpr, An, Bu, P, Lt); River Danjab, 75–80 km, (A, G); River Bore, 100–105 km, (Bpr, Bs, small planorbids); River Hedie (=R. Guder) near Guder, 140–145 km, 20.0 C, (Bpr, An, A, G, P); River Awaro near Ambo, 120 km, 22.5 C, (Bpr, small planorbids); River Sorbie, 115–120 km, 26 C, (Bpr); River Meti, 110–115 km, 19.5 C, (Bs); River Bukisa, 110–115 km, 24.5 C, (Lt, An); River Gova (?), 30 km, 16.0 C, pH 7.2, (Lt, Bpr, G, Bs, Bu); A stream in a stock farm, about the midst of Holeta River and Holeta Village, 16.0 C, pH 7.2, (Bpr, Bs, An, P); River Chancho, 30–35 km, 18.0 C, pH 5.4, (Lt); River Wolemera, 37 km, 16.0 C, pH 5.6, Swampy habitats near the stream, (P); River Suba, 10 km from Holeta on a road branching off from the main road, (Bs, A, Lt); River Awaro in Awaro Chabe Village, 115–120 km, 17.0 C, pH 5.6, (Bu, G, P, F); River Sorbe, 115 km, in Ambo (Agere Hiwot) Village, 22.0 C, pH 6.2, (Bpr); River Mete, 115 km, 15.5 C, pH 5.4, (Bpr, Bs, G, Bu); River Boren, 100–105 km, 26.0 C, pH 5.8, (Bpr); River Janjab, 75–80 km, 18.5 C, pH 5.4, (G, A); River Kere near Wolencomi, 70–75 km, 14.0 C, pH 5.6, (Bpr, G); River Nacho-Janketobara near Ihud Gebeya, 70–75 km, 14.0 C, pH 5.6, (Bpr); River Abadebela, 65–70 km, 13.0 C, pH 5.8, (Bpr, Lt); River Berga, 55–65 km (?), 16.0 C, pH 6.6, (A); A stream, 50–55 km, 16.0 C, pH 5.4, (P); A small stream in Jakob Stock Farm near the Imperial Central Laboratory & Research Institute, Addis Ababa, (Bu); River Bomba, (Bpr, Lt)

エチオピア産淡水貝類の研究，特に人寄生住血吸虫の中間宿主貝の同定，分布および生態

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1969年1月より1971年1月にわたる期間にエチオピア各地で，マンソン住血吸虫およびビルハルツ住血吸虫の中間宿主貝に重点をおいて数度にわたる採集をおこない，これらの中間宿主貝の分布および生息条件を調査した。採集された腹足類（巻貝類）は2目（前鰓目および有肺目）からなり，前鰓目には3科が含まれ，有肺目には4科が認められた。また，斧足類（二枚貝類）には4科が含まれていた(Table 1)。エチオピアにおいては *Biomphalaria pfeifferi rueppellii* と *Bulinus* (*Physopsis*) *abyssinicus* とが，それぞれマンソン住血吸虫およびビルハルツ住血吸虫の最も重要な中間宿主である。エチオピアにおける淡水貝類の分布には2つの型がみられ，1つは全土に広く分布するもの（広布種）と他は散在するものである。*Biom. pfeifferi rueppellii* と *Bulinus* (*Bulinus*) *sericinus* とは前者の型に属し(Figs. 2, 3), *Biomphalaria sudanica* や *Bulinus* 属の *Physopsis* 亜属に属する各種など(Figs. 5, 6) は後者にはいる。淡水貝の同定は困難なことが多く，住血吸虫症の研究者のためにエチオピア産淡水貝の検索表(Tables 3, 4, 5, 6) および検索図(Figs. 12, 13, 14, 15) を作成した。淡水貝の分布を左右する生態学的条件としては，降雨および生息地の栄養的条件（餌料の多少）が水温やpHなどよりも重要であり，また水流も重要な条件となることがある。地域の開発にともなう人の移住によって水域が排泄物や家庭廃水によって軽度に汚染されると中間宿主貝にとって好適な生息場所を提供することとなり，これらの地点が新しい住血吸虫症の流行地となる可能性がある。

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AN EPIDEMIOLOGICAL STUDY OF HOOKWORM INFECTION IN OKINAWA ISLAND

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Abstract: This is a survey of the incidence of hookworm infection and a study of infection process in a rural village named Haebaru, Okinawa Prefecture. Mass treatment of hookworm carriers was repeated for six years from 1963 to 1968, and its effect on the whole incidence and new and re-infections was studied extensively. In addition, the biological behaviour and resistance of *N. americanus* and *A. duodenale* have been investigated. Incidence of hookworm infection in this community was 32.4%, mostly caused by *N. americanus*. The incidence was highest among the farmers as judged by the age and distribution of infected persons in regard to farming, indicating the correlation between the length of farm work hours and the incidence of hookworm infection. The infection took place mainly in the fields where sugar cane, white gourd melon and Chinese cabbage were planted, according to the investigation of plant distribution, mode of fertilization and the incidence of hookworm dermatitis. Infection was not affected by seasonal difference. The factors contributing to the high incidence of hookworm infection in this area must be the warm humid climate, the unique agricultural pattern of producing sugar cane and other vegetables, and improper disposal of the night soil. Six years of mass treatment of hookworm infection resulted in the decrease of the infection rate, markedly among the aged and children, but insignificantly among the farmers who showed frequent new and re-infections. *A. duodenale* infection decreased markedly, far more than *N. americanus* infection. The incidence of new infection was highest among the adult females, mostly infected by *N. americanus*. The survival time of hookworms in distilled water was longer at low temperature and shorter at high temperature, and the hatching rate was highest at 27 C. *A. duodenale* survived longer than *N. americanus* at high as well as low temperature. Toxicity of fertilizers tested was strongest with calcium cyanamide, followed by ammonium sulfate, superphosphate, and potassium chloride. They affected *N. americanus* much more than *A. duodenale*. Three tested pesticides, Dieldrin, Sumithion and Malathion demonstrated weak toxicity on hookworms. Hookworms, *N. americanus* in particular, proved little resistance to nitric acid, phosphoric acid and lactic acid in the order listed, and also to alkali such as sodium hydroxide, ammonium hydroxide and sodium carbonate. The larvae were vulnerable to sodium nitrite, carbon disulfide and distilled water, showing no difference due to pH.

INTRODUCTION

A number of investigators have reported in the literature their various studies on epidemiology, biology, pathology and clinical aspects of hookworms. As the re-

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sults of these studies, the incidence of hookworm infection declined year after year in this country. Yet, there exist those areas where the incidence is higher than 20%. It was revealed that a high degree of damage has been inflicted in those humid districts, particularly in Kyushu where high temperature and high humidity prevail and notoriously high incidence of hookworm infection is still a major public health problem. Previous studies, however, were concerned merely in the study of incidence based on stool examinations or in the result of mass treatment. There have been few studies concerning the cause of prevalence, environmental conditions and socio-economical factors in order to understand the mode and behaviour of hookworm infection. In fact, it is obvious that hookworm infection is inseparably related to the climate, landscape, soil contents and other given natural environment as well as to socio-biological factors including the life, occupation, age, race and others. Therefore, the study of these various factors is mandatory in establishing an effective eradication programme against hookworm infection in rural districts. On the other hand, the night soil used as a fertilizer is the source of hookworm larvae. Concentration and variety of the hookworm larvae in the night soil and their life cycle play important roles in hookworm infection. The survival and resistance of hookworms in nature and in vivo have been studied by many scholars who agree in weaker resistance of hookworms as compared with roundworms. Because of complex natural conditions, each community has its own individual environment. An extremely unique environment is found in Okinawa Island, which is located in the southern end of Japanese Islands and exposed to a subtropical climate, consequently being affected seriously by hookworm infection which requires an effective eradication programme.

The author selected Haebaru village, Okinawa Prefecture, as a sample of typical Okinawa villages with their specific environment, and studied the mode of hookworm infection there, including the place and time of hookworm infection, since 1963. Mass treatment of hookworm infection was repeated for six years from 1963 to 1968, and the change of infection rate, new cases of infection as well as reinfection and many other consequences were investigated annually. The investigation also included a series of experiments to test hatching and survival of hookworms in vivo and to evaluate their resistance to pesticides and other chemical agents.

Ground of Survey

The ground of this survey, Haebaru village, is located in the South of Okinawa Island as shown in Fig. 1, consisted of 12 small communities including Yamakawa community which was most thoroughly investigated. The total population of the village was 9,858, with 1,863 families. The size of the village is 8,911 km^2, bounded by Shuri-nishihara village in the north, by Yonabaru town in the east, and by Naha City and Tomigusuku village in the west. It has thrived as an old farming village with its numerous roads crossing all over. Regular buslines run on the four national highways running through the village, Route 44, 46, 5 and 40, indicating a convenient location for transportation. Another advantage is its close distance to Naha City, the largest city in Okinawa. Therefore, the increase of industrial plants and their workers has been noted in the recent years, adding suburban features to the village.

Of 1,395 farmer families, which consist 74% of total families, only 278, or 20%, are engaged in full-time farming. The rest of farmers have other occupations such as government officials, business firm employees, construction laborers and military institution workers, beside off-time farming. The sugar cane fields consist 79% of all farms, representing the unique Okinawa agriculture. The small community of Yamakawa, where most of our studies were made, has a population of 695 and 120 families, with a large number of full-time farmers. The landscape is flat and the soil is rich in this community which is ideal for agriculture with its good supply of water. Vegetable production has increased lately, owing to modernized farming technique, making many farmers prosperous.

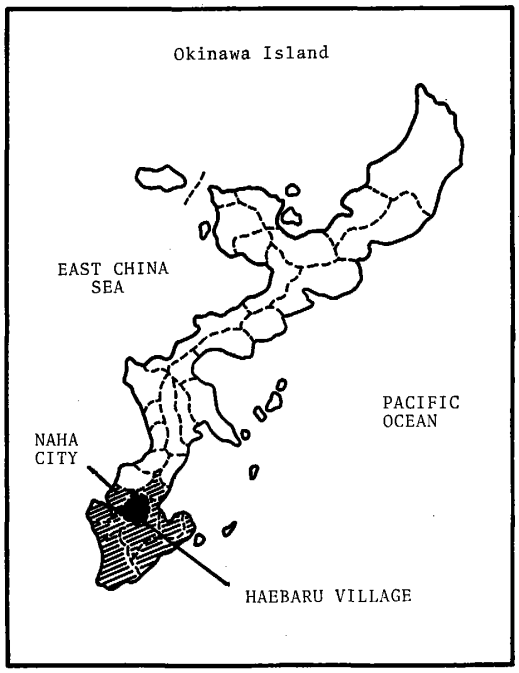

Fig. 1 A map of Okinawa Island showing the areas of the survey.

Method of Survey

1. Stools of all school children and other inhabitants were examined for hookworms in 10 communities of the Southern districts of Okinawa Island for 7 years from 1960 to 1967, applying three methods of test, the thin layer smear, egg concentration, and cultivation.

2. In the community of Yamakawa, stools were examined for parasite eggs on 615 of 695 inhabitants, using the thin layer smear (double cover glass 18×18 mm) and magnesium sulfate centrifugal floatation technique, and the species of hookworms were differentiated using the filter paper cultivation method.

3. Every family in the community was interviewed to investigate on the size of farms, process of farming, disposal of the night soil, history of hookworm dermatitis in relation to the place and season of infection, and other informations helpful to learn

the contributing factors of hookworm infection.

4. For six consecutive years from 1963 to 1968, except the year of 1965, mass stool examinations were conducted annually, and bephenium hydroxynaphthoate and 1-bromo-naphthol were administered to the hookworm-egg positive individuals. *A. duodenale* was treated with bephenium hydroxynaphthoate, 5.0 g per day for adults and 2.5 g per day for children, and *N. americanus* was treated with 1-bromo-naphthol, 6.0 g for adults, 4.5 g for high school children, 3.0 g for primary school children, and 2.0 g for younger children. Two drugs were administered together for mixed infection.

5. After three weeks of medication, stools were examined for negative change and to differentiate the species of hookworms, if they still existed, applying the filter paper cultivation method.

6. Hatching and survival of hookworms were studied under the microscope on the hookworm-egg containing samples cultivated on the filter paper at various temperatures ranging from 4 to 37 C.

7. After cultivating 10 days at 27 C, the infective stage larvae (F-larvae) were dropped in the solutions containing various chemical and medical agents, and their survival time under 27 C incubation was studied. When the survival was questionable, negative response to pin prick was interpreted as dead. Each medium was changed anew every three days.

Results

I. Incidence of Parasite Infection in the Southern District of Okinawa Island

Every year from 1960 to 1967, stool examinations were carried out on 10,531 inhabitants, 5,062 males and 5,469 females, of the 10 communities in the Southern District of Okinawa Island, including school children as well as general public. Tables 1 and 2 are the results of this survey based on three testing methods, thin layer smear, egg concentration and cultivation. The highest incidence of parasite-egg positive carriers was found in Tomigusuku, 39.5% in 1961–1962, followed by Osato, 38.2% in 1961, Kochinda, 33.3% in 1961, Tokashiki, 32.1% in 1960, and Zamami, 28.2% in 1960. The city of Naha showed the lowest incidence of 8.7% in 1961 and 1967.

Among the identified parasite eggs, the hookworm eggs were found most frequently, in 1,757 or 16.7 percent. The incidences of other eggs were; *Ascaris lumbricoides* 315 (3.0%), *Trichuris trichiura* 248 (2.4%), *Strongyloides stercoralis* 93 (0.9%), *Rhabditis hominis* 54 (0.5%), *Hymenolepis nana* 6 (0.1%) and others 130 (12.3%), showing very low incidences of roundworms and whipworms.

II. Mode of Hookworm Infection

In Yamakawa, Haebaru village, stool examinations have been carried out on 615 inhabitants, nearly all residents, using three above-mentioned techniques. As shown in Table 3, there were found 276 parasite-egg carriers (35.1%), including ten among the children younger than 6 years (8.2%), 35 in the 7 to 12 years age group (30.0%), 19 in the 13 to 15 years age group (23.8%), and 152 in the group older than 16 years (51.0%), confirming the high incidence among the adults. The sexual

TABLE 1 Result of stool examination for parasite eggs among the inhabitants in the Southern District of Okinawa Island (1960–1967)

Location	No. of persons		Hookworm	Roundworm	Whipworm	Strongyl.	Rhabditis	Dwarf tapeworm	Others
	examined	positive							
Naha City	1,894	164 (8.7)	56 (3.0)	30	42	11	7		27
Itoman	582	134 (23.0)	115 (19.8)	5	5	10	1		6
Kochinda	1,311	436 (33.3)	318 (24.3)	73	66	28	19		13
Gushikami	759	113 (14.9)	52 (6.9)	33	8	2	15		14
Tamagusuku	950	99 (10.4)	36 (3.8)	29	13	4			3
Osato	152	58 (38.2)	54 (35.5)	2	2	2			1
Haebaru	2,438	537 (22.0)	330 (13.3)	89	71	22	8	1	24
Tokashiki	190	61 (32.1)	47 (24.7)	2	7	3	2		9
Zamami	333	94 (28.2)	48 (14.4)	38	7	2	1		8
Tomigusuku	1,922	760 (39.5)	701 (36.5)	14	27	9	1	5	24
Total	10,531	2,456 (23.3)	1,757 (16.7)	315 (3.0)	248 (2.4)	93 (0.9)	54 (0.5)	6 (0.1)	123 (1.2)
Male	5,062	1,408 (27.8)	1,001 (19.8)	160	137	49	16	1	57
Female	5,469	1,048 (19.2)	756 (13.8)	155	111	44	38	5	73

( ): %

TABLE 2 Number and percentage of hookworm egg carriers

Location	No. of examined subject	Egg carriers			
		A. duodenale	*N. americanus*	Both	Unidentified
Naha City	56		46 (82.6)		10
Itoman	115	3 (2.6)	89 (77.4)	3 (2.6)	20
Kochinda	318	32 (10.1)	245 (77.0)	19 (6.0)	22
Gushikami	52	3 (5.8)	39 (75.0)		10
Tamagusuku	36	3 (8.3)	33 (91.7)		
Osato	54	1 (1.9)	45 (83.3)		8
Haebaru	330	27 (8.2)	235 (71.2)	12 (3.6)	33
Tokashiki	47	9 (19.8)	24 (51.1)	6 (12.8)	8
Zamami	48	12 (25.0)	27 (56.3)	2 (4.2)	7
Tomigusuku	701	88 (12.6)	486 (69.4)	38 (5.4)	89
Total	1,757	178 (10.1)	1,269 (72.2)	80 (4.6)	207
Male	1,001	116 (11.6)	699 (69.8)	48 (4.8)	
Female	756	62 (8.3)	570 (75.4)	32 (4.2)	

( ): %

ratio was 110 males (37.7%) versus 106 females (32.4%), slightly higher among male population. As to the species of parasites, hookworm eggs were found in 199 (32.4%), roundworm eggs in 3 (0.5%), whipworm eggs in 11 (1.8%), strongyloides eggs in 4 (0.7%), and others in one (0.2%), confirming the fact that majority are hookworm-egg carriers.

TABLE 3 Age and sex distribution of parasite egg carriers at Yamakawa in 1963

Age group	No. of persons		Hookworm	Roundworm	Whipworm	Strongyl.	Others
	examined	positive					
0– 6	122	10 (8.2)	9 (7.4)		1		
7–12	115	35 (30.0)	28 (24.3)	1	5	1	1
13–15	80	19 (23.8)	18 (22.5)		1		
16–	298	152 (51.0)	144 (48.3)	2	4	3	
Total	615	216 (35.1)	199 (32.4)	3 (0.5)	11 (1.8)	4 (0.7)	1 (0.2)
Male	292	110 (37.7)	104 (35.6)	2	10		
Female	323	106 (32.8)	95 (29.4)	1	1	4	1

( ): %

1. Variety of hookworms and the state of infection

Table 4 shows the result of filter paper cultivation of stools among 199 hookworm carriers. *Ankylostoma duodenale* was found in 8 (4.0%), *Necator americanus* in 146

TABLE 4 Age and sex distribution of hookworm egg carriers at Yamakawa in 1963

Age group	No. of persons examined	positive			Unidentified
		A. duodenale	*N. americanus*	Mixed infection	
0– 6	9		5 (55.6)		4
7–12	28		18 (64.3)	1 (3.6)	9
13–15	18		12 (66.7)	1 (5.6)	5
16–	144	8 (5.4)	111 (77.1)	10 (6.9)	15
Total	199	8 (4.0)	146 (73.4)	12 (6.0)	33
Male	104	5 (4.8)	73 (70.2)	6 (5.8)	20
Female	95	3 (3.2)	73 (76.8)	6 (6.3)	13

( ): %

(73.4%), and both were found in 12 (6.0%). The ratio between *A. duodenale* and *N. americanus* was 20 versus 158, *N. americanus* outnumbering *A. duodenale*. Slight sex affinity was found between *A. duodenale* and the male population and also between *N. americanus* and the female population. Age distribution of hookworm-egg carriers is shown in Figs. 2 and 3. The incidence is lowest, 7.4% among the children younger

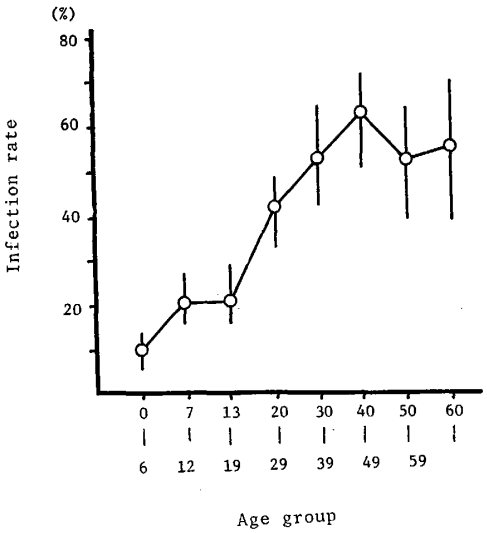

Fig. 2 Age distribution curve of hookworm infection rate at Yamakawa in 1963.

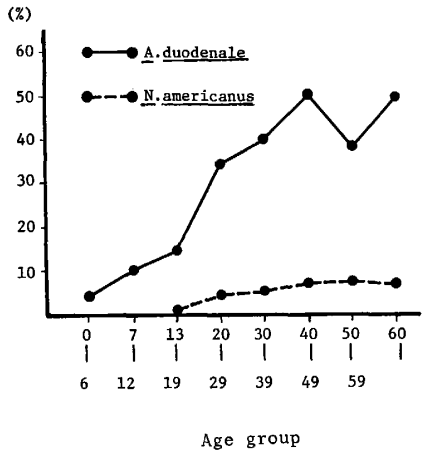

Fig. 3 Age distribution curve of *A. duodenale* and *N. americanus* infection rates at Yamakawa in 1963.

than age 6, but it increases with the age, reaching the maximum 61.1% among the forties, declining thereafter gradually. There was found no significant difference of age distribution between the two species, the peak being in the forties with *N. americanus* (56.0%) and in the fifties with *A. duodenale* (7.8%). In younger generations, the age

distribution curve is evenly low with *A. duodenale*, zero among infants, 0.8% among primary school children, 1.3% among high school children, and 5.9% among the twenties, but it shows a sharp increase with *N. americanus*, 4.1%, 16.5%, 16.3% and 40.6% respectively.

As shown in Table 5, occupations of total 120 families were divided into 88 full-time farming families (483 members), 14 part-time farming families (78 members), and 18 non-farming families (54 members), with their hookworm infection rates of 33.9%, 29.5%, and 22.2% respectively, the rate being higher among the farming families.

TABLE 5 Occupations and hookworm infection rates at Yamakawa in 1963

Occupation		No. of families	No. of persons	
			examined	positive (%)
Farmer	Main job	88	483	164 (33.9)
	Side job	14	78	23 (29.5)
Non-farmer	Office worker	3	54	12 (22.2)
	Carpenter	1		
	Dressmaker	3		
	Shopkeeper	4		
	Driver	1		
	Teacher	4		
	Others	2		
Total		120	615	199 (32.4)

2. Farm activities and hookworm infection

(a) Farm work and its time in regard to hookworm infection rate: This small community has 27.2 hectares of sugar cane fields and 29.4 hectares of vegetable fields. The average size of the field owned by a family is 22.6 ares of sugar cane field and 24.5 ares of vegetable field. There is no rice field in this area. All families were classified into four groups according to the size of farm per person, below 4.0, 4.1 to 7.0, 7.1 to 10.0 and over 10.1 ares. The corresponding hookworm infection rates in these groups were 22.2%, 28.6%, 27.6% and 37.7% respectively, with a proportional correlation of higher incidence of hookworm infection to larger size of farm per family member. The farmer families have higher infection rates than non-farmer families, indicating a close relationship between the farm work and hookworm infection. Duration of farm activities was also classified into five groups, full-time, half-time, intermittent, harvest time only, and none. Hookworm infection rates of first three groups were 71.8%, 60.3% and 40.0%, indicating higher infection rates in the longer farming time groups. When the first four groups were compared with the non-farming Group V, the incidence of the former is much higher, 58.1%, than the latter, 17.3%, as shown in Table 6.

(b) Night soil and its disposal: The disposal of the night soil was also inves-

tigated on 102 farming families. In 66 families (64.7%), the night soil is still a fertilizer, and one-third of the night soil is carried to the field directly from the cesspool. Storage time of the indirectly manured night soil is less than a week in the half of them. This means that most of the night soil is raw when it is manured into the field, suggesting high opportunities for hookworm-eggs to infest the field.

TABLE 6 Comparison of hookworm infection rate between farmers and the others

	No. of persons	
	examined	positive (%)
Farmers	227	132 (58.1)
Others	388	67 (17.3)

(c) Farm work and hookworm dermatitis: In the last five years 25 cases of "Koemake" dermatitis with erythema and quaddel were found and studied. All patients were farmers and 16 of them (64.0%) were full-time farmers. As shown in Table 7, infection occurred in all seasons, 5 in February–March, 10 in April–May, 5 in July–August, and 5 in September–November. The places of infection were, 9 in the sugar cane field, 6 in the white gourd melon field, 4 in the Chinese cabbage field, 2 in the sweet potato field, one in the green pepper and celery field and another one in the bitter cucumber and snake gourd field. There are seasonal prevelences, the Chinese cabbage field in February–March, the sugar cane and white gourd melon fields in April–May, the sugar cane field in July–August, and the Chinese cabbage and cabbage fields in September–November. The sugar cane field seems to be the favorite place of infection throughout a year because of its long growth time. There are three peaks of manure fertilization in a year, the spring, the summer and the

TABLE 7 Season and farm field in relation to "Koemake" dermatitis at Yamakawa (1963 to 1967)

	Season	Feb. | Mar.	Apr. | May.	Jul. | Aug.	Sept. | Nov.	Total
Fields of infection	Sugar cane		6	3		9
	White gourd melon	1	4	1		6
	Chinese cabbage	2			2	4
	Sweet potato	1		1		2
	Cabbage				2	2
	Green pepper and celery				1	1
	Bitter cucumber and snake gourd	1				1
Total		5	10	5	5	25

autumn. And hookworm dermatitis occurred most frequently in April and May when the night soil was added most often, particularly to the sugar cane and white gourd melon fields. Yet, dermatitis is not infrequent in other months. February and March are the months to plant spring vegetables, July and August for summer sugar cane, and September to November for autumn vegetables. These findings indicate the importance of sugar cane and white gourd melon fields as the place of hookworm infection in Okinawa.

3. Change of hookworm infection rate following mass treatment

Mass treatment of hookworm infection was attempted annually for six years from 1963 to 1968 in a model community of Yamakawa, and the subsequent change of infection rate was evaluated. Table 8 shows the number of the tested inhabitants, incidence of hookworm-egg carriers, the number of treated carriers, their rate of negative change, the number of positive remainders, and the species of hookworm discovered. As the result of mass treatment project, the incidence of hookworm infection decreased from 32.4% in 1963 to 16.1% in 1964, 12.7% in 1966, 15.2% in 1967, and 15.2% in 1968. There were 199 hookworm-egg carriers in 1963, and 114 remained positive after the first treatment, but they decreased to 31 in 1964, 41 in 1966, 48 in 1967 and 39 in 1968, approximately one-fifth of the initial number. The numbers of *A. duodenale* infections in the respective years were 3, 2, 0, 0, 0, and those of *N. americanus* infections were 94, 25, 37, 47 and 36, and those of mixed infections were 5, 2, 3, 0 and 1. In the final year, left were almost only cases of *N. americanus*. As shown in Fig. 4, the eggs disappeared from feces after mass treatment, more significantly among the aged and children than among the other adults, though the latter had a higher rate of infection.

TABLE 8 Yearly change of hookworm infection rate under repeated mass treatment of hookworm carriers at Yamakawa

	Year	1963	1964	1966	1967	1968
	examined	615	413	401	416	394
	positive	199	66	51	63	51
	(%)	(32.4)	(16.1)	(12.7)	(15.2)	(15.2)
	treated	164	63	41	53	45
No. of	turned to negative	85	35	10	15	12
persons	(%)	(51.8)	(55.6)	(24.4)	(28.3)	(26.7)
	remained positive (1)	79	28	31	38	33
	untreated (2)	35	3	10	10	6
	(1)+(2)	114	31	41	48	39
	A. duodenale	3	2	0	0	0
	N. americanus	94	25	37	47	36
	Mixed infection	5	2	3	0	1
	Unidentified	12	2	1	1	2

Remarks: Stool examination and mass treatment were not made in 1965

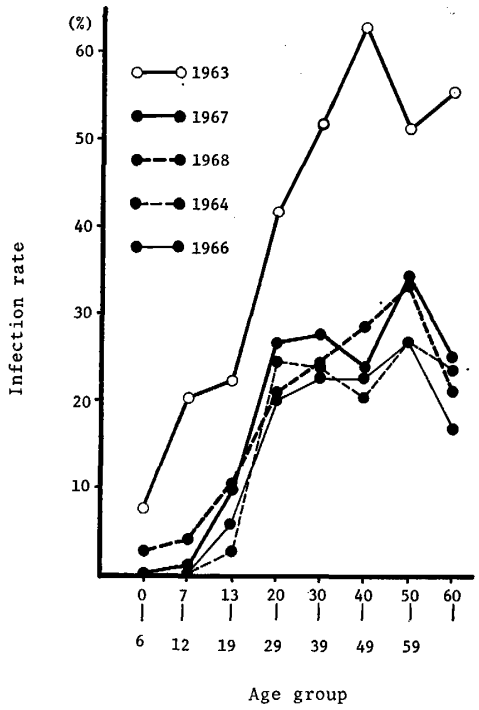

Fig. 4 Yearly change of age distribution curve of infection rates with hookworm eggs at Yamakawa.

Teble 9 shows a follow-up study made for 6 years on these 352 patients to evaluate the trend of hookworm infection, particularly of new and reinfection. The subjects of the study included 170 males and 182 females. Hookworm-eggs were found in 119 of them, 60 males and 59 females, before the treatment. No eggs were found in the remaining 233, 110 males and 123 females. As the results of the treatment, 14

TABLE 9 Six-year follow-up of hookworm carriers at Yamakawa under repeated mass treatment

1963	1964	1966	1967	1968	Male	Female	Total	Remarks
+	+	+	+	+	10	4	14	
+	+	+	+	−	38	44	82	
+	+	−	−	+	4	6	10	
+	−	−	+	−	8	4	12	
+	−	+	−	+	0	1	1	Reinfection
−	+	−	+	−	0	1	1	
−	+	+	−	+	2	1	3	
−	−	+	+	−	2	6	8	New infection
−	−	−	−	+	1	4	5	
−	−	−	−	−	105	111	216	
		Total			170	182	352	

TABLE 10 Yearly change of hookworm infection rate under repeated mass treatment, in relation to age, sex and species

Age group	No. of persons examined	positive in 1963	1964	1966	1967	1968
0– 6	61	4 (6.6)	2 (3.3)	0	0	0
7–15	67	15 (22.4)	3 (4.5)	0	0	0
16–18	34	9 (26.5)	3 (8.8)	2 (5.9)	2 (5.9)	1 (2.9)
19–	190	92 (48.8)	49 (25.8) [1]	39 (20.5) [8]	45 (23.7) [7]	44 (23.2) [1]
Total	352	120 (34.1)	57 (16.1) [1]	41 (11.6) [8]	47 (13.4) [7]	45 (12.8) [1]
Male	170	59 (34.7)	32 (18.8)	21 (12.4) [3]	20 (11.8) [2]	22 (12.9)
Female	182	61 (33.5)	25 (13.7) [1]	20 (11.0) [5]	27 (14.8) [5]	23 (12.6) [1]
A. duodenale	120	15	4	4	2	2
N. americanus		101	54	41 [8]	47 [7]	45 [1]
Unidentified		12	1 [1]	0	0	0

( ): %, [ ]: New infection

(10 males and 4 females) remained as positive carriers, 82 (38 males and 44 females) changed from positive to negative, 11 (4 males and 7 females) changed from positive to negative and again to positive, but 12 (8 males and 4 females) from positive to negative, to positive again, and to negative at last. Nine others (2 males and 7 females) changed from negative to positive and again to negative, and 8 (3 males and 5 females) from negative to positive, whereas the rest, 216 (105 males and 111 females), remained negative. This means that there were 17 (5 males and 12 females) new infection cases and 27 (14 males and 13 females) reinfection cases during 6 years. Yearly distribution of these infection cases according to age, sex and species of hookworms is tabulated in Table 10. New infection cases were found one in 1964, 8 in 1966, 7 in 1967, and one in 1968. They were all adults, mostly carriers of *N. americanus*. It seems extremely difficult to eradicate hookworms by mass medical treatment alone in the endemic community.

III. Resistance of Eggs and Larvae

1. Influence of temperature to maturation: Hookworm-egg containing stools were cultivated in the filter paper at various temperatures from 4 to 37 C at every fifth grade. The results are shown in Table 11. The larvae were born at the temperature ranging from 18 to 37 C. The rate of hatching varied depending on the species of hookworms. The rate with *N. americanus* was 3.5% at 18 C, 8.8% at 22 C, 31.0% at 27 C, 32.8% at 32 C, and 0.3% at 37 C. With *A. duodenale,* the rate was 4.0%,

8.7%, 73.3%, 90.6% and 58.0% respectively, a higher maturation rate than *N. americanus* at any given temperature, with a significant difference at high temperature. There was found no difference in the speed of larva maturation, changing to the infective-stage larvae (F-larvae) in 11 days at 27 C and in 8 days at 32 and 37 C.

2. Temperature and survival time: The survival time of the larvae after hatching was studied at the temperature from 18 C to 37 C, as shown in Table 12. The average survival time at various temperatures was as follows: *N. americanus* survived 66 days at 18 C, 69 days at 22 C, 44 days at 27 C, 27 days at 32 C and 13 days at 37 C. *A. duodenale* survived 74 days, 73 days, 63 days, 45 days and 27 days at the respective temperatures. They survived longer at the lower temperature, regardless the species of hookworms. The longest survival of *N. americanus* at the respective temperatures were 75 days, 75 days, 85 days, 33 days and 16 days, and those of *A. duodenale* were 80 days, 82 days, 70 days, 61 days and 39 days. At the higher temperature hookworms died at the stage of R-larvae.

IV. Resistance of the Infective-stage Larvae (F-Larvae) to Various Chemical Solutions

The survival time of F-larvae kept in the various concentrations of chemical fertilizers, pesticides, acid and alkali, and ovicides was as shown in Table 13. All experiments were conducted at 27 C.

1. Survival time in the distilled water: The survival time of hookworms in the distilled water was studied at the temperature range of 18 to 37 C. The average survival time of *A. duodenale* larvae was 86 days at 18 C, 88 days at 22 C, 69 days at 27 C, 46 days at 32 C, and 12 days at 37 C, and that of *N. americanus* larvae was 74 days, 79 days, 47 days, 17 days and 6 days at the respective temperatures, showing the longer survival at the higher temperature, markedly with *A. duodenale.* Fig. 5 shows the death curve at the different temperatures. The death curve of F-larvae was analyzed to compare the destiny of *N. americanus* with *A. duodenale.* When the horizontal plane is log X and the vertical plane is probit M, then the death curve takes a shape as illustrated in Fig. 6. $Y=4.18\times-1.55$ in *A. duodenale,* suggesting a different death curve due to species. The following study of resistance of the two species of hookworm larvae was conducted at 27 C.

2. Survival time of larvae in the diluted fertilizers: The average survival time of *A. duodenale* larvae in the diluted superphosphate was 18 days in 200× dilution, 19 days in 400×, 29 days in 800×, and 27 days in 1,600×. The average survival time of *N. americanus* larvae was 12 days, 13 days, 15 days and 15 days respectively, significantly shorter than the survival time in the distilled water. The longest survival time of *N. americanus* was shorter than that of *A. duodenale.*

The average survival time of *A. duodenale* in calcium cyanamide solution was 9 days in 200×, 12 days in 400×, 13 days in 800× and 17 days in 1,600× dilutions, whereas that of *N. americanus* was 3 days, 4 days, 7 days and 11 days respectively, much shorter than the former owing to its poorer resistance. In ammonium sulfate solution, *A. duodenale* larvae survived 14 days in 200× dilution, 17 days in 400×, 19 days in 800×, and 23 days in 1,600× on the average, whereas *N. americanus* larvae survived 13 days, 14 days, 16 days and 15 days respectively, suggesting the inhibiting effect of the agent to hookworms, especially to *N. americanus.* In potassium

TABLE 11 Influence of temperature on hatching of hookworm eggs in distilled water

Species	Temp. (C)	EPG*	No. of larva hatched after each incubation period																	
			3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19 (days)	Total (%)
N. americanus	4	200																		0
	8	200																		0
	13	200																		0
	18	600						4	7	1	2	4			1	1			1	21 (3.5)
	22	600					6	25	8	1		3		5	1	1		1	2	53 (8.8)
	27	200		1	3	8	13	7	3	24	2									62 (31.0)
	32	600		153	24	9	8	1	1	1										197 (32.8)
	37	600					2													2 (0.3)
A. duodenale	4	800																		0
	8	800																		0
	13	800																		0
	18	300										1	1		4		3	3		12 (4.0)
	22	300					8	2	4	6	3	3								26 (8.7)
	27	800		48	241	88	28	36	24	12	16	7	5	4	6	6	9	5	2	586 (73.3)
	32	800	238	276	117	43	19	19	4	9										725 (90.6)
	37	800	172	106	98	63	20	20	4											464 (58.0)

* Estimated numbers of eggs in 1 g of the stool used for the experiment

TABLE 12 Influence of temperature on survival of hookworm larvae in distilled water

Sp.	Temp. (C)	EPG*	No. of larvae hatched	No. of dead larvae after each incubation period																												Total(%)	Dead infective larvae (%)	Survival period (days)	
				7			10			15			20						25									30 (days)								Average	Max.
A. duodenale	4	200	0																																
	8	200	0																																
	13	200	0																																
	18	200	21																													0	0	66	75
	22	200	53																													0	0	69	75
	27	200	62	1	3											1						2	1	4	2							14 (22.6)	4 (6.5)	44	85
	32	600	197		2	1					1		1	2	1	6	4					22	58	66	17							181 (91.9)	2 (1.0)	27	33
	37	600	2			1				1																						2 (100.0)	0	13	16
N. americanus	4	800	0																																
	8	800	0																																
	13	800	0																																
	18	300	12																													0	0	74	80
	22	300	26																													0	0	73	82
	27	800	586																													0	0	63	70
	32	800	725	1		1							1				2							1	1							7 (1.0)	1 (0.1)	45	61
	37	800	464	1	1			1		2	1	1	1	2	6	10	16	12	13	11		4	60	111	52			19				324 (69.8)	8 (0.4)	27	39

* Estimated numbers of eggs in 1 g of the stool used for the experiment

TABLE 13 Survival time on infective-stage larvae in various chemical solutions

	Chemical agent	Dilution	Survival of larvae (days)	
			A. duodenale	*N. americanus*
Chemical fertilizer	Superphosphate	200×	14–23 (17.6)	10–13 (11.8)
		400×	12–25 (18.6)	12–16 (13.1)
		800×	18–33 (29.1)	14–19 (15.3)
		1,600×	18–30 (27.4)	14–20 (15.1)
	Calcium Cyanamide	200×	8–10 (9.2)	3 (3.0)
		400×	8–14 (11.5)	3– 6 (3.8)
		800×	8–18 (13.2)	6– 9 (7.2)
		1,600×	9–24 (16.9)	10–11 (10.8)
	Ammonium Sulfate	200×	11–21 (14.2)	12–15 (13.2)
		400×	12–22 (17.2)	13–15 (14.1)
		800×	12–29 (18.5)	14–22 (15.9)
		1,600×	9–26 (23.4)	14–21 (15.1)
	Potassium Chloride	200×	29–43 (36.3)	22–28 (25.1)
		400×	28–45 (38.4)	27–36 (32.7)
		800×	33–55 (45.0)	27–48 (39.4)
		1,600×	39–51 (40.7)	40–52 (46.7)
Pesticide	Dieldrin	100 ppm	27–39 (32.7)	17–28 (20.0)
		10	29–52 (40.6)	19–27 (21.5)
		1	29–55 (41.6)	19–33 (23.9)
		0.1	28–53 (42.9)	20–49 (29.1)
	Sumithion	100 ppm	19–24 (22.0)	13–19 (15.8)
		10	20–35 (30.4)	14–32 (21.1)
		1	21–37 (30.5)	18–33 (23.9)
		0.1	19–42 (38.2)	20–38 (26.8)
	Malathion	100 ppm	23–28 (24.9)	16–19 (17.1)
		10	27–48 (41.2)	19–53 (27.6)
		1	28–45 (37.7)	22–49 (39.5)
		0.1	34–51 (45.7)	20–52 (38.5)
Acid	Nitric Acid	200×	16–20 (17.2)	2– 3 (2.5)
		400×	16–24 (21.2)	5–11 (7.5)
		800×	23–31 (28.6)	10–18 (13.3)
		1,600×	27–43 (34.9)	17–30 (20.9)
	Phosphoric Acid	200×	22–36 (27.4)	5–12 (7.7)
		400×	16–38 (29.1)	12–16 (13.9)
		800×	16–41 (31.6)	15–22 (17.6)
		1,600×	24–50 (35.2)	18–49 (31.0)
	Lactic Acid	200×	23–38 (31.1)	15–22 (17.3)
		400×	23–41 (32.9)	18–31 (20.8)
		800×	26–43 (36.5)	18–38 (23.5)
		1,600×	34–46 (44.9)	22–55 (38.3)

Alkali	Ammonium Hydroxide	200×	22–30 (27.7)	12–13 (12.6)
		400×	20–32 (28.8)	12–14 (13.1)
		800×	29–35 (31.0)	13–16 (13.9)
		1,600×	26–36 (31.1)	13–22 (15.1)
	Sodium Hydroxide	200×	15–31 (24.6)	3– 4 (3.7)
		400×	29–40 (35.7)	14–16 (14.7)
		800×	36–44 (42.7)	14–32 (16.6)
		1,600×	30–51 (45.3)	25–49 (39.8)
	Sodium Carbonate	200×	31–49 (40.7)	25–43 (33.2)
		400×	30–50 (42.4)	25–45 (40.6)
		800×	39–51 (50.2)	25–49 (39.0)
		1,600×	44–55 (52.1)	31–47 (39.1)
Ovicide	Sodium Nitrite	1,000×	17–40 (29.0)	22–30 (24.4)
		2,000×	29–44 (37.2)	22–44 (30.4)
		4,000×	24–42 (35.5)	23–39 (28.2)
		8,000×	23–46 (32.8)	22–41 (27.1)
		16,000×	23–50 (39.3)	22–47 (29.6)
	Carbon Disulfide	200×	27–40 (34.7)	20–29 (24.5)
		400×	15–41 (31.1)	20–33 (25.6)
		800×	17–41 (31.1)	20–43 (28.8)
		1,600×	18–49 (35.0)	20–42 (27.9)
		3,200×	19–42 (34.9)	20–43 (26.5)
	Distilled water	18 C	(86.1)	(73.8)
		22 C	(88.1)	(79.3)
		27 C	(68.6)	(47.2)
		32 C	(45.5)	(17.4)
		37 C	(11.8)	(6.2)
	pH	4	6–33 (29.0)	6–14 (7.7)
		6	23–34 (29.4)	8–16 (10.0)
		8	26–33 (27.6)	13–18 (14.8)
		10	25–33 (28.9)	15–30 (25.9)
	Saline solution	1.3%	16–32 (20.8)	12–25 (17.4)
		1.1	14–34 (20.8)	16–29 (20.7)
		0.9	18–33 (23.0)	18–29 (23.7)
		0.7	16–35 (27.1)	19–33 (26.5)
		0.5	17–37 (31.6)	20–34 (24.7)

( ): Average

chloride solution, *A. duodenale* larvae survived 36 days in 200× dilution, 38 days in 400×, 45 days in 800×, and 41 days in 1,600×, whereas *N. americanus* larvae survived 25 days, 33 days, 39 days and 47 days respectively. In summary, the survival time of hookworm larvae was shortest in calcium cyanamide solution, and prolonged in superphosphate and potassium chloride solutions, demonstrating the strongest parasiticidal action of calcium cyanamide. *N. americanus* larvae proved poorer resistance

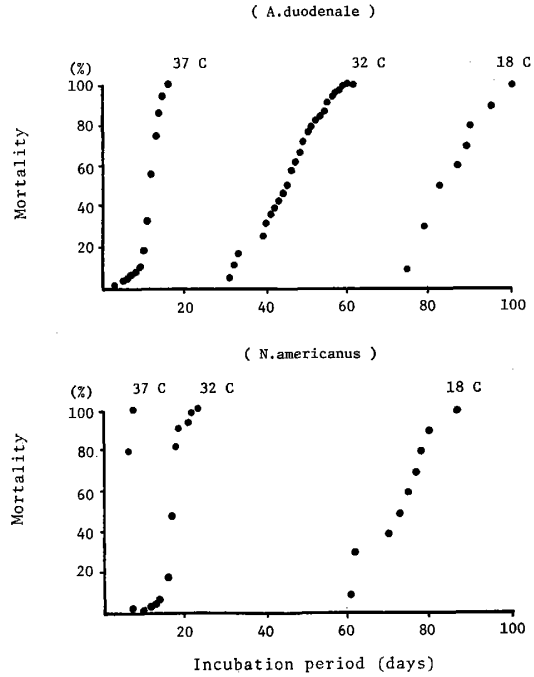

Fig. 5 Survival time of infective stage larvae in distilled water at various temperatures.

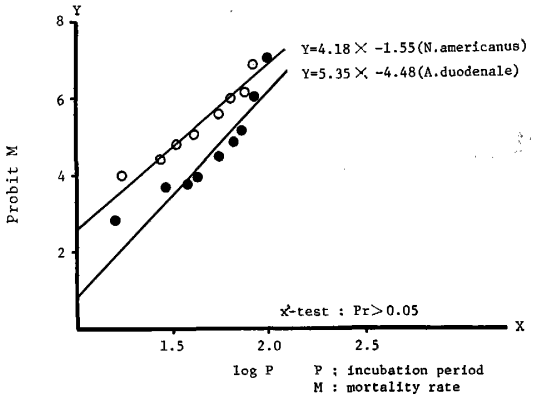

Fig. 6. Cummulative mortality of infective stage larvae in distilled water at 27 C.

than *A. duodenale.*

3. Survival time of hookworm larvae in the pesticides: The survival time of hookworm larvae was tested first in the Dieldrin solution. *A. duodenale* larvae survived 33 days in 100 ppm of Dieldrin, 41 days in 10 ppm, 42 days in one ppm and 43 days in 0.1 ppm, whereas *N. americanus* larvae survived 20 days, 22 days, 24 days and 29 days respectively. In the Sumithion solution, the survival time of *A. duodenale* larvae was 22 days in 100 ppm, 30 days in 10 ppm, 31 days in one ppm and 38 days in 0.1 ppm, whereas that of *N. americanus* larvae was 16 days, 21 days, 24 days and 27 days respectively, the shortest survival among the pesticides tested. In the Malathion solution, *A. duodenale* larvae survived 25 days in 100 ppm, 41 days in 10 ppm, 38 days in one ppm and 46 days in 0.1 ppm on the average, whereas *N. americanus* larvae survived 17 days, 28 days, 40 days and 39 days respectively. Thus, Sumithion is the most potent pesticide followed by Dieldrin and Malathion.

4. Survival time of larvae in acid solutions: The survival time of *A. duodenale* larvae in the nitric acid solution was 17 days in 200× dilution, 21 days in 400×, 29 days in 800×, and 35 days in 1,600×, whereas that of *N. americanus* larvae was 3 days, 8 days, 13 days and 21 days respectively. In the phosphoric acid solution *A. duodenale* larvae survived 27 days in 200× dilution, 29 days in 400×, 32 days in 800×, and 35 days in 1,600×, whereas *N. americanus* larvae survived 8 days, 14 days, 18 days and 31 days respectively, similar to the survival in the nitric acid solution. In the lactic acid solution, *A. duodenale* larvae survived 31 days in 200× dilution, 33 days in 400×, 37 days in 800× and 45 days in 1,600×, whereas *N. americanus* larvae survived 17 days, 21 days, 24 days and 38 days respectively. There-

fore, the pesticidal action is highest with nitric acid and lowest with lactic acid among the three acid solutions tested.

5. Survival time of hookworm larvae in alkali solutions: *A. duodenale* larvae survived in the ammonium hydroxide solution, 28 days in 200× dilution, 29 days in 400×, 31 days in 800×, and 31 days in 1,600×, whereas *N. americanus* larvae survived 13 days, 13 days, 14 days, and 15 days respectively, revealing no significant difference due to dilution, but significantly short survival of *N. americanus* larvae. In the sodium hydroxide solution, *A. duodenale* larvae survived 25 days in 200× dilution, 36 days in 400×, 43 days in 800×, and 45 days in 1,600×, whereas *N. americanus* larvae survived 4 days, 15 days, 17 days and 40 days respectively, obviously less resistant than *A. duodenale* larvae. The average survival time of *A. duodenale* in the sodium carbonate solution was, 41 days in 200× dilution, 42 days in 400×, 50 days in 800×, and 52 days in 1,600×, whereas *N. americanus* larvae survived 33 days, 41 days, 39 days, and 39 days respectively. The parasitocidal action of sodium carbonate was weaker than other two alkali with little difference due to dilution. Therefore, the order of larva-cidal potency of the three alkali is: sodium hydroxide, ammonium hydroxide and sodium carbonate.

6. Survival time of hookworm larvae in ovicides: The average survival time of *A. duodenale* larvae in the sodium nitrite solution was 29 days in 1,000× dilution, 37 days in 2,000×, 36 days in 4,000×, 33 days in 8,000× and 39 days in 16,000×, whereas *N. americanus* larvae survived 24 days, 30 days, 28 days, 27 days, and 30 days respectively, shorter than the controls, with no significant difference due to concentration. In the carbon disulfide solution, the survival time of *A. duodenale* larvae was 35 days in 200× dilution, 31 days in 400×, 31 days in 800×, 35 days in 1,600× and 35 days in 3,200×, whereas *N. americanus* larvae survived 25 days, 26 days, 29 days, 28 days and 27 days respectively, resembling to the result with sodium nitrite.

7. Survival time of hookworm larvae and pH of culture media: The average survival time of *A. duodenale* larvae varied depending on pH of the media: 29 days in pH 4, 29 days in pH 6, 28 days in pH 8, and 29 days in pH 10, whereas *N. americanus* larvae survived 8 days, 10 days, 15 days and 26 days respectively, showing an insignificant difference due to pH.

8. Survival time of hookworm larvae in various dilutions of saline water: Five different concentrations of saline water were used to test the survival time of the larvae. The average survival time of *A. duodenale* larvae was 21 days in 1.3% saline solution, 21 days in 1.1%, 23 days in 0.9%, 27 days in 0.7%, and 32 days in 0.5%, whereas that of *N. americanus* was 17 days, 21 days, 24 days, 27 days, and 25 days respectively, longer survival in lower concentration.

Discussion

Extensive epidemiological surveys of hookworm infection have been carried out by various scholars in many areas of Japan, and their reports have been published one after another. According to these reports, the incidence of hookworm infection is generally high in every prefecture in Kyushu which is known for its subtropical environment. Higher than 50% of infection rate was found in several communities.

In Okinawa, however, the study concerning hookworm infection had been rare before the War. The only report to be mentioned was an article entitled as "A Survey of Parasites in Okinawa and the Result of Eradication Programme" by the Health Center, Okinawa Prefecture. After the War, however, a number of investigations of larger scales have been launched and reported by many authors including Kuniyoshi (1945, 1947, 1949, 1962), Major Hunter III (1949), Shiroma (1956, 1960), Sasa *et al.* (1958), Sato *et al.* (1958), Kuniyoshi *et al.* (1959), Heishiki (1961), Katamine *et al.* (1962), Kawashima *et al.* (1963), Yamamoto *et al.* (1964), Miyahara *et al.* (1964), Kato (1967, 1968), and Tada *et al.* (1968). All these reports confirmed the much higher incidence of hookworm infection in Okinawa in comparison with the other parts of Japan, even higher than the rate of 28.6% reported by the Health Center, Okinawa Prefecture, in 1938. This high incidence is due to mass stool examinations frequently conducted after the War on school children and adult inhabitants and also due to improved technique of egg collection and cultivation. The result of our mass survey in 10 communities of the Southern districts of Okinawa Island, 10,531 individuals in total, showed various incidences of hookworm infection from 3.0 to 64.0%, 16.7% on the average.

Considering the fact that majority of our subjects were primary and secondary school children, the real incidence of the general population must be much higher, although the infection rates of the roundworm and whipworm were as low as 3.0 and 2.4 percent.

All former reports stated the dominance of *N. americanus* among hookworms found in Okinawa (Sasa *et al.*, 1958; Kuniyoshi, 1959; Kinjo *et al.*, 1959; Heishiki, 1961; Yamamoto *et al.*, 1964; Kuniyoshi *et al.*, 1963; and Tada *et al.*, 1968). Our present survey also confirmed the predominance of *N. americanus*. There is no doubt that *A. duodenale* is uncommon in Okinawa. It is noteworthy that *N. americanus* prevalence is more or less limited to the Southern sections of Japan, including Okinawa Prefecture, the Southern half of Miyazaki Prefecture (Shinkado, 1959), and a district in Kagoshima Prefecture, although it has been believed that *N. americanus* was found more in the South than in the North, and more in the lower lands than in the higher altitudes.

After confirming the prevalence of *N. americanus* in Okinawa, the author attempted to study the mode of hookworm infection in its relation to farming, the place and season of infection in particular, in a community called Yamakawa, Haebaru village. The incidence of hookworm infection in Yamakawa was 32.4%, mostly due to *N. americanus,* representing a typical farm community in Okinawa. The incidence was 7.4% among the infant, considerably high for the age, 24.3% among the school children, and 48.3% among the adults, showing gradual increase as the age advanced. A steep increase curve was found at the age range of twenties, i.e. after finishing school and starting as farmers. The highest peak comes at the age of forties, 61.1 percent. The full-time farming families showed higher incidence than the part-time farming families, 33.9 versus 22.2%, suggesting a close relation of infection to farm activities. No difference of incidence was found between male and female population, as expected from the fact that both men and women share the same farm labors.

The farmers consist 31.1% of the whole population, but their incidence of hook-

worm infection was 59.0%, strikingly higher than the rest of the population, 17.3 percent. The incidence is higher among the full-time farmers than part-time farmers. The larger the size of farm, the higher was the incidence of hookworm infection. All the figures indicate that hookworm infection is related to farm work, to frequency and duration of contact with farms.

Treatment of the night soil was investigated on 102 farm houses, and it was found that the night soil was used as a fertilizer in 64.7% of these farm houses, and the raw night soil was poured into the field directly in one-third of the cases. Even when the night soil was stored before the use, half of the stored manure was used within one week of storage. There exist the alarming opportunities of heavy contamination of the fields with fresh hookworm-eggs.

Next come the place and season of hookworm infection in this community. Hookworm dermatitis due to percutaneous infection occurred in 23 farmers, of whom 16 were full-time farmers. There were 10 cases of hookworm dermatitis in April–May, but only five in February–March, as in July–August and September–November. Infection seems to occur throughout the year. The peak of hookworm infection was in May and early September at Saitama Prefecture according to Komiya *et al.* (1953), in April–June at Kagawa Prefecture according to Yoshida (1956), in April to August except June at a village in the suburb of Tokyo according to Suzuki (1959), and in May–June and September–October at Nagasaki Prefecture according to Sakaguchi (1961). It is obvious that the season of infection varies depending upon the agricultural practice, the kind of products and the different climates. The sugar cane field had the highest chance of infection, followed by white gourd melon, Chinese cabbage, green pepper and celery, bitter cucumber and snake gourd fields. The factors contributing to the high incidence of hookworm infection in this district are, therefore, humid warm climate, the type of agriculture producing sugar cane and vegetables, and improper treatment of the night soil.

The most effective method to control hookworm infection in the endemic areas is to conduct a mass treatment continuously. There have been, however, only a few reports of repeated mass treatment in the literature; Matsubayashi *et al.* (1952), Komiya *et al.* (1953), Isoda (1958) and Uchida (1958). That is why the author continued a mass treatment programme annualy from 1963 to 1969 at Yamakawa, a community in the Southern district of Okinawa Island, and studied the change of infection trend, incidence of new cases in regard to age and sex, and comparison of two hookworm species. As stated already, the incidence of hookworm infection prior to the mass treatment programme was 32.4%, mostly caused by *N. americanus*. The incidence dropped to 16.1% in 1964, one year after the first mass treatment, but no further decrease followed in spite of repeated community-wide anti-hookworm medical treatment, owing probably to the egg-positive carriers who failed to take medicine and also due to new infections. Therefore, a follow-up study was made on 352 cases which were examined annually for 6 years. Negative change followed medical treatment among the young and the aged who had relatively lower incidence of carriers, more often among *A. duodenale* carriers than among *N. americanus* carriers. The newly infected individuals during the programme were all adults, as were the 327 reinfected individuals. Concerning the sex, there were more females among the

newly infected cases. In 1968, as the result of 6-year-long mass treatment programme, hookworm infection was found only among the adults of age 20 to 59, and *A. duodenale* has virtually disappeared. It should be emphasized that a full-scale control programme is necessary to eliminate hookworms in an endemic area, although there is no doubt that a mass treatment with anthelmintics is an effective means to prevent and eradicate hookworm infection. It would be ideal if the hookworm larvae could be killed in the soil in the endemic areas.

In the past, the resistance of hookworm-eggs and larvae has been studied by various scholars including Inatome (1932), Sasada (1934), Sano (1952), Kobayashi *et al.* (1955), Kutsumi *et al.* (1955), Yasuda (1957) and Kozai (1960, 1961). All the authors agreed in the low resistance of *N. americanus*. The present author also investigated the resistance of hookworm larvae to various chemical fertilizers, pesticides and other agents, with particular consideration to the practical concentrations applicable to field sprays.

The control survival time of the hookworm larvae in the distilled water was shortest at 37 C, but it was prolonged at 32 C, 27 C, and 18 C progressively, demonstrating the significant influence of temperature. Of the two species of hookworms, *A. duodenale* larvae have stronger resistance and survived longer at any temperature than *N. americanus* larvae.

Both species did not survive long in the fertilizers. Calcium cyanamide proved to be the most potent larva-cide, followed by ammonium sulfate and potassium chloride. Calcium cyanamide of 1,600× dilution can kill *A. duodenale* larvae in 17 days, and *N. americanus* larvae in only 10.8 days on the average. This means that the ordinary concentration of fertilizers is potent enough to eliminate hookworm larvae in the fields, most effectively with calcium cyanamide.

The pesticides can also destroy the hookworm larvae, but none of them was as potent as calcium cyanamide, although only Sumithion, Dieldrin and Malathion were tested among the pesticides.

Among the acid chemical agents, nitric acid had the most powerful action to kill the two species of hookworms, followed by phosphoric acid and lactic acid. Among alkali, sodium hydroxide was most powerful, followed by ammonium hydroxide and sodium carbonate, with a significantly strong effect on *N. americanus*. However, the difference due to concentration was not significant. Both sodium nitrite and carbon disulfide seemed to possess larva-cidal action as well as ovicidal action, because the survival of larvae were shorter in these ovicides than the control. The survival time was prolonged in lower concentrations, although *N. americanus* had a weaker resistance to the ovicides. It can be assumed that spray of chemical fertilizers and pesticides is a most effective means to eliminate hookworm larvae. Therefore, a dramatic eradication can be achieved by using chemical fertilizers in the place of the night soil which is the major source of hookworm infection.

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沖縄に於ける鉤虫症の疫学的研究

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沖縄南部の１農村で鉤虫の感染状況と感染成立の動態要因を観察，６年間を通じて集団駆虫を行って，感染率の推移を追究した。又 *Necator americanus* と *Ancylostoma duodenale* 両種鉤虫の生態と抵抗性について実験的観察を行った。対象となった南風原村の住民の鉤虫感染率は32.4%で大部分は *N. americanus* である。感染率は農耕従事者に画然と高く，しかも農耕作業時間と密接な関係がある。鉤虫皮膚炎の発生からみて最も主要な感染の場所は沖縄特有の甘蔗畑で，冬瓜畑，白菜畑がこれに次ぎ，１月の最寒期を除いて年間を通じて感染が成立しているものと思われる。また農業の形態と屎尿の高率な使用が鉤虫蔓延を助長している。６カ年の集団治療により著明な感染率の低下がみられるが，農業専従者には毎年，新旧両感染がくりかえされている。しかも成人女性に多く，大部分は *N. americanus* である。両種鉤虫感染幼虫の蒸留水中での生存期間は高温で短く，低温で長いが，いずれの温度でも *A. duodenale* で長い。虫卵孵化率は27C 前後で最も高い。各種化学肥料（石灰窒素，硫酸アンモニア，過燐酸石灰，塩化カリ），農薬として用いられる各種殺虫剤（スミチオン，デルドリン，マラサイオン）は一定濃度以上で両種鉤虫感染幼虫の生存に対して影響を与えるが，いずれも *N. americanus* で著明である。又，色々の濃度の酸，アルカリ，（硝酸，リン酸，乳酸，アンモニア，炭酸ソーダ，亜硝酸ソーダ，二硫化炭素）や殺卵剤，食塩水の殺虫作用についても観察されたが，常に *N. americanus* が *A. duodenale* に比較して抵抗力が弱いことが証明された。

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CRYO-PRESERVATION OF THE PARASITIC PROTOZOA

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Abstract: In the present paper, about 200 literatures on the cryo-preservation of the parasitic protozoa have been surveyed, and the following problems have been discussed: cooling rate, storage periods at various temperatures, effects of cryo-protective substances in relation to equilibration time or temperatures, and biological properties before and after freezing. This paper is composed of three main chapters, and at first, the history of cryo-preservation is reviewed in details. In the second chapter, other literatures, which were not cited in the first chapter, are introduced under each genera or species of the protozoa. In the last chapter, various factors on cryo-preservation mentioned above are discussed by using the author's data and other papers in which various interesting problems were described. The following conclusions have been obtained in this study: Before preservation at the lowest storage temperature, it appears preferable that samples are pre-cooled slowly at the rate of 1 C per minute until the temperature falls to −25 to −30 C. The cooling rate might be obtained by the cooling samples for 60 to 90 minutes at −25 to −30 C freezer. For storage, however, lower temperatures as low as possible are better for prolonged storage of the samples. Many workers recommended preservation of the samples in liquid nitrogen or in its vapor, but the storage in a dry ice cabinet or a mechanical freezer is also adequate, if the samples are used within several weeks or at least several months. Cryo-protective substances such as glycerol or DMSO are highly effective to keep higher survival rate of the protozoa in frozen state. Most workers recommended to use 10% glycerol or 5 to 7.5% DMSO for this purpose. For the use of glycerol, at least 30 to 60 minutes of equilibration at temperatures as high as 37 C, is necessary to produce satisfactory results, because at 25 C or lower temperatures, the cryo-protective action of glycerol becomes insufficient. In DMSO, however, samples should be cooled as soon as possible after adding the substance into protozoan suspension. Prolonged equilibration with DMSO is apparently toxic to trichomonad. Many workers pointed out that biological properties of the protozoa such as infectivity, virulence, antigenicity, and drug resistance, were not changed by prolonged period of preservation at the low temperature. These aspects are greatly advantageous for cryo-preservation. By adopting the cryo-preservation technique, furthermore, we save expenses for the maintenance through animal passages or in vitro culture, and thus we can store much more protozoan species or strains in the laboratory. Finally, the author proposed to build the cryo-preservation center of protozoan strains, because in this country we have no adequate center to deposit our strains. Even if nobody use a certain strain for experiment at the moment, yet we can preserve them for the future need. Other laboratories also have many strains, and some of them may be used frequently, but others are not. If all strains be collected in one center, and if the center supplies each strain in case of need, we could save a lot of expense to preserve strains in each laboratory.

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Before the advent of cryo-preservation technique, various strains of the parasitic protozoa had been preserved by animal inoculation or by cultivation in laboratories. For instance, maintenance of *Toxoplasma gondii* (RH strain) or *Trypanosoma gambiense* necessitates storage in mice every 3 to 4 days, and *Trichomonas vaginalis* requires to sub-culture every 48 hours. In our laboratory, we preserve the following parasites by animal passage: *Plasmodium gallinaceum, P. berghei, Trypanosoma gambiense,* and two strains of *Toxoplasma gondii.* Solely to preserve those parasites, 640 mice and 120 chickens are necessary in every year, costing us about 113,200 yen (374 dollars) for the animals. Moreover, we have several strains of *Entamoeba, Leishmania,* and *Trichomonas* in culture media. At a moderate estimation the total expense for time, culture materials, cages or feeds for the animals, might cost more than 300,000 yen (1,000 dollars) a year, which is equivalent about 10% of the total budget per year for our laboratory (6 researchers).

Since the cryo-preservation was adopted widely in various laboratories, it has become possible that not only we could save money but also we could store more various species and strains of the parasitic protozoa in a frozen state, and even some of "type specimens" preserved by other workers can be re-examined in a living state. Furthermore, in many strains, it was often reported that pathogenicity and biological properties of parasite changed gradually during the long period of animal passage or sub-culture. In cryo-preservation, however, such properties of each strain can be preserved without apparent loss or change.

Lumsden and Hardy (1965) and Lumsden (1972) proposed the terms *stabilate* and *stabilation* for population of organisms stored in frozen state. According to them the term *stabilate* means a population of organisms viably preserved "on a unique occasion", and *stabilation* is the process of preparing a *stabilate.* In cryo-preservation, organisms arrest "the continuous reproduction of a population" and thus "avoid the continuous selection" without change of its biological properties such as infectivity, drug sensitivity, and antigenicity. The term *strain* which means organismal populations maintained by serial passage is distinguished from *stabilate.* A *strain* might change during a period of serial passage in animals or in culture media. It is a great advantage to adopt cryo-preservation for workers.

During past 30 years, more than 200 papers concerning cryo-preservation of the parasitic protozoa at various temperatures (−70 to −190 C) were published and most of them were reviewed by Mühlpfordt (1960), A. U. Smith (1961), Diamond (1964), and Dalgliesh (1972). Even today, however, there are still many problems to be solved. The present paper will introduce and discuss the problems in more details from a historical view point.

I. Historical Review

According to A. U. Smith (1961), from the time of Antony van Leeuwenhoek (1676–1677), who first observed cryo-resistance of the protozoa, to the beginning of this century, there are several reports concerning the life and death of the protozoa after freezing and thawing. On the parasitic protozoa, Laveran and Mesnil (1904) showed that trypanosomes survived and retained virulence after exposure for several

minutes to liquid air at −191 C. For example, *Trypanosoma lewisi* survived after 75 minutes at that temperature, and still could infect rats. Gaylord (1908) reported that *Trypanosoma gambiense* survived even after freezing in liquid air for 20 minutes, but could not survive after about 40 minutes, and De Jong (1922) also observed that *Trypanosoma equiperdum* survived for 21 days in liquid air (−191 C).

After these pioneer's trials, Coggeshall (1939) succeeded to preserve *Plasmodium knowlesi* and *P. inui* in a −76 C dry ice box for 70 days, and the parasites were still infective to fresh hosts. His attempt was the first important step in cryo-preservation of the parasitic protozoa. Since his report, many workers have attempted to store their materials at −70 to −190 C, and approximately 200 papers have been reported on cryo-preservation of various protozoa.

1) Coggeshall's technique of cryo-preservation and its modifications

Some early trials of cryo-preservation of pathogenic microorganisms such as spirochetes, *Treponema* spp. or filtrable viruses at −75 C were carried out by several workers including T. B. Turner (1938) during the ten years from 1930 to 1940. These studies have encouraged protozoologists, and Coggeshall (1939) wrote a paper on preservation of malaria parasites. He used the following freezing and thawing technique. One m*l* of the blood containing *Plasmodium knowlesi* or *P. inui* was placed in screw-capped celluloid tube of 2 cm in diameter. With whirling motion each tube was immersed in a mixture of solid CO_2 and alcohol. Then, as soon as the material is frozen, it was rapidly transfered to a low temperature storage cabinet, which was maintained at temperatures between −72 and −80 C by solid CO_2. For testing, the tube was thawed as rapidly as possible in a water bath at 37.5 C. Monkeys were inoculated with 1 m*l* of the infected blood stored at −76 C for 70 days, and both malaria parasites were able to infect to the monkeys. According to Horsfall (1940), Coggeshall's unpublished studies proved that *P. knowlesi* had remained infectious for 140 days, *P. inui* for 151 days, *P. cathemerium* for 35 days, *Trypanosoma brucei* for 135 days, *T. duttoni* for 34 days, and *T. equiperdum* for 62 days. Furthermore, Horsfall described an ideal dry ice cabinet for preservation of microorganisms, and the cabinet had been used by Coggeshall (1939) before Horsfall's report published.

Modifications of Coggeshall's technique were adopted by Archetii (1941) to preserve *Plasmodium gallinaceum* for 50 days at −75 C, and Manwell and Jeffery (1942), and Manwell (1943) who kept avian malaria parasites of 10 different species in the infected blood at −55 to −78 C for a maximum period of 212 days. Manwell and Edgett (1943) described some modifications of cooling method in which they used the tubes with thinner wall varied between 0.018 and 0.030 inches in thickness to increase the rate of cooling and thawing in the storage of *Plasmodium lophurae*. The tubes were rotating during cooling. According to their paper, the superiority of the thinner tubes was also a logical inference from the fact that *Toxoplasma gondii* (may be RH strain) was found to be still viable after freezing and thawing in such tubes, whereas it had never been possible to preserve this species in the thicker tubes (about double thickness of the thinner tube) previously used. They concluded that the temperature for freezing was not so important as the temperature of storage, and it was possible to preserve *P. lophurae* in viable condition after freezing at a temperature as high as −10 C. For storage, lower temperatures were better because

P. lophurae survived for 244 days at −75 C. According to Manwell *et al.* (1945), *T. gondii* was kept frozen only a few minutes in a mixture of dry ice and ethyl alcohol using the thinner tube, but long period of preservation was not reported. F. Wolfson (1945) also preserved *P. lophurae, P. cathemerium,* and *P. relictum* for period varying from 2 days to one year at −75 C. Saunders and Scott (1947) were the first to preserve the human malaria parasite, *Plasmodium vivax.* About 2 to 3 m*l* of the citrated human blood infected with *P. vivax* were put into small vials and the blood was rapidly frozen at −75 C, then stored in dry ice box at −50 C. The parasites survived up to 16 days or longer and, in a case, up to 37 days, and the blood was capable to transmit malaria and induce fever in the patients who have been treated for neurosyphilis by this means. Boyd (1949) also reported that *Plasmodium falciparum* survived for 156 days at −72 to −80 C.

Weinman and McAllister (1947) have studied to preserve many species of the pathogenic protozoa at −70 C. They reported that better results were obtained when the material was first frozen at −15 C and then stored at −70 C than when it was immediately brought to a temperature of −70 C. There were survivors with both procedures, but in the case of *Trypanosoma rhodesiense* or *T. cruzi,* the number of survivors using the former procedure increased 2 to 10 times of the latter. This modified cooling technique is the first proof that slow cooling is less distructive than rapid cooling of the protozoa. According to their paper, following results were included; *Trypanosoma gambiense* survived for 561 days at −70 C, *T. rhodesiense* for 595 days, *T. cruzi* for 653 days, *T. lewisi* for 531 days, *Leishmania tropica* for 794 days, *L. donovani* for 276 days, *Pentatrichomonas hominis* for 407 days, and *Plasmodium lophurae* for 235 days, but *Trichomonas vaginalis, Chilomastix mesnili, Balantidium coli, Tetrahymena geleii* (non parasitic ciliate), and *Entamoeba histolytica* did not survive under the same condition. In the case of *Trichomonas vaginalis,* only 2 out of 10 samples frozen at −15 C and stored at −70 C contained living organisms when thawed. *Toxoplasma gondii* also could survive after freezing, but could not be stored. Later, Weinman (1958) reported that *T. rhodesiense* survived for 8 years at −70 C, and the parasite was still infectious to fresh host. Levaditi (1952) also preserved *Trypanosoma congolense* for 100 days and *Plasmodium berghei* for 15 days at −70 C. The malaria parasite was usually stored as the infected host blood in a frozen state. Only Brumpt and Bao Van Ty (1945) recorded unsuccessful experiment in which blood parasites and sporozoites of *Plasmodium gallinaceum* were rapidly frozen to −25 C, and stored at the temperature, but no viable malaria parasite was observed when thawed. The freezing and storing temperature might have been inadequate. The success of preservation of malaria sporozoites owes to Jeffery and Rendtroff (1955), who reported that viable human malaria sporozoites were obtained after cryo-preservation at −70 C. The longest survival records of each malaria parasite at this temperature were as follows: *Plasmodium vivax* for 375 days, *P. falciparum* for 180 days, and *P. ovale* for 70 days. In 1957, Jeffery again recorded longer survival of sporozoites of *P. ovale* for 997 days under the same condition described above.

The attempts of cryo-preservation, before the discovery of cryo-protective action of glycerol to living cells by Polge *et al.* (1949), were discussed already. As a rule, *Plasmodium* spp., *Trypanosoma* spp., and *Leishmania* spp. are the easiest parasites to

be stored in frozen state without any cryo-protectant. The successful preservation for prolonged period seems to depend upon constant maintenance of temperature of the storage cabinet. In early days, most workers used dry ice cabinet to preserve their materials, because mechanical freezers were not available, and the temperature within the freezers sometimes fluctuated often. Even now, many workers (Lumsden, 1972, and others) recommended to use chemical refrigeration (dry ice or liquid nitrogen) for cryo-preservation of the protozoa rather than mechanical refrigeration.

2) Discovery of cryo-protective effect of glycerol

In 1938, T. B. Turner described that viruses placed in glycerol and maintained at refrigerator temperature will remain virulent for a number of weeks. In this case, glycerol have been used only as suspended media of the material. The effect of glycerol as a cryo-protectant was not recognized until Polge *et al.* (1949) found that glycerol had a protective action to the bull semen in cryo-preservation. Stimulated by their finding, many workers tried to use glycerol in cryo-preservation of the parasitic protozoa (Fulton and A. U. Smith, 1953, may be the first). At that time, one of the most important problems was how to avoid contamination of *Tritrichomonas foetus* to the bull semen, because artificial insemination may spread trichomonad infection among cattles. Since the bull semen can be stored safely at −79 C in the presence of glycerol (Polge and Lovelock, 1952), the possibility was examined by workers if *T. foetus* also can survive with spermatozoa after freezing and thawing procedure. Joyner (1954) answered that *T. foetus* did not survive at some concentrations of glycerol tolerated by bovine spermatozoa: they could be eliminated from infected semen by overnight storage at 5 C in 29% glycerol, or preferably, by freezing to −79 C in the presence of 10% glycerol (cited from Shorb, 1964). Soon McWade and Williams (1954) reported that *T. foetus* could survive in prepared milk semen extender in the presence of 7.5% glycerol at −79 C for 125 days. Leidl and Mahrla (1954) also pointed out that the trichomonads were tolerated in the horse serum broth with 10% glycerol frozen at −99 C. Joyner and Bennett (1956) again confirmed that *T. foetus* failed to survive freezing and thawing in the presence of 10% glycerol (incubation for overnight at 5 C) in egg yolk citrate diluent under the same condition which was adequate for sperm survival. They believed that *T. foetus* were particularly sensitive to the toxic effects of glycerol when suspended in other media such as egg yolk phosphate or milk.

Prior to Joyner's and other papers, McEntegart (1954) reported that the most satisfactory condition of cryo-preservation of trichomonads was found to be storage at −79 C following slow cooling recommended by Fulton and A. U. Smith (1953), the trichomonads being suspended in medium containing 10% glycerol. The "slow cooling" was carried out by the "slow addition" of powdered solid CO_2 into alcohol bath in which samples in ampoule were immersed, and from 0 to −15 C cooling was in the rate of 1 C per minute, below −15 C in the rate of 4 C per minute. According to McEntegart, *Trichomonas gallinae* was easily preserved and survived for 6 months or more even without glycerol, and *T. vaginalis* remained viable for 4 months but died out after 6 months. *Pentatrichomonas hominis* in the presence of 10% glycerol survived for 6 months, but with 5% glycerol did not survive beyond 4 months, and *Tritrichomonas foetus* could not survive under any of the conditions tested. More

recently McEntegart (1959) has reported that the survival period of *T. vaginalis* at −79 C in the presence of 5% glycerol was prolonged to 26 months, and after rapid thawing, a few sluggishly motile trichomonads were still observed microscopically.

Levine and his co-workers studied intensely on cryo-preservation of *T. foetus*, and their first paper appeared in 1954. The paper was only a short abstract (Levine and Marquardt, 1954), but soon they published its detail in the next year (Levine and Marquardt, 1955). According to their paper, they adopted slow cooling method (1 C per minute) similar to McEntegart (1954), and *T. foetus* survived more than 128 days at −76 C in the presence of 1.0 to 1.1 M glycerol (9 to 10%). They also tested the effect of several compounds related to glycerol such as ethylene glycol, 1, 2, 3, 4-butanetetrol, 1, 2-propanediol, and others, and concluded that while glycerol was the most effective compound studied, several related compounds also protected the protozoa against the injurious effects of freezing. In the next experiment, Levine *et al.* (1958) wanted to determine some of the factors which affect the protective action of glycerol both against the immediate effects of freezing and against the subsequent slow deterioration during the storage in a −20 C freezer in the presence of 1.0 M glycerol. They thought that if *T. foetus* died of slow metabolism during prolonged preservation at this temperature, it might be possible to prevent the death by adding of the antimetabolites such as malonic acid, sodium fluoride and sodium iodoacetate. For this purpose, their selected concentration of the antimetabolites failed to lower the metabolism of trichomonads at 37 C, but when the medium frozen, remaining liquid changes to a high concentration of these antimetabolites sufficient to prevent slow metabolism. However, they found that the addition of antimetabolites did not improve the survival. They also experimented the addition of lechitin to the medium in the hope of decreasing the brittleness of the cell membrane, but survival rate did not increase. Increase of the sodium chloride concentration of the medium decreased survival rate of *T. foetus*. They also examined optimal concentration of glycerol in freezing at −21 C, and reported that it was 1.5 M. According to the author's experience (Miyata, 1973a) at −20 C, the medium similar to the one used by Levine *et al.* sometimes did not freeze in the presence of 10% glycerol (about 1.0 M), and the medium maintained a super-cooled state, in which trichomonad still survived. It was not clear whether the medium of Levine *et al.* froze or not, and also even if the medium already froze, question remained whether trichomonad froze or not. Salt concentration was examined by the author (Miyata, 1973a), and somewhat different result was obtained from Levine *et al.*: in *T. vaginalis* it might be said the survival rates in salt concentration of 0.9 to 1.5% were higher than those in lower concentrations.

In other experiment, Levine *et al.* (1959) froze *T. foetus* at −21 C in the presence of 1.0 M glycerol at different phases of the growth curve, and they concluded that when the trichomonad froze during initial and logarithmic phases, they did not survive, and optimum survival came after at the peak of the growth curve.

In the papers written by Fitzgerald and Levine (1957 and 1961), the term *equilibration* was used, and they also checked the temperature fluctuation inside the tubes of frozen medium themselves with thermocouples. When equilibration

with glycerol was carried out at 24 C, survival after freezing and thawing was better following rapid equilibration (glycerol was added all at once, and one hour equilibration) than following slow equilibration (1/6 of final amount of glycerol was added each hour for 6 hours, the equilibration time, 7 hours), but survival of *T. foetus* was extremely poor following either rapid or slow equilibration at 4 C, and glycerol might become toxic at this temperature. They also reported that buffering the storage medium to pH 6.4 to 7.1 with glycylglycine increased survival upon freezing, but triethanoamine had no significant effect.

Levine *et al.* (1962) reported that *T. foetus* survived much longer in the extended storage at −95 C than at −28 C, because at −28 C trichomonads die off slowly, whereas survivors remained constant at −95 C for 128 to 256 days. The survival was much better in the original Diamond's medium (1957) in which trichomonads had been grown than when they were frozen in a physiological salt solution or in the fresh Diamond's medium. They thought that presumably some product or products of the trichomonad's metabolism have an additional protective action which supplements that of glycerol.

3) Discovery of the effect of DMSO as a cryo-protectant

In 1959, Lovelock and Bishop found that dimethyl sulfoxide (DMSO) had a protective action similar to glycerol to living cells in cryo-preservation. This finding also stimulated many protozoologists. Walker and Ashwood-Smith (1961) showed that 5% DMSO and 10% glycerol were equally effective for cryo-preservation of *Trypanosoma rhodesiense* and *T. congolense* at −79 C, and they concluded that the lower toxicity of DMSO both to the host and to the parasite, as compared with glycerol in cryo-preservation. Collins and Jeffery (1963) also compared 5% DMSO and 10% glycerol in low-temperature preservation of blood stage of *Plasmodium berghei* and *P. gallinaceum* at −70 C, and they reported that both substances were effective to those malaria parasites, and although 10% glycerol gave rise to superior survivals, the lower toxicity of DMSO to the parasites made it a useful alternative for preservation.

4) Preservation in liquid nitrogen

Although the possibility of preservation of the living protozoa in liquid nitrogen was suggested by early workers, the use of liquid nitrogen for dry ice or mechanical refrigeration is relatively new. The temperature in the dry ice cabinet or mechanical freezers was maintained from −70 to −95 C at the lowest, and at such relatively high temperatures, the cryo-preservation of the protozoa is not permanent but only for several months or a few years. The cause of death of the protozoa during preservation period is thought that ice crystal can form and grow in the protozoan cells. The ice crystal, however, does not form and grow below the recrystallization point of water (−130 C). Therefore, the temperature for preservation of living cells might be better kept below this point. Diamond *et al.* (1961 and 1963) reported that use of liquid nitrogen refrigerator for the preservation of *Entamoeba histolytica* (monoxenic culture grown in association with a *Crithidia* sp.), *Tritrichomonas foetus, Trichomonas gallinae, T. vaginalis, Pentatrichomonas hominis, Trypanosoma cruzi,* and *T. ranarum* in the presence of 5% DMSO. Before cooling, trichomonads and amoebae-*Crithidia* suspensions were allowed to equilibrate for 15 and 30 minutes, respectively, at 35 C, and the trypanosomes for 15 minutes at room temperature, the samples

were then cooled by two-step cooling technique, in which the samples were cooled at first at the rate of 1 C per minute from 0 to −35 C, then they were plunged directly into liquid nitrogen. To examine the survival, aliquots of the samples were momentarily immersed and rapidly swirled about in a 45 C water bath containing a few drops of 25% Aerosol O. T. (American Cynamid Co.) which was added to improve heat transfer. According to their results, cultures of *E. histolytica* were obtained from samples stored as long as 14 months, and also viable cultures were obtained from *T. gallinae* and *T. vaginalis* stored for 10 months and *T. foetus* and *P. hominis* stored for 5 months. *T. cruzi* and *T. ranarum* survived up to 4 months. No difference in numbers was found between samples thawed 24 hours after freezing and those thawed after the longest period of storage. The result indicated the absence of gradual death during extended period of storage in liquid nitrogen, whereas such death was commonly reported at dry ice temperature by many workers.

Herbert *et al.* (1968) also reported the storage of *Trypanosoma brucei* subgroup at −196 C by using rapid cooling method, and without glycerol or DMSO they obtained the excellent result. Reather and Seidenath (1972) successfully stored a total of 28 species of the parasitic protozoa containing 10 genera, *Entamoeba, Pentatrichomonas, Trichomonas, Tritrichomonas, Crithidia, Leishmania, Trypanosoma, Plasmodium, Babesia,* and *Eimeria,* for 720 to 2,662 days at −196 C. Their cooling procedure is as follows: The samples containing DMSO or glycerol are stored in plastic ampoules for 30 minutes at 4 C, then they cooled at 0.4 C per minute from 4 to −10 C, at 2 C per minute from −10 to −30 C, at 5 C per minute from −30 to −60 C, and finally when reached −60 C, the samples are stored in liquid nitrogen. Their paper shows the possibility of extremely prolonged preservation in liquid nitrogen, and this is the longest storage record in the frozen state reported in the literature. The record, however, does not mean the maximum and more extension may be possible in future.

II. Other Literatures

1) Amoebae

In the history of cryo-preservation of the parasitic protozoa, glycerol as a cryoprotectant was used at first by Fulton and A. U. Smith (1953) for the storage of *Entamoeba histolytica* at −79 C. Their success stimulated many workers. Fulton and A. U. Smith used *E. histolytica* which cultured monoxenically with *Bacillus coli,* and in the presence of 5 to 10% glycerol, the amoebae were cooled by two step technique; 0 to −15 C for 20 minutes, −15 to −70 C for 30 minutes, and storage at −79 C. Before cooling, the samples were allowed to equilibrate with glycerol for 30 minutes at 37 C. Thawing after 65 days of freezing, the samples were inoculated to fresh media, and the survived amoebae multipled in the media.

Kasprzak and Rydzewski (1970) reported that *E. histolytica* (3 strains), *E. invadens,* and free-living *E. moshkovskii* survived for 80 days, 30 days, and 450 days, respectively, at −75 C in the presence of 10% glycerol by slow cooling.

Gordon *et al.* (1969) using DMSO, preserved axenic *E. histolytica* for 5 months at −196 C in liquid nitrogen. They cooled the samples at the rate of 1 C per minute untill temperature reached −70 C, then the samples were stored in liquid nitrogen.

Bosh and Frank (1972) observed that *E. invadens* and *E. histolytica* could survive freezing in liquid nitrogen using 10% DMSO. Neal *et al.* (1974) also reported their successful storage of 6 species of the genus *Entamoeba* in liquid nitrogen in the presence of DMSO. Other papers concerning the preservation of *Entamoeba* spp. in liquid nitrogen were introduced in page 167 of this review.

In the papers introduced above, trophozoite stage samples were used for cryo-preservation, but Halpern and Dolkart (1954) and Chang (1955) examined the cyst of *E. histolytica* at about −30 C without cryo-protectant, but their results were unsuccessful except for several hour survival after freezing.

Molinari (1955, 1956a, and 1956b) wrote several papers on the effect of low temperature (4, −20, −79, or −180 C) on survival of trophozoites of *E. histolytica* without using cryo-protectant.

According to the author's own experiments (Miyata, 1973b), the cryo-preservation of Laredo strain of *E. histolytica* at −75 C did not succeed even in the presence of glycerol or DMSO, and the main reason of this failure could be explained that rice powders which were contained in the medium became injurious, because under freezing condition, amoeba decreased the cell volume, and rice powders ingested inside the cell might be destroyed by mechanical pressure to organelles such as nucleus or cell membrane.

2) Trypanosomatids

Polge and Soltys (1957) studied cryo-preservation of trypanosomes with or without glycerol by two different cooling methods; (a) *Quick freezing*: the samples were frozen directly in a mixture of dry ice and absolute alcohol at −79 C. (b) *Two stage freezing*: samples were first placed in alcohol at room temperature, then cooled slowly (2 C per minute) by adding small pieces of dry ice untill the temperature reached −20 C. After the desired temperature was obtained, the samples were continued to cool at 5 C per minute to −79 C.

According to their paper, both the whole horse serum and the yolk citrate diluent proved unsatisfactory without glycerol. In Alsever's solution (glucose 4.66 g, sodium chloride 1.05 g, sodium citrate 2.0 g, and distilled water 200 m*l*) and sperm-Ringer or other diluents, but the best results (about 50% survival) were obtained with the fastest rate of cooling without glycerol, and survived trypanosomes decreased when the cooling rate was slower. In contrast, in the media containing glycerol, the viability after thawing was poor after quick freezing and was much better after slow cooling. The best results in all diluents (about 80% survival) were obtained with slow cooling and with 5 or 10% glycerol in the medium.

Lymph tubes, instead of test tubes or vials, were used by Cunningham *et al.* (1963b) for preservation of trypanosomes at −80 C. The trypanosome suspensions containing 7.5% glycerol were distributed in "about 25 mg quantities" to the tubes, and after sealing, they were cooled slowly. By the use of these small tubes, many materials could be preserved in a small space.

The following authors also studied cryo-preservation or cryo-resistance of trypanosomes at various temperatures: Reuße (1956), Molinari and Montézin (1956a and 1956b), Molinari (1960b), Gordon (1961), Flück (1962), Berson (1962), Cunningham and Harley (1962), Cunningham *et al.* (1963a and 1965), Resseler *et al.*

(1965), Mieth (1966), Lapierre and Vinh Hien (1968a and 1968b), Dar *et al.* (1972), Miyata (1973b), and Filardi and Brener (1975).

Leishmania spp. were easily preserved by Foner (1963), Most *et al.* (1964), and Heymen and Monsour (1962). Foner reported that *Leptomonas culicidarum* and *Herpetomonas muscidarum* survived for 13 months at −60 C in the presence of 10% glycerol.

3) Trichomonads

The following papers are not cited in this review: Blackshaw and Beattie (1955), Honigberg and King (1962), Honigberg *et al.* (1965), Resseler *et al.* (1965), Lumsden *et al.* (1965 and 1966), Müller (1966), Kasprzak and Rydzewski (1970), and Miyata (1973a, 1974, 1975a, and 1975b).

4) *Giardia* spp.

As far as the author knows, there are only three papers containing cryo-preservation of *Giardia* spp. Bemrick (1961) reported that trophozoites of *Giardia muris* could survive for 40 days in frozen state (at −38 C?) in the presence of glycerol. At −20, −38 and −70 C, the parasites would survive at least 24 hours if glycerol were used. Slow freezing in cotton insulated tubes (a modification of Levine and Marquardt, 1955) resulted in a higher survival rate than quick freezing, and if 1% Tween 80 was added into basal medium containing glycerol, the number of survived parasites after freezing and thawing was greater than that where the Tween 80 was not present. The temperature of −38 C appeared to be the most favorable for the survival of *G. muris,* and at the temperautre, optimum concentration of glycerol was 14.2 percent. But *G. muris* may survive quick freezing at −70 C for several months.

Meyer and Chadd (1967) preserved trophozites of *Giardia* spp. obtained from culture at −70 C for at least 2 years in the presence of 7% glycerol. They compared various concentrations of glycerol (1, 3, 5, 7, 9, and 15%) and DMSO (1, 3, 5, 7, and 9%). All samples were frozen slowly in four steps: room temperature for 15 minutes, −12 to −15 C for 30 minutes, and −22 C until frozen (45 to 90 minutes); then they were stored at −70 C. The parasites survived after freezing and thawing (in a 37 C water bath) at every glycerol and DMSO concentration examined, but most viable parasites were recovered after using 7% DMSO. The parasite did not survive in cultures frozen without cryo-protectants.

The paper written by Le Corroller *et al.* (1970) also dealt with preservation of *G. muris* as cited in page 177 of this review.

5) *Dientamoeba fragilis*

According to Dwyer and Honigberg (1971), *Dientamoeba fragilis* could survive for 6 months after storage in liquid nitrogen by using slow-cooling technique in the presence of DMSO.

6) Ciliates

As far as the author knows, only one paper (Weinman and McAllister, 1947) was published on the cryo-preservation of *Balantidium coli* at −70 C with negative result cited in page 164 of this review. After discovery of the effect of cryo-protectants such as glycerol or DMSO, there are still few papers on the study of low-temperature preservation of ciliate.

Hwang *et al.* (1964) reported that *Tetrahymena pyriformis* could survive for 3 months

after freezing at −196 C in the presence of 10% DMSO. The samples were cooled for 20 minutes in a −20 C freezer (2 C per minute), then they were stored in liquid nitrogen. The number of survived ciliate after freezing and thawing reduced to about one fourth of that before cooling.

Wang and Marquardt (1966) also preserved *T. pyriformis* for 112 days in a −95 C deepfreezer using 10% DMSO, and the ciliate still survived. They also adopted two step freezing; the samples were first cooled in a −27 C freezer for 20 minutes (4.5 C per minute), and those were transfered into a −95 C alcohol bath, then stored in the freezer. Furthermore, they reported that *Paramecium aurelia* survived for 7 days at −27 C or −196 C in the presence of 6.0 to 7.5% DMSO. Unfortunately the survival ciliates, however, did not multiply in the inoculated fresh media, except for the storage for 20 minutes at −27 C. According to Marquardt *et al.* (1963), ciliates (*Colpoda steinii*) and amoebae (*Vahlkampfia* sp.) were found from sands or dusts collected in an ice tunnel of Greenland, and cyst of both species could survive at the low temperature of −28 or −95 C, but trophozoites of either protozoa did not.

C. Wolfson (1935) observed *Paramecium* by using a cryo-microscope which was designed by himself. Other papers on the study of cryo-biology of ciliate are as follows; Efimoff (1924), Altavilla *et al.* (1971), Matsusaka (1971), and Tanno (1972).

7) Malaria parasites

Plasmodium spp. seem to be one of the easiest parasites for cryo-preservation in dry ice, mechanical freezers, or liquid nitrogen in the presence or absence of cryo-protectants already mentioned above. The freezing methods adopted by various workers are simillar to each other, and it is needless to repeat the details of the papers written by following authors: Vargues (1952), Molinari and Montézin (1956b), Molinari (1960a and 1961), Molinari and Tebibzadeh (1961), Jeffery (1962 and 1965), Shute and Maryon (1962), Yoeli *et al.* (1963), Collins *et al.* (1963), Vincke *et al.* (1965), Jacobs (1965), Warhurst (1966), Bafort *et al.* (1966), Bafort (1968), Killick-Kendrick and Bruce-Chawatt (1969), Booden and Geiman (1970 and 1973), Allen (1970), Miyata (1973b), and so on.

Among them, Molinari (1961), Shute and Maryon (1962), and Bafort (1968), reported on cryo-preservation of sporozoites in the salivary gland of vector mosquitoes.

Estimation of the number of alive malaria parasites before and after freezing was rather difficult. Warhurst (1966), however, pointed out that percentage survival of 2 strains of *P. berghei* after freezing at −78 C in 7% DMSO might be measured by application of a lineal relationship between log inoculum size and *pre-two percent period* (the period between intravenous inoculation of parasites and infection of two percent of the red cells). The measurement of the effect of low-temperature on the protozoa by titration was reported by Overdulve and Antonisse (1970a, 1970b and 1970c).

8) *Toxoplasma gondii*

Toxoplasma gondii was one of the difficult species to preserve at low temperature without cryo-protectants. Recently, Fabio *et al.* (1967), however, described that *T. gondii* (RH strain) which was prepared in physiological saline could survive for 47 days at −70 C. Miyata (1973b) also reported that *T. gondii* (RH starin) was

easily preserved in a −75 C deepfreezer for at least 30 days without using any cryo-protectant.

By using such cryo-protectants, at least RH strain of the parasite is more easily stored in dry ice, deepfreezers, or liquid nitrogen. For example, Chandler and Weinman (1956) preserved two toxoplasma strains at −70 C for 184 days by using mouse peritoneal exudate for freezing in the presence of 10% glycerol. Eyles *et al.* (1956) also preserved *T. gondii* by slow freezing in glycerol solution for 209 days at −70 C. They observed that very slow cooling using 5 to 10% glycerol was the best, but even with the optimum methods, the initial loss of viability after freezing was great, whereas storage at below −70 C resulted in little further loss of the viability over the period examined. Recently, Stewart and Feldman (1955) described the use of rollar-tube tissue cultures of *T. gondii* as a source of parasites for cryo-preservation by two step cooling in dry ice, and after 360 day preservation, the cultures became positive after one blind passage.

In liquid nitrogen, according to Franchi and Hahn (1968a and 1968b), *T. gondii* survived in the presence of 10% glycerol after slow cooling. The samples were kept at 4 C for 2 to 3 hours, then they were cooled at the rate of 1 C per minute. After pre-cooling, the samples were stored at −196 C. R. Smith (1973) stored safely RH strain of *T. gondii* which was obtained from tissue culture, in liquid nitrogen with the fetal calf serum as a cryo-protectant. He preserved samples directly in liquid nitrogen. Before reaching this ideal method, he attempted to use DMSO or glycerol by slow cooling at the rate of 1 C per minute to −70 C, then they were cooled and stored in liquid nitrogen.

All the authors of the papers cited above used proliferative form of *T. gondii*, but following two authors showed the possibility of preservation of cysts or parasites within cysts. Kwantes *et al.* (1967) preserved cyst-cyst type strains which were isolated from various human tissues or tissue fluid. After storage at −70 C the strains produced cysts in mice and their virulence did not change during the preservation. Roble (1965) also observed that cysts survived for 200 days in fresh milk at −76 C. Miyata (1973b), however, reported a negative results on cysts of Beverley strain in preservation at −75 C with or without glycerol. According to this paper, even in those negative samples, a few cysts of normal appearance was detected microscopically without damage, but they have already lost the infectivity to mouse.

Frenkel and Dubey (1973) observed that sporulated oocysts of *T. gondii* could survive for 28 days at −20 C without using cryo-protectants.

Gartner and Theile (1970) studied on deep-frozen toxoplasm with electron microscope. According to them, in the process of rapid cooling, water penetrated into cell through the conoid by pressure differences between the outside and the inside of the cell, and after freezing the water changed to ice which destroyed the cell. They also described that in slow cooling, dehydration of the cell was a cause of cell damage, while mechanical destructions were diminished and the parasite could survive. Before the publication of their interesting paper, this kind of study has not been reported by protozoologists, but the direction suggested by Gartner and Theile would be followed by other workers in near future. Other papers on cryo-preservation of *T. gondii* were as follows; F. and A. Roger (1957), Fabio *et al.* (1967), Bugiar-

dini *et al.* (1967), Bollinger *et al.* (1974), and Dumas (1974a, 1974b and 1974c).

9) *Eimeria, Leucocytozoon, Babesia, Eperythrozoon, Nosema, Anaplasma,* and *Theileria*

Cryo-preservation of above genera was studied by following workers: *Babesia berbera* by Pipano and Senft (1966), *B. bigemina* by Waddell (1963), Pipano and Senft (1966), and Barnett (1964); *B. trautmani* by Barnett (1964); *B. calballi* by Frerichs *et al.* (1968) and Wayne *et al.* (1968); *B. equi* by Frerichs *et al.* (1968); *B. rodhaini* by Overdulve and Antonisse (1970b), and Mieth (1966); *B. canis* by Reuße (1956) and Mieth (1966); *Leucocytozoon simondi* by Kocan *et al.* (1967); *Eimeria tenella* by Kouwenhoven (1967), Doran and Vetterling (1968 and 1969), Doran (1969a and 1970), and Norton and Joyner (1968); *E. acervulina* by Norton *et al.* (1968); *E. meleagrimitis* by Doran and Vetterling (1968 and 1969) and Doran (1969a); *E. adenoeides* by Doran (1970) and Norton and Joyner (1968); *E. ahsata, E. arloinigi, E. duodenalis, E. maxima, E. phasiani,* and *E. stiedae* by Norton and Joyner (1968); *Eimeria* spp. by Landers (1953); *Eperythrozoon wenyoni* by Ishihara and Minami (1968); *Anaplasma centrale* and *A. marginale* by Barnett (1964), Pipano and Senft (1966), and Ishihara and Minami (1968); *Theileria* sp. (2 strains) by Ishihara and Minami (1968); *Nosema cuniculi* by Bedrnik and Vavra (1971).

According to Doran and Vetterling (1969), and Doran (1969a), oocysts, sporocysts released from oocysts, and excysted sporozoites of *E. meleagrimitis* and *E. tenella* in media containing 7% DMSO were frozen at −80 C (1 C per minute), and stored in liquid nitrogen vapor for 4 months. After thawing, oocysts could not infect fresh hosts, although these samples appeared in an excellent condition under microscope. After 3 months, sporocysts of both species could infect, but in a less extent than those infected by fresh oocysts. Frozen sporozoites caused infection to fresh hosts comparable to those caused by unfrozen fresh oocysts. Those results are somewhat similar to those of the cyst of Beverey strain of *Toxoplasma gondii* (Miyata, 1973b). According to the author's opinion, since water in the inside of the oocyst is unable to pass the oocyst membrane during the freezing procedure, the water would form ice crystals within the oocyst, and the ice becomes injurious to sporozoites inside of the oocyst. Water penetration to the sporocyst membrane appeares faster than that to the oocyst membrane.

Doran (1969b) studied freezing of excysted sporozoites of *Eimeria* spp. According to his results, survival of sporozoites was better at the lower concentration of DMSO before freezing, but after freezing and thawing, survival was better at the higher concentration of DMSO. With 10 to 12.5% DMSO, various equilibration periods and cooling rates were compared, and the best results were obtained where cooling rate from the freezing point to −30 C was 1 C per minute. In the concentrations of 2.5, 5.0 and 7.5% DMSO, various equilibration periods up to 2.5 hours were examined, but the survival rate of sporozoites was not improved.

10) Other protozoa

The following authors studied on cryo-biology of the protozoa: A. U. Smith *et al.* (1951), Gehenio and Luyet (1953), Sharf (1954), Altavilla *et al.* (1971), and Klein (1972).

Annear (1956) preserved *Strigomonas oncopelti* for 12 months in dried state *in vacuo* at 4 or 20 C. The contents of ampoules yielded heavy growths of the flagellates

after 12 month storage.

11) Helminths

Weinman and McAllister (1947) studied on the cryo-preservation of helminth at −70 or −15 C. Microfilariae of *Wuchereria bancrofti* and *Dirofilaria immitis* could survive at −70 C without cryo-protectant, and infective larvae of *Ancylostoma caninum* also survived for 107 days at −15 C and 44 days at −70 C, respectively.

Recently, several papers were published on the attempts to preserve helminth larvae at low temperatures in the presence of cryo-protectant. Microfilariae or mosquito stages are easily preserved. The following authors published their studies on cryo-preservation or cryo-biology of helminths including some papers concerning free living nematodes: De Coninck (1951), J. H. Turner (1953), Gustafson (1953), Poole (1956), Asahina (1959), Taylor (1960), Beye and Lawless (1961), Bemrick *et al.* (1965), Restani (1968), Ogunba (1969), Parfitt (1971), Obiamiwe and Macdonald (1971), Campbell *et al.* (1972 and 1973), Isenstein and Herlich (1972), Campbell and Thomson (1973), McCall *et al.* (1975), and James (1975).

III. Various Factors on Cryo-preservation

Various factors involved in cryo-preservation of the parasitic protozoa were introduced and discussed briefly in this chapter.

1) Cooling rate

Before preserving the parasitic protozoa in the frozen state, most workers cooled the samples at a rate of about 1 C per minute to temperature around −30 C. To obtain the cooling rate, the samples were pre-cooled at −20 to −30 C for 30 to 90 minutes, then they were stored at −70 to −80 C or −170 to −190 C. The author compared five different cooling rates between 0.25 to 5 C per minute in preservation of *Trichomonas vaginalis* (Miyata, 1974). Each group of 10 samples in the presence of 10% glycerol was pre-cooled in each freezer of 5 different temperatures for 90 minutes, then the samples were stored in a −75 C freezer. According to the results, the highest survival rate of *T. vaginalis* was obtained at the cooling rate of 0.78 C per minute by the use of a −30 C freezer (Figs. 1 and 2). The survival rate at 5 C per minute was about 20 percent lower than the highest survival rate.

Diamond (1964) reported that 30 to 40 percent of *Entamoeba invadens* protected with 15% DMSO and 4.5% glucose have survived when cooled at 1 C per minute, but there were almost no survivors in the samples cooled at 8 C per minute.

The cooling and thawing process of the parasitic protozoa in cryo-preservation can be explained in Fig. 3 (after Miyata, 1973a). The sample is cooled at about −30 C for one to two hours. The temperature of the sample drops without freezing, and the super-cooled state continues for several minutes. The survival rate of the cells does not change during super-cooled state, but when the state is broken, the temperature slightly goes up by liberation of the latent heat in crystallization, then the temperature of the sample again drops slowly. The point at the beginning of freezing (F in Fig. 3) is called the freezing point or the freezing temperature of the sample. The freezing temperature is lower in higher concentration of the cryo-protectant as shown in Fig. 4. From the point to −30 C or lower, dehydration of the cells is carried out by

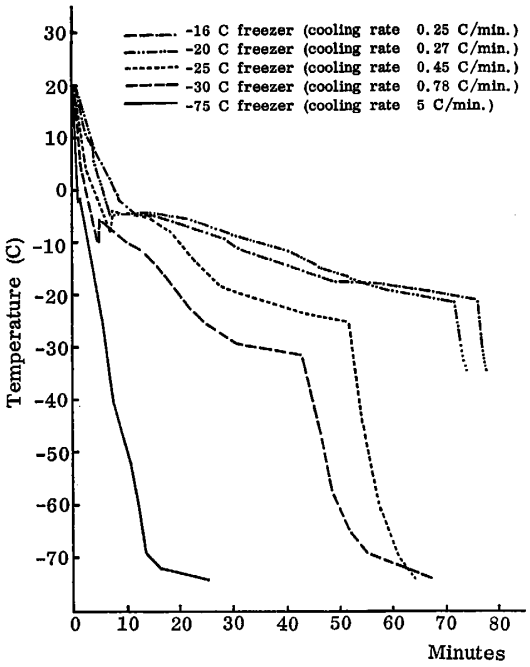

Fig. 1 The cooling curves of pre-cooling in various freezers (Miyata, 1974).
Note: Cooling curves were only traced until temperature reached the lowest temperature in each freezer and after transfered into the −75 C freezer.

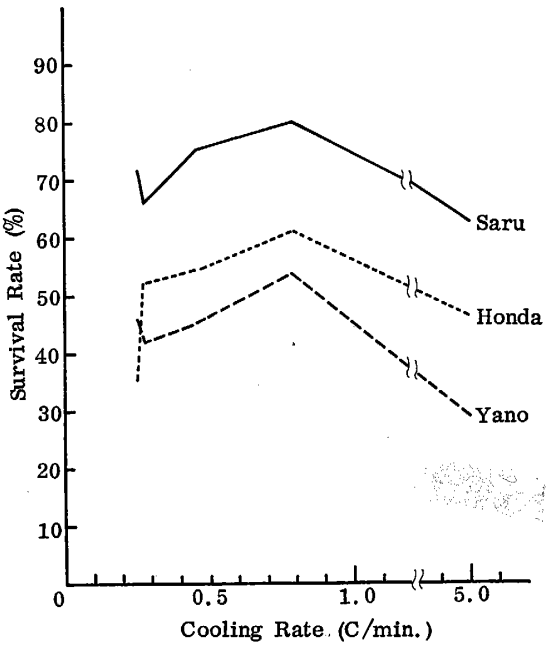

Fig. 2 The survival rate of trichomonad in each cooling rate after 1 to 5 day frozen storage in −75 C freezer (modified from Miyata, 1974).
Note: Three strains (Saru, Honda, Yano) of *Trichomonas vaginalis* were used in this experiment.

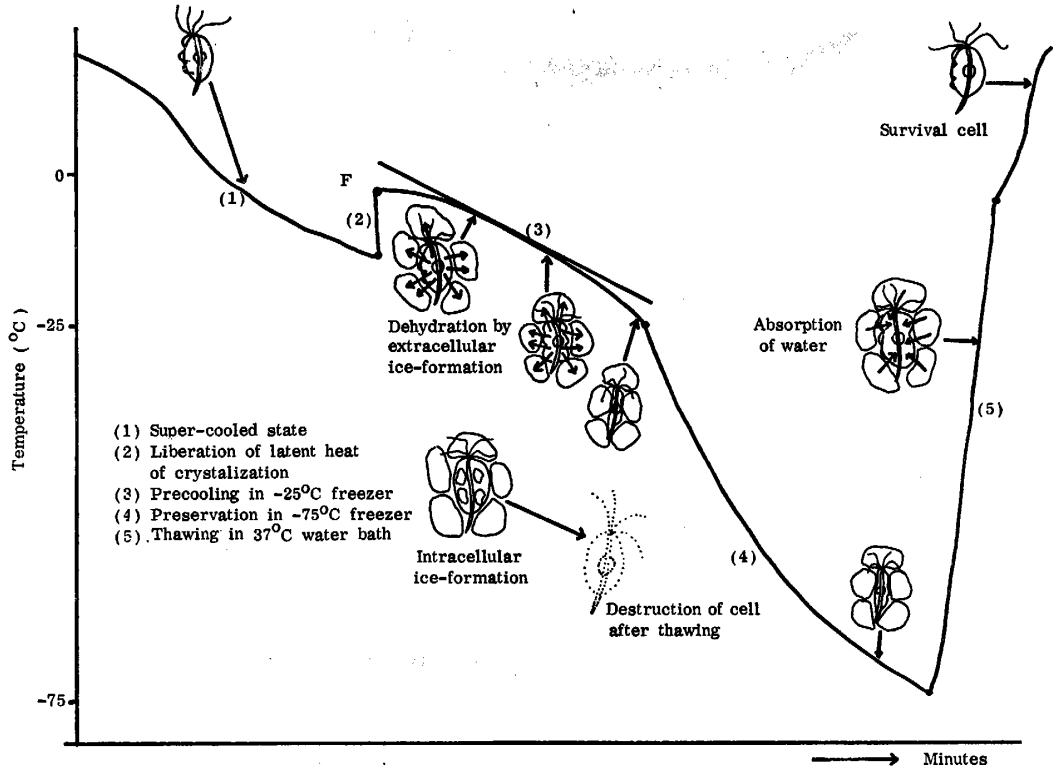

Fig. 3 The schematic explanation of the pre-cooling method (Miyata, 1973a).

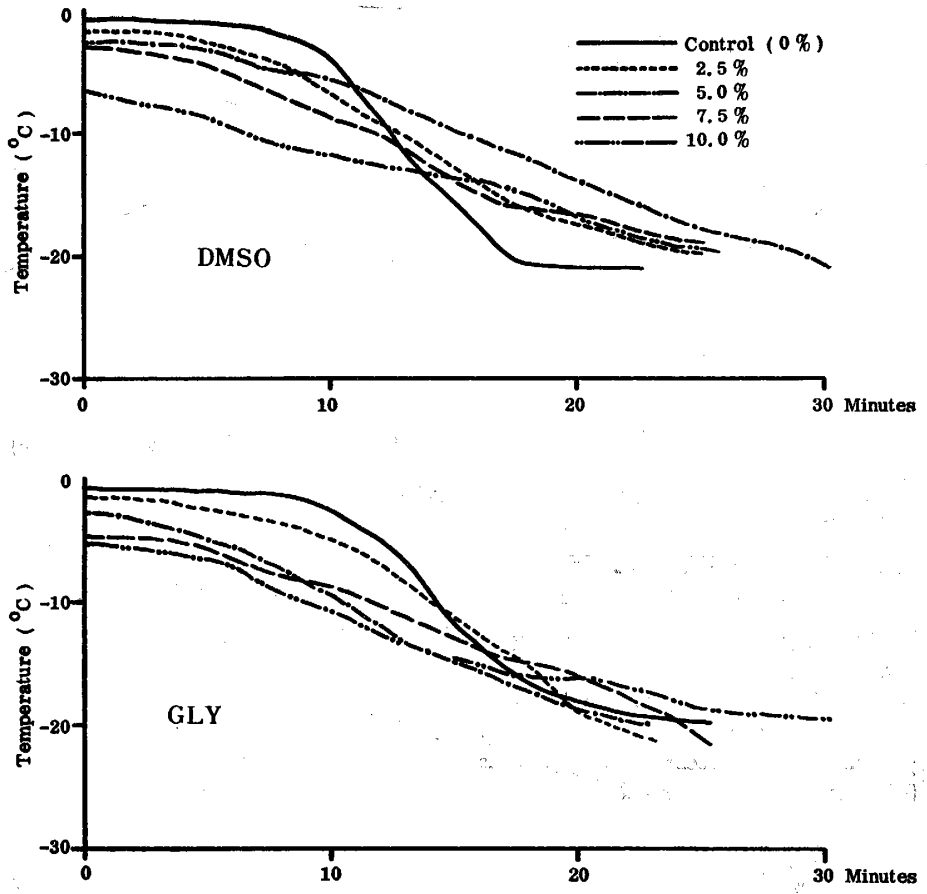

Fig. 4 The cooling curves of various concentrations of glycerol (GLY) or DMSO in a −25 C freezer (Miyata, 1973a).
Note: Each curve is shown from the beginning of freezing, and super-cooled state is excluded.

formation and growth of extracellular ice crystals. Dehydration of the cells must be thorough, because if the water remains in the cell, the water may be crystallized later and destroy the cell mechanically. After sufficient dehydration, the sample must be cooled rapidly to the storage temperature. In the rapidly cooled samples without pre-cooling, the water in the cells can not go out, and changes to ice crystal in the cell. This is the reason to explain why slow cooling is better than rapid cooling. In the case of very slow cooling, salt concentration could be effective. Several workers reported that some species or strains of the parasitic protozoa could survive after freezing directly in dry ice alcohol or liquid nitrogen. In such cases, many ice crystals may be formed within the cell, but the size of each crystal is too small to injure the cell.

Most workers recommended that the frozen samples must be thawed in a water bath at about 40 C as soon as possible after taking out from a storage cabinet, because recrystallization of ice can be avoided by such rapid thawing. Diamond (1964) and Dalgliesh (1972) pointed out that the survival rate increased in proportion to the warming rate.

2) Storage period at various temperatures

In Table 1, storage periods of the parasitic protozoa (24 genera including about 90 species) at various temperatures were summarized. The longest preservation period of each species at different temperatures in the presence or absence of cryo-protectants was sought from many literatures as far as the author knows. Several species could survive after preservation at relatively high temperatures as −20 to −30 C, but the storage period is rather short. According to such authors, gradual death occured during preservation period at such a high temperatures, and finally all organisms died. At −70 to −90 C, survival period prolonged to months or often years, but at this temperature still living parasites decreased gradually with the increase of storage period. At the temperatures of liquid nitrogen itself or its vapor, the protozoa could survive for an extremely long period. At present, the longest survival record so far tested is longer than 2,500 days (Raether and Seidenath, 1972), but the period might have been extend infinitely. It was said by many workers that before cooling and after storage for several years in liquid nitrogen, there were no significant loss of survival number and of their infectivities to the host.

3) Use of chemical compounds for cryo-preservation

Both glycerol and DMSO are widely used by workers as cryo-protectants, whereas recently, DMSO instead of glycerol was recommended by several workers (Dalgliesh, 1972; Walker and Ashwood-Smith, 1961; Collins and Jeffery, 1963; Diamond, 1964; and Miyata, 1973a), because of its rapid equilibration and lower toxicity. In addition to these two chemicals, some other substances were reported having the cryo-protective action to living cells, for example, dimethylacetamide by Djerassi *et al.* (1971) and pyridine N-oxide by Nash (1961). Dobbler (1966) reviewed and discussed of structure and function of cryo-protective compounds.

As a protozoologist, O'Connel and his co-workers (1968) examined the cryo-protective action of 83 chemicals such as alcohol, sugars, amines, and others, for preservation of *Crithidia fascilatus* at −20 C. According to their results, the best protectant among the chemicals tested was glycerol at 10 percent (w/v), and as a group the alcohols were most effective, and several sugars also showed some protective activities. Levine and Marquardt (1954) also tested several compounds related to glycerol in cryo-preservation of *Tritrichomonas foetus* introduced in page 166 of this review.

The protective action of the serum in cryo-preservation of the protozoa was reported by several workers (Jeffery and Rendtroff, 1955; Polge and Soltys, 1957; R. Smith, 1973; and Miyata, 1973b).

More recently, Le Corroller *et al.* (1970) examined macromolecular, *extracellular* cryo-protectants such as polysaccarides and polyvinyl pyrrolidone instead of micromolecular, *intracellular* cryo-protectants such as glycerol and DMSO, because the toxicity of *intracellular* cryo-protectants were pointed out by various workers. They prepared a mixture containing 15 g of dextran sorbitol (Rheomacrodex) and 15 g of polyvinyl pyrrolidone in 100 m*l* distilled water. Protozoan suspensions were mixed in equal volume of the cryo-protectant mixture. The samples which distributed in one to 4 m*l* in ampoules were cooled and stored at −70 C. *Trypanosoma lewisi, T. cruzi, T. congolense, T. brucei, T. gambiense, Tritrichomonas muris, Trichomonas tenax, T. vaginalis, Pentatrichomonas hominis, Giardia muris, G. intestinalis, Toxoplasma gondii,* and

TABLE 1 The longest preservation records in each parasitic protozoa at various temperatures with or without cryo-protectant

Species	Stage of parasite	Storage temperature (C)	Cryo-protectant	Storage period (days)	Author
Entamoeba coli	trophozoite	−170	DMSO	115*	Neal *et al.*, 1974
E. hartmanni	〃	−170	DMSO	1,043*	〃
E. histolytica	cyst	−28	none	(7.5 hrs)	Chang, 1955
〃	trophozoite	−75	glycerol	80	Kasprzak & Rydzewski, 1970
〃	〃	−170	DMSO	2,675*	Neal *et al.*, 1974
E. invadens	〃	−75	glycerol	30	Kasprzak & Rydzewski, 1970
〃	〃	−170	glycerol or DMSO	105	Diamond, 1964
〃	〃	−170	DMSO	1,909*	Neal *et al.*, 1974
E. moshkovskii	〃	−75	glycerol	405	Kasprzak & Rydzewski, 1970
〃	〃	−170	DMSO	1,902*	Neal *et al.*, 1974
E. ranarum	〃	−170	DMSO	504*	〃
E. terrapinae	〃	−170	DMSO	1,162*	〃
Dientamoeba fragilis	〃	−170	DMSO	1,435*	〃
〃	〃	−196	DMSO	180*	Dwyer & Honigberg, 1971
Tritrichomonas augusta	〃	−79	glycerol	91	Müller, 1966
Tritrichomonas foetus	〃	−16	glycerol	196	Reuße, 1956
〃	〃	−28	glycerol	128	Levine & Andersen, 1966
〃	〃	−95	glycerol	2,048	〃
〃	〃	−196	DMSO	1,383	Raether & Seidenath, 1972
Tritrichomonas muris	〃	−70	P+P†	730*	Le Corroller *et al.*, 1970
Tritrichomonas sp. (*suis* ?)	〃	−79	glycerol	91	Müller, 1966
Trichomonas gallinae	〃	−19	glycerol	133*	Stabler *et al.*, 1964
〃	〃	−72	glycerol	364*	〃
〃	〃	−196	DMSO	1,383	Raether & Seidenath, 1972

Trichomonas teiax	trophozoite	−75	glycerol	450	Kasprzak & Rydzewski, 1970
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
Trichomonas vaginalis	〃	−25	DMSO	14*	Miyata, 1973a
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−75	glycerol	780*	McEntegart, 1959
〃	〃	−170	DMSO	1,154	Diamond, 1964
Trichomitus fecalis	〃	−196	DMSO	1,383	Raether & Seidenath, 1972
Pentatrichomonas hominis	〃	−70	none	407	Weinman & McAllister, 1947
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−79	glycerol	487	Müller, 1966
〃	〃	−196	DMSO	1,383	Raether & Seidenath, 1972
Monocercomonas sp.	〃	−170	DMSO	196	Diamond, 1964
Giardia intestinalis	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
Giardia muris	〃	−38	glycerol	27	Bemrick, 1961
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
Giardia spp.	〃	−70	glycerol	730*	Meyer & Chadd, 1967
Herpetomonas muscidarum	culture	−60	glycerol	390*	Foner, 1963
Leptomonas culicidarum	〃	−60	glycerol	390*	〃
Crithidia fasciculata	〃	−20	glycerol	21*	O'Connell *et al.*, 1968
〃	〃	−170	DMSO	155	Diamond, 1964
Crithidia sp. (ReF–1: PRR)	〃	−196	DMSO	1,260	Raether & Seidenath, 1972
Crithidia sp. (ReF–2)	〃	−196	DMSO	1,260	〃
Leishmania adleri	〃	−60	glycerol	390*	Foner, 1963
Leishmania agamae	〃	−60	glycerol	390*	〃
Leishmania braziliensis	〃	−60	glycerol	390*	〃
〃	〃	−196	DMSO	420*	Resseler *et al.*, 1965
Leishmania donovani	tissue	−64	glycerol	365*	Allain, 1964
〃	〃	−70	none	276	Weinman & McAllister, 1947

Leishmania donovani	culture	−64	glycerol	180*	Allain, 1964
〃	〃	−196	DMSO	420*	Resseler *et al.*, 1965
〃	tissue	−196	glycerol	2,643	Raether & Seidenath, 1972
Leishmania enrietti	〃	−196	glycerol	2,484	〃
〃	culture	−196	DMSO	420*	Resseler *et al.*, 1965
Leishmania infantum	〃	−60	glycerol	390*	Foner, 1963
Leishmania tarentolae	〃	−60	glycerol	390*	〃
Leishmania tropica	〃	−70	none	794	Weinman & McAllister, 1947
〃	〃	−60	glycerol	390*	Foner, 1963
〃	〃	−196	DMSO	450*	Resseler *et al.*, 1965
Trypanosoma brucei	blood	−76	none	135	Horsfall, 1940
〃	〃	−80	glycerol	850*	Cunningham *et al.*, 1963b
〃	〃	−80	glycerol	850*	〃
〃	fly	−80	glycerol	850*	〃
〃	blood	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−196	DMSO	330*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,661	Raether & Seidenath, 1972
Trypanosoma congolense	〃	−70	none	100	Levaditi, 1952
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−80	glycerol	852*	Cunningham *et al.*, 1963b
〃	〃	−196	DMSO	90*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,650	Raether & Seidenath, 1972
Trypanosoma conorhini	culture	−20	glycerol	28*	O'Connell *et al.*, 1968
Trypanosoma cruzi	blood	−70	none	234	Weinman & McAllister, 1947
〃	culture	−70	none	653	〃
〃	〃	−80	glycerol	852*	Cunningham *et al.*, 1963b
〃	blood?	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	blood	−196	glycerol	2,627	Raether & Seidenath, 1972
〃	culture	−170	DMSO	987	Diamond, 1964
Trypanosoma duttoni	blood	−76	none	34	Horsfall, 1940
〃	culture	−64	glycerol	540*	Allain, 1964

Trypanosoma equinum	blood	−196	glycerol	2,640	Raether & Seidenath, 1972
Trypanosoma equiperdum	〃	−76	none	62	Horsfall, 1940
〃	〃	−75	glycerol	520	Kasprzak & Rydzewski, 1970
〃	〃	−190	none	21	De Jong, 1922
〃	〃	−196	DMSO	270*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,656	Raether & Seidenath, 1972
Trypanosoma evansi	〃	−76	none	62	Horsfall, 1940
〃	〃	−79	glycerol	618	Ishihara & Minami, 1968
〃	〃	−196	glycerol	2,654	Raether & Seidenath, 1972
Trypanosoma gambiense	〃	−20	glycerol	3	Miyata, 1973b
〃	culture	−70	none	189	Weinman & McAllister, 1947
〃	blood	−70	none	561	〃
〃	〃	−79	glycerol	250	Polge & Soltys, 1957
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−196	DMSO	90*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,662	Raether & Seidenath, 1972
Trypanosoma lewisi	culture	−70	none	185	Weinman & McAllister, 1947
〃	blood	−70	none	531	〃
〃	blood?	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	blood?	−196	DMSO	30*	Resseler *et al.*, 1965
〃	blood	−196	glycerol	2,648	Raether & Seidenath, 1972
Trypanosoma pipistrelli	blood?	−196	DMSO	420*	Resseler *et al.*, 1965
Trypanosoma ranarum	culture	−170	DMSO	987	Diamond, 1964
Trypanosoma rangeli	blood?	−196	DMSO	7	Resseler *et al.*, 1965
Trypanosoma rhodesiense	blood	−70	none	2,920*	Weinman, 1958
〃	〃	−79	DMSO	30	Walker & Ashwood-Smith, 1961
〃	〃	−196	DMSO	420*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,650	Raether & Seidenath, 1972
Trypanosoma rotatorium	culture?	−196	DMSO	1	Resseler *et al.*, 1965
Trypanosoma theileri	culture?	−196	DMSO	7	〃
Trypanosoma vivax	culture?	−80	glycerol	852*	Cunningham *et al.*, 1963b

Tetrahymena pyriformis	trophozoite	−95	DMSO	112	Wang & Marquardt, 1966
〃	〃	−196	DMSO	90*	Hwang *et al.*, 1964
Paramecium aurelia	〃	−27	DMSO	7	Wang & Marquardt, 1966
〃	〃	−196	DMSO	7	〃
Plasmodium berghei	blood	−20	none‡	1	Miyata, 1973b
〃	〃	−20	serum‡	7	〃
〃	〃	−20	glycerol‡	49	〃
〃	〃	−70	none	15	Levaditi, 1952
〃	〃	−75	glycerol	520	Kasprzak & Rydzewski, 1970
〃	〃	−70	DMSO	168	Collins & Jeffery, 1963
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	sporozoite	−70	DMSO	45	Bafort, 1968
〃	blood	−170	DMSO	302	Diamond, 1964
〃	〃	−196	glycerol	2,622	Raether & Seidenath, 1972
Plasmodium cathemerium	〃	−78	none	156	Manwell, 1943§
Plasmodium circumflexum	〃	−78	none	137	〃
Plasmodium falciparum	〃	−70	none	1,921	Collins *et al.*, 1963
〃	sporozoite	−70	none	183	Jeffery & Rendtroff, 1955
〃	blood	−190	DMSO	52	Booden & Geiman, 1970
Plasmodium gallinaceum	〃	−75	none	50	Archetti, 1941
〃	〃	−70	glycerol	281	Jeffery, 1962
〃	〃	−70	DMSO	168	Collins & Jeffery, 1963
〃	sporozoite	−196	none	767	Weathersby & McCall, 1967
Plasmodium hexamerium	blood	−78	none	185	Manwell, 1943§
Plasmodium inui	〃	−76	none	151	Horsfall, 1940
Plasmodium knowlesi	〃	−76	none	140	〃
〃	〃	−190	DMSO	52	Booden & Geiman, 1970
Plasmodium lophurae	〃	−75	none	244	Manwell & Edgett, 1943
Plasmodium malariae	〃	−70	none	60	Jeffery & Rendtroff, 1955
Plasmodium nucleophilum	〃	−78	none	212	Manwell, 1943§

Plasmodium oti	blood	−78	none	127	〃
Plasmodium ovale	〃	−70	none	234	Jeffery & Rendtroff, 1955
〃	sporozoite	−78	none	997	Jeffery, 1957
Plasmodium relictum	blood	−78	none	158	Manwell, 1943§
〃 (*P. praecox*)	〃	−196	glycerol	2,641	Raether & Seidenath, 1972
Plasmodium routix	〃	−78	none	175	Manwell, 1943§
Plasmodium vaughani	〃	−78	none	46	〃
Plasmodium vinckei	〃	−75	?	414	Bafort *et al.*, 1966
Plasmodium vivax	sporozoite	−70	none	958	Jeffery & Rendtroff, 1955
〃	blood	−75	none	37	Saunder & Scott, 1947
Leucocytozoon simondii	〃	−196	glycerol	210*	Kocan *et al.*, 1967
Babesia argentina	〃	−80	DMSO	1,225	Dalgliesh, 1972‖
Babesia berbera	〃	−70	glycerol	215	Pipano & Senft, 1966
〃	〃	−70	glucose	15	〃
Babesia bigemina	〃	−79	glycerol	626	Ishihara & Minami, 1968
〃	〃	−70	glucose	240	Pipano & Senft, 1966
Babesia caballi	〃	−196	glycerol	1,045	Frerichs *et al.*, 1968
Babesia canis	〃	−76	glycerol	weeks	Reuße, 1956
〃	〃	−196	glycerol	2,621	Raether & Seidenath, 1972
Babesia equi	〃	−196	glycerol	603	Frerichs *et al.*, 1968
Babesia rodhaini	〃	−196	glycerol	2,627	Raether & Seidenath, 1972
Eimeria adenoides	sporozoite	−196	DMSO	853	〃
Eimeria brunetti	〃	−196	DMSO	1,120	〃
Eimeria dispersa	〃	−196	DMSO	853	〃
Eimeria gallopavonis	〃	−196	DMSO	853	〃
Eimeria meleagrimitis	〃	−196	DMSO	853	〃
Eimeria tenella	〃	−196	DMSO	1,112	〃
Toxoplasma gondii (RH)	prolif.	−20	glycerol	75	Miyata, 1973b
〃 (RH)	〃	−20	none	1	〃
〃	oocyst	−20	none	28	Frenkel & Dubey, 1973

Toxoplasma gondii		cyst	−20	none	200	Robl, 1965
〃		prolif.	−70	glycerol	711	Mackie, 1972
〃		〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	(RH)	〃	−75	none	30	Miyata, 1973b
〃		cyst	−75	DMSO	730*	Kwantes *et al.*, 1967
〃		prolif.	−75	DMSO	730*	〃
〃		〃	−196	DMSO	450*	Resseler *et al.*, 1965
〃		〃	−196	serum	160	Smith, 1973
Nosema cuniculi		spore	−196	DMSO	60*	Bedrnik & Vavra, 1971
Eperythrozoon wenyoni		tissue	−79	glycerol	764	Ishihara & Minami, 1968
Anaplasma centrale		blood	−70	glycerol	439	Pipano & Senft, 1966
Anaplasma marginale		〃	−79	glycerol	495	Ishihara & Minami, 1968
Theileria sp.		〃	−79	glycerol	764	〃
Borrelia hispanica		culture ?	−196	DMSO	330*	Resseler *et al.*, 1965

* roughly estimated from date shown in weeks, months, or years

† polysaccarides+polyvinyl pyrrolidone (see page 177 in this paper)

‡ none: blood diluted in same volume of physiological saline
serum: blood diluted in same volume of bovine serum
glycerol: blood diluted in same volume of distilled water containing 15% glycerol

§ temperature varied between −55 to −78 C

‖ temperature varied between −70 to −80 C

trophozoite: trophozoite stage (mostly culture form)

culture: culture form

blood: stage in blood

fly: metacyclic form or other forms found in tsetse fly

sporozoite: sporozoites (in *Eimeria,* excysted sporozoites)

cyst: cyst itself or, in *Toxoplasma,* parasites which can produce cyst.

prolif.: proliferative form, parasites obtained from peritoneal exudate or from tissue culture

Plasmodium berghei survived for more than 2 years at the temperature without losing their virulence.

(a) Equilibration

In the use of cryo-protectants, samples should be kept or incubated for several minutes at least at temperatures higher than sub-zero temperature before cooling. The adequate temperature and period in the incubation was reported by some workers (Levine *et al.*, 1958; Dalgliesh, 1972; Miyata, 1974, 1975a and 1975b). They preferred the term *equilibration* or *to equilibrate* to *incubation* or *to incubate*. As one of the authors using the term, the reason of the choice should be explained. In the previous papers (Miyata, 1974, 1975a, and 1975b), the author showed several figures, and in the present paper, some of them are presented in Figs. 5 and 6. In the Fig. 5, after incubation at 25 C or 37 C for various intervals between 0 to 180 minutes, the samples (*Trichomonas vaginalis*, 5 strains) were cooled in a −30 C freezer (cooling rate, 1 C per minute) in the presence of glycerol, then the samples were stored at −75 C. The survival rate of *T. vaginalis* increased gradually with the prolonged incubation period, and after several minutes, the maximum survival rate has appeared, and no more incubation was needed. The effect of glycerol as a cryo-protectant on survival is enhanced with prolonged incubation, which is necessary to equilibrate protozoan cells to glycerol.

The temperature for equilibration is also important, as shown in Fig. 6. In this experiment, the samples were allowed to equilibrate with 10% glycerol for 100 minutes at various temperatures, and they were stored at −75 C, after pre-cooling in the −30 C freezer. The survival rate was markedly improved at higher temperatures. Incubation at 0 C was not effective, but even at this temperature, the survival rate was still very high, if compared with the samples without glycerol. The survival rate for non-glycerol samples was less than one percent, or sometimes no survivor was observed microscopically. Fitzgerald and Levine (1961), and Dalgliesh (1972) pointed out that glycerol was toxic at around 4 C. The author's opinion, however, is quite different from them, and he believes that glycerol is not toxic at 4 C, but at this temperature, cryo-protective action does not work sufficiently.

These observations clearly show the importance of the temperature and the period for the equilibration between cells and glycerol. According to Lovelock (1953), glycerol must penetrate into the cells, and for the penetration, time and temperature must be important. At least, in the present time, the author is uncertain whether or not glycerol is really present within trichomonad cells. Therefore, the volume of glycerol within cells must be measured at various temperatures and in various equilibration periods. According to literatures surveyed so far, there has been no report dealing with such problems in the protozoological field.

The action of DMSO is very different from that of glycerol as shown in Figs. 6 and 7. These experiments were carried out by the same method mentioned above. The good effectiveness of DMSO as a cryo-protectant was demonstrated at lower temperatures (Fig. 6), and the lowest survival rate of *T. vaginalis* was obtained from the samples which were incubated at 37 C. The effect of equilibration time at 25 C or 37 C on the survival was also very different. At either temperature, survival rate decreased with the prolonged equilibration time, and the highest survival rate was

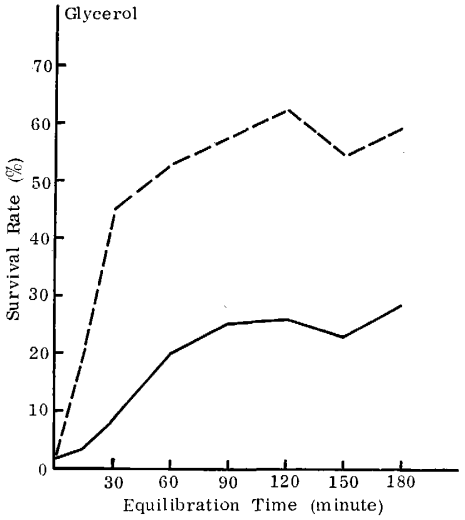

Fig. 5 The effect of the equilibration time on trichomonad survival with 10% glycerol at 25 C or 37 C (Miyata, 1975a).

Notes for Figs. 5 to 9:
---- at 37 C
—— at 25 C
After the equilibration, samples were pre-cooled at a −30 C freezer for 90 minutes, then they were stored in a −75 C freezer for 1 to 5 days. After thawing in a 37 C water bath, survival rate was examined. The pooled survival rate of 5 strains of *Trichomonas vaginalis* was shown in the figures. The details of these experiments were seen in Miyata, 1975b.

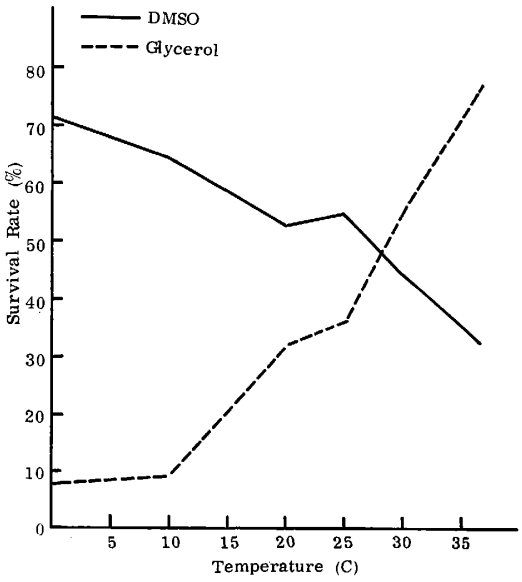

Fig. 6 The effect of the equilibration temperature on trichomonad survival with 10% glycerol or 7.5% DMSO for 100 minutes (modified from Miyata, 1975a).

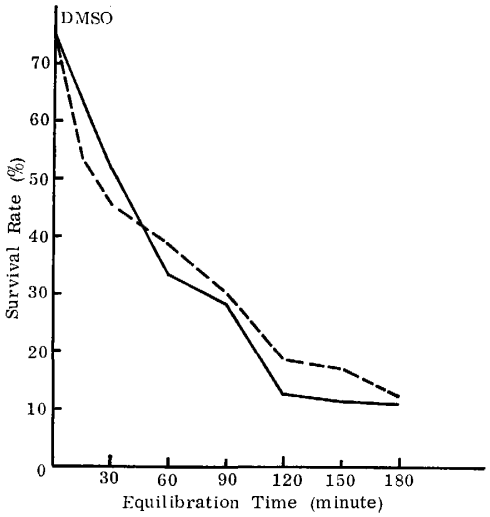

Fig. 7 The effect of the equilibration time on trichomonad survival with 7.5% DMSO at 25 C or 37 C (Miyata, 1975a).

obtained in the sample without equilibration. According to Lovelock's theory (1953), the time for penetration of DMSO into cells must be required. The author's results, however, show that penetration of DMSO is rather injurious, and if this substance is used, the samples should be cooled as soon as possible after addition of DMSO. In the samples without cooling, living trichomonads did not change in

the number during equilibration period, even after 3 hour incubation. This shows that injurious action of DMSO to cell is caused not by prolonged incubation but by cooling and thawing after prolonged incubation. The action of DMSO and glycerol varied among the species or strains used as reported by various workers.

Meyer and Chadd (1967) also pointed out that both glycerol and DMSO proved toxic to the parasites in cultivation at 37 C. DMSO are clearly more toxic; 3.5% DMSO killed more than 99% *Giardia* in 2 days while 3.5% glycerol killed about 50% of the parasites in the same period. Therefore, they recommended that the protective agent should be diluted as quickly as possible after thawing.

(b) Optimal concentration of cryo-protectants

The optimal concentration of glycerol or DMSO were studied by most workers who attempted to preserve the protozoa in a frozen state. Their opinion varied, but most of them recommended to use 10% glycerol and 5 to 7.5% DMSO. According to the author's experiment (Miyata, 1975a and 1975b), the optimal concentration of glycerol in *Trichomonas vaginalis* changed with equilibration period and temperature. For example, the optimal concentration was higher at 25 C than 37 C in equilibration for 100 minutes (Fig. 8). In the case of DMSO, the optimal concentration is fixed between 5 to 10% at various temperatures (Fig. 9). The incubation period is important, and in higher concentration many parasites may die during prolonged equilibration as pointed by Doran (1969b).

Diamond (1964) used 5% DMSO, because this concentration gave him satisfactory results for many protozoa. By using 5% DMSO, *Entamoeba invadens* survived by slow cooling and storage at −170 C, but recoveries of viable organisms were

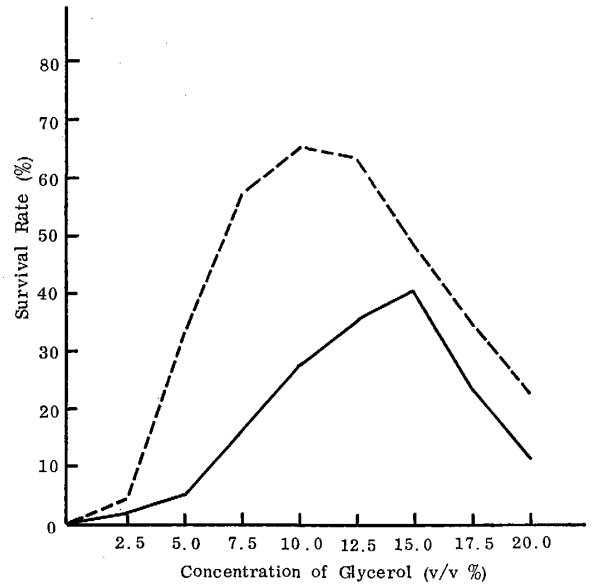

Fig. 8 The effect of the concentration of glycerol on trichomonad survival after 100 minutes equilibration at 25 C or 37 C (original).

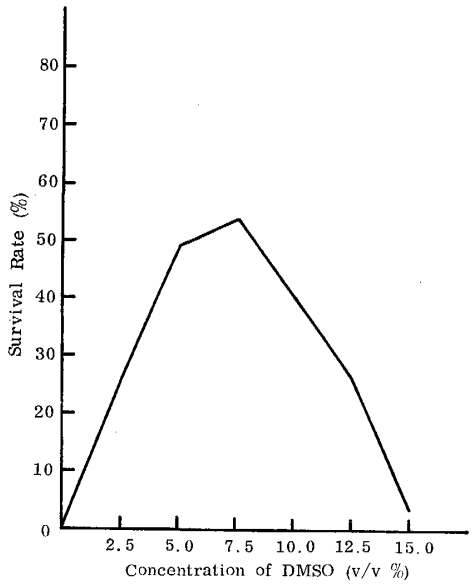

Fig. 9 The effect of the concentration of DMSO on trichomonad survival after 60 minute equilibration at 25 C (original).

only one percent. Then, the concentration of DMSO was increased to 15 percent, and this gave rise to a maximum recovery of about 25 percent. Stimulated by Djerassi and Roy (1963) who employed DMSO and sugar in freezing preservation of blood platelets of the rat, Diamond attempted to add 4.5% glucose to sample containing 15% DMSO, and 45 percent of amoebae recovered, whereas no protection was shown with glucose alone at concentrations from 2.25 to 9% (w/v).

4) Maintenance of biological properties of the parasitic protozoa in frozen state

It is well known that biological properties such as virulence, antigenicity, and drug resistance of various parasites change gradually by prolonged passage in animals or culture media. Some workers reported that several strains have lost their pathogenicity to inoculated mice after prolonged storage in a frozen state. For example, Allain (1964) observed the loss of pathogenicity in one of 9 strains of *Trypanosoma cruzi* stored at −64 C for 6 months, but other 8 strains retained their original virulences and viabilities. Many workers, however, claimed that the properties did not change by using cryo-preservation.

(a) Virulence

According to Stabler *et al.* (1964), the highly virulent strain of *Trichomonas gallinae* (Jones' Barn strain) survived for 52 weeks at −72 C by addition of 1.0 M glycerol (1 hour equilibration at room temperature), and its virulence did not change during the prolonged preservation period, but the continued in vitro cultivation at 37.5 C resulted in the gradual loss of pathogenicity of the trichomonad to domestic pigeons.

Lindgren and Ivey (1964) also reported that the virulence of *Trichomonas vaginalis* (12 strains) did not change during a period of 8 week preservation at −43 C in the presence of 10% glycerol, whereas the virulence decreased in after 2 to 3 months cultivation at 37 C. Jeffries and Harris (1967) obtained the result similar to Lindgren and Ivey on *T. vaginalis* and *Tritrichomonas foetus*. Diamond *et al.* (1965) examined the virulence of *T. vaginalis* to mice after 730 day storage at −170 C by using 5% DMSO, they found that the virulence did not change singnificantly by such long period storage.

Weathersby and McCall (1967) reported that sporozoites of *Plasmodium gallinaceum* were preserved in the vector, *Aedes aegypti*, for 767 days in liquid nitrogen without apparent loss of viability or infectivity. Minter and Goedbloed (1971) obtained live trypanosomatid parasites from the frozen tsetse flies and sandflies kept in liquid nitrogen with no obvious loss of viability or infectivity. Filardi and Brener (1975) pointed out that prolonged preservation at −196 C apparently did not change the biological characteristics of different strains of *Trypanosoma cruzi*.

(b) Gametogenesis

Many workers observed the disappearance of gametocytes of certain malaria parasites such as *Plasmodium* spp. by serial blood transferes in vertebrate hosts. By the application of cryo-preservation, the original nature to produce gametocytes in various blood parasites might be maintained. This is another advantage of cryo-preservation. Furthermore, according to Bafort *et al.* (1966), the ability to reproduce gametocytes in *P. vinckei* which had lost the power by continuous animal passages recovered in the parasites which were kept in frozen state at −75 C.

(c) Drug resistance

Schneider *et al.* (1968) examined the survival time and retention of antimalarial resistance of malaria parasites (*Plasmodium berghei* and *P. gallinaceum*) in liquid nitrogen in the presence of 10% glycerol. The samples were cooled at 1.3 C per minute for 60 minutes, then rapidly cooled in liquid nitrogen. One of the strain of *P. berghei* survived for 639 days and *P. gallinaceum* also for 62 days. There was no remarkable difference of antimalarial resistance between frozen samples and unfrozen ones. Since the antimalarial resistance of the malaria parasite is reduced if the parasites were maintained from host to host without medication, the cryo-preservation might be useful to keep such characters of the parasites.

(d) Antigenicity

According to Gordon *et al.* (1969), no permanent antigen change had occured as a result of 5 month preservation of *Entamoeba histolytica* at −196 C.

(e) Loss of kinetoplast

According to Overdulve *et al.* (1970c), one strain of *Trypanosoma evansi* lost its kinetoplast after storage at −76 C and at −196 C, and it did not reappear in subsequent mouse passage. This kind of observation, however, was not reported by other workers who preserved trypanosomes in a frozen state.

IV. Conclusion

In the introduction of this paper, the author has already pointed out the advantages of cryo-preservation of the parasitic protozoa, and he would not repeat the subject again. In cryo-preservation, biological properties such as infectivity, virulence, antigenicity, drug resistance, or to produce gametocytes, might be maintained without apparent loss or change as reported by many workers. Of course, several undesirable results were described by some workers, for example, loss of pathogenicity or even loss of kinetoplast permanently in parasites after freezing. Even considered those minus results, still cryo-preservation is a useful method to maintain our materials because during prolonged period of animal passages or in vitro cultivation, original natures of parasites frequently changes in infectivity, virulence, or other properties as pointed out by many workers. To avoid such undesirable changes, we must improve our preservation technique furthermore, and there is no doubt that problems will be solved in future. The advantages of cryo-preservation are apparently greater than those in animal passage or serial cultivation, and especially the preservation period is extremely prolonged by the use of liquid nitrogen. At present, however, the following problems are not yet solved clearly, for instance, fate of ice crystals in the frozen cell, optimal cooling rates, action of cryo-protectants, effect of salt concentration (or other substances present in suspended media), or penetration rates of cryo-protectants into the cells.

Today, cryo-preservation of the protozoa is popular all over the world, and various new techniques were proposed and discussed by many workers, but the standard methods might be summarized as follows:

(1) For most parasitic protozoa, cryo-protectants such as DMSO or glycerol are useful. Several workers recommended to use DMSO (5 to 7.5%) rather than

glycerol (about 10%) because equilibration is unnecessary. Glycerol, however, seems also an useful protectant because DMSO is rather toxic than glycerol in higher concentration and in prolonged equilibration. The equilibration with glycerol must be carried out for 30 to 60 minutes at a higher temperature as 37 C. DMSO, however, should be added at a lower temperature and the materials must be cooled as soon as possible after adding the substance.

(2) Slow cooling in the rate of 1 C per minute is ideal in most parasites, and this cooling rate can be obtained by cooling at −25 to −30 C, or by slow addition of small pieces of dry ice into alcohol bath.

(3) For cryo-preservation, the lower temperature is better, and storage in liquid nitrogen or its vapor is the best.

(4) Frozen materials should be thawed as rapidly as possible to avoid recrystallization of the ice during warming, and for that purpose, 37 to 40 C water bath is useful.

In our country, we have no center in which various protozoa strains are stored to supply to other researchers. In one laboratory, some strains might be used routinely for experiments, but other many strains are not. For example, in our laboratory, nobody uses *Trypanosoma gambiense, Entamoeba histolytica, Leishmania donovani,* and *Toxoplasma gondii* (2 strains) at present. Other laboratories may have some strains out of use. If those strains were deposited certain center, the center could supply the strains soon whenever the need for them arose. For these reason, the author would like to propose to build such a preservation center of protozoan strains in this country. If we could have the center, it would benefit all researchers who have interest in protozoology and tropical medicine.

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寄生原虫類の凍結保存

宮　田　　彬

最近30年間に発表された原虫の凍結保存に関する論文は，200篇を越えている。そこでこの論文では，それらのうち主な論文を紹介するとともに凍結保存が可能な原虫類の保存方法や保存期間などを総括し，さらに今後の問題点を論じた。また今までに十分な検討を加えずに用いられていた凍害保護剤について，特にグリセリンと DMSO の用い方，平衡時間などについて，著者の研究を中心に紹介した。原虫類の最適保存法及びこの論文の論旨は次の通りである。1）原虫は，適当な保護剤を含む溶液あるいは培地中に攪拌し，試験管またはアンプルに分注する。2）保護剤の濃度は，グリセリンは10% 前後，DMSO は，7.5%前後が適当である。グリセリンの場合は，比較的高い温度（例えば 37 C）で 30—60 分平衡させる。高温に耐えない原虫は，25 C 前後で60—90分平衡させる。DMSO は，低い温度（例えば 0C）で加え，平衡時間をおかず直ちに凍結する。3）凍結は2段階を用いる。すなわち，−30 C 前後のフリーザー中で約90分予備凍結し（この時冷却率は約 1 C, 1 分），ついで保存温度へ移す。4）保存温度としては，液体窒素のような超低温が好ましいが，−75 C でも数カ月程度は保存可能である。5）凍結材料は，37〜40 C の恒温槽中で急速融解し，融解後は，すみやかに動物あるいは培地へ接種する。6）原虫の種類によっては，もっと簡単に保存できる。原虫ごとに予備試験を行い，目的の保存温度に数日保存して高い生存率の得られる方法を採用するとよい。7）今後の問題点としては，保存原虫の性質（薬剤耐性，抗原性，感染性など）の長期保存における安定性を検討することと純低温生物学的な立場から超低温下における細胞の生死のメカニズムを解明することである。前者については，多くの研究者が凍結保存による実験株の性質の変化は認められないと指摘している。8）終りに数多くの実験株を保存し，研究者に提供する低温保存センターの設置の必要性を提案した。

長崎大学熱帯医学研究所疫学部門

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