INTRODUCTION

Aedes aegypti (Linn.) is one of the most medically important mosquitoes in Thailand, because it transmits the viruses causing chickungunya and dengue fever (Herms and James, 1961; James and Harwood, 1969). Efforts to reduce the transmission of these diseases have concentrated on control of the vector, because no vaccine or therapeutic agent for the diseases is available. Various chemical insecticides have traditionally been used in attempts to control the vector. However, use of chemical insecticides has several disadvantages: (1) many insects, including mosquitoes, develop resistance to them, (2) they are relatively non-specific and often kill predatory organisms that are important to the natural control of the target species, (3) many of them persist and become widely distributed in the environment.

Because of these disadvantages of chemical insecticides, it is desirable to seek alternative methods of mosquito control (Beirne, 1962; Burges and Hussey, 1971; Cameron, 1963). Biological control with insect pathogens represents one such possible alternative. Insect pathogens include viruses, rickettsiae, bacteria, fungi, protozoa and nematodes. Representatives of each of these groups have distinctive biological and physical characteristics that give them some potential as biological control agents (Burges and Hussey, 1971; Chapman, 1974; Franz, 1961). For instance, many insect pathogenic protozoa possess the following desirable characteristics: (1) they occur commonly

among insects, (2) many are highly host specific, (3) many produce a spore that is relatively resistant to environmental degradation, (4) some are vertically transmitted and, thus, have the ability to persist and spread within a target population, and (5) many easily overcome the defense mechanisms of the host (Franz, 1961; Weiser, 1963).

A protozoan pathogen that is highly lethal to A. aegypti has been found in Thailand (Hembree, personal communication), and efforts to evaluate its biological control potential for A. aegypti are underway at the SEATO Medical Research Laboratory. This pathogen appears identical to Helicosporidium parasiticum Keilin, 1921.

Review of Literature on *Helicosporidium parasiticum*

Taxonomy

Genus *Helicosporidium* belongs to Order Helicosporidia, Class Sporozoa, Phylum Protozoa (Steinhaus, 1967). The taxonomic status of this order is presently uncertain and its possible relationships have been discussed by several authors.

Keilin (1921) and Weiser (1970) explained that there was no real affinity between Order Helicosporidia and the Cnidosporida, because they differed in some stages of development and in the structure of the spore and the filament. For the same reasons, Keilin (1921) also suggested there was no similarity between Order Helicosporidia and the Haplosporidia or the Mycetozoa. Later, Weiser (1970) proposed to transfer Order Helicosporidia from the protozoa to the lower fungi, because of similarity in the morphology of some developmental stages and because of his perception of its mode of infection. Keilen and Lindegren (1974) initially agreed with Weiser's proposal, but they were uncertain of the possible relationship between this order and the primitive ascomycetes. There was no evidence of mycelium and certain reproductive activities characteristic of the fungi were absent.

Finally, Lindegren and Hoffmann (1976) studied the ultrastructure of developmental stages of *Helicosporidium* sp., which had been experimentally injected into fifteen-day old larvae of the navel orangeworm, *Paramyelois transitella*. Because of the presence of well defined golgi bodies and a mitotic nuclear

division, characteristics of animal cells, they suggested that this pathogen was not an ascomycete, as had been previously reported, but was a protozoan. Fukuda et al. (1976) agreed with Lindegren and Hoffmann (1976), since they found no evidence to the contrary.

Life cycle

Helicosporidium parasiticum, a parasite of the Ceratopogonid larva, Dasyhelea obscura, was originally described by Keilin (1921). His observations indicated there were both schizogonic and sporogonic stages in the life cycle, of which he provided a diagram. This observation was supported by Weiser (1970).

According to Keilin, the youngest stage in schizogony was a small round cell of 2-3μ in diameter, with homogenous protoplasm and small spherical nucleus. After two successive divisions, the parasite developed to a small schizont composed of four cells disposed in a tetrahedral configuration. These schizonts either broke up into four merozoites or underwent a third division, producing eight merozoites. The schizogonic part of its life cycle was very active and resulted in the formation of an enormous number of merozoites.

Because of the smallness of the parasite, it was difficult to follow the detail of sporogony. During spore formation, merozoites resulting from the schizogony developed to a tetrahedral morula after two successive divisions. Of the four cells, one

developed to a filament surrounding the other three cells, which were sporozoites. Then, an external membrane, the sporocyst, was secreted. This enveloped the whole group of four cells, thus forming a spore of 5-6μ in diameter. When elongate, the filament was 60-65μ long and pointed at both ends (Figure 8).

Kellen and Lindegren (1974) also studied the ultrastructure of the life of what they believed to be H. parasiticum from the navel orangeworm, Paramyelois transitella. Two stages of multiplication were observed as described by Keilin (1921). In schizogony, they found pellicle formation around the merozoites that was not mentioned by Keilin (1921). In sporogony they observed that the spore wall formed before the differentiation of a filament and the three sporoplasms.

Host range

Keilin (1921) suggested that H. parasiticum had a wide natural host range. Although it was a common parasite of Dasyhelea obscura larvae, it also infected other dipterous larvae, such as Mycetobia pallipes, and non-insectan arthropods, such as the tyroglyphid mite, Hericia hericia. Helicosporidium sp. in mosquitoes was first recorded by Chapman from field collected larvae of Culex territans in Louisiana (Chapman, 1974). Subsequently, Fukuda et al. (1976) found what seemed to be H. parasiticum in larvae of Culex nigripalpus collected from polluted water near Lake Charles, Louisiana. An Helicosporidium sp., also appearing to be H. parasiticum, was found in naturally

infecting larvae of A. aegypti in Thailand in 1974 by Hembree (personal communication).

Experimental transmission of Helicosporidium has been reported by several investigators. Kellen and Lindegren (1973) experimentally infected various species of insects and mites by feeding them H. parasiticum spores. They recorded the following new hosts of this pathogen: 10 species of Coleoptera (4 families), 5 species of Lepidoptera (1 family), a mosquito (Culex pipiens quinquefasciatus), and 3 species of mites (1 family). Chapman also noted that larvae of C. territans were experimentally infected by per oral exposure to an Helicosporidium sp. that was isolated from infected navel orangeworm, Paramyelois transitella (Chapman, 1974). Finally, Fukuda et al. (1976) found that Helicosporidium sp., isolated from field collected larvae of C. nigripalpus, could infect 14 species of mosquito larvae in 6 genera and also 6 other species of Diptera, Coleoptera and Lepidoptera. The most susceptible mosquito hosts were Anopheles albimanus, Anopheles quadrimaculatus and Culex salinarius. These records of new hosts of Helicosporidium supported Keilin's statement that this pathogen had a broad host range.

Pathology

Keilin (1921) proposed that H. parasiticum infection in D. obscura occurred by ingestion of spores. After being swallowed by a host, the spores were ruptured by the straightening of the

coil filament, releasing the sporoplasms in the gut lumen. The sporoplasms were regarded as the infectious stage, the filament serving only to open the sporocyst. The sporozoites, subsequently penetrated through the wall of the alimentary canal into the body cavity and then began their schizogonic cycle. This observation was supported by Kellen and Lindegren (1974) and Fukuda et al. (1976). On the contrary, Weiser (1970) suggested that transmission of Helicosporidium occurred by penetration through the cuticle, as is typical of the fungi. Kellen and Lindegren (1974) believed that the habitual mode of invasion was via the gut, although transmission of spores via cuticular puncture wound might occur.

Keilin (1921) observed that, when larvae of D. obscura were infected with H. parasiticum, they were opaque and milky, while normal larvae were white and translucent. When parasitized larvae were examined under the microscope, their body cavities were filled with small, round, parasitic corpuscles, which circulated freely and later concentrated at the posterior part of the host, which became turbid. In advanced infections hosts lost their mobility and became fragile. In recent infections, the parasites were found in the cells of either the fat body or the nerve ganglia. The former was rapidly destroyed, and the parasites attached to the fat droplets and escaped into the body cavities. The nerve ganglia were reduced to the neurolemma by destruction of the glial cells. It was interesting to note that the parasites were never found in the nerve commissures. Infections were usually terminal in the larval stage, although

occasionally infected pupae were found. No infected adults were found. Infected tissues were autolysed before the death of the hosts.

Weiser (1970) histologically studied a caterpillar of a hepialid moth, Hepialis palipus, which was infected by H. parasiticum. He observed that the parasites stimulated cyst formation and local melanization in the host. Within the cyst, there were several stages of H. parasiticum. Melanization occurred mainly in the early stage of cyst formation. The host reaction locally prevented increase in numbers of the parasite. The principal infected tissue of the hepialid was the fat body, with no damage occurring in other tissues and no free parasites found in the hemolymph.

Fukuda et al. (1976) found that beetle Helicosporidium transmitted to mosquito larvae produced a localized infection and a definite host melanization, like Weiser (1970) observed, but mosquitoes exposed to Helicosporidium recovered from natural mosquito infections acquired a more general infection. There was no evidence of host melanization, and spores flowed freely in the hemolymph as in D. obscura observed by Keilin (1921). The mortality of infected larvae usually occurred during the fourth stage, but some mortality also occurred in pupae and in emerging adults that were heavily infected. Contrary to Keilin's observation in D. obscura, they found spores of Helicosporidium in surviving adult mosquitoes, and they suggested that mosquito Helicosporidium might be transmitted transovarially.

Review of Literature on Storage Methods

Many microorganisms have been stored alive for various periods of time by several methods, such as storage at low temperature, deep freezing and lyophilization (Bailey, 1972; Minter and Goedbloed, 1971; Ogunba, 1969; Pilley, 1976). A lot of compounds and mixtures, such as dimethyl sulfoxide (DMSO), egg yolk, glycerol and skim milk, were demonstrated to be good protectants against the damaging effect of freezing (First, 1971; Ogunba, 1969). Different storage methods and protectants were suitable to different species (Entwistle and Martin, 1972; Ott and Horton, 1971), and the ability to withstand prolonged storage was considered to be species dependent by Henry and Oma (1974).

Storage of pathogenic and parasitic protozoa at low temperature has been reported by several authors. For example, Henry and Oma (1974) successfully stored spores of Nosema locustae in water and in cavaders at about -10°C, and the spores were still infective for more than one year. Other Nosema spp. have been stored at -23°C, 4°C and 2-5°C (Milner, 1972; Moffett and Wilson, 1971; Ohshima, 1964; Revell, 1960). Blood parasites such as Plasmodium spp. and Trypanosoma cruzi remained viable and infective after storage at -70°C and -79°C, respectively (Minter and Goedbloed, 1971; Shute and Maryon, 1962).

Deep freezing is one of the most effective methods for preservation of living organisms, either directly or in the presence of a protectant. Weathersby and McCall (1967) demonstrated

that the infectivity of Plasmodium gallinaceum, frozen in whole, experimentally infected A. aegypti, was retained after deep freezing in liquid nitrogen (+196°C) for more than two years. Other parasitic protozoa, Trypanosoma spp., for example, were also well preserved by this method, either in the presence or in the absence of protective agents (Herbert et al., 1968; Minter and Goedbloed, 1971). Obiamiwe and Macdonald (1971) observed that Brugia pahangi was still infective after being frozen at -196°C, with 10 percent DMSO as a protectant, for up to two months. Both trophozoites of Entamoeba histolytica and sporozoites of Plasmodium spp. were effectively preserved in liquid nitrogen with DMSO as a protectant (Bafort, 1968; Diamond et al., 1961). Glycerol was considered to be a good protectant for cryopreservation of Trypanosoma sp. and Plasmodium spp. at -196°C (Cunningham et al., 1963; Jeffery, 1962).

Bailey (1972) found that infectivity of Nosema apis, lyophilized in freshly killed bees, was slightly decreased after 1 month of storage at 18°C. Lewis and Lynch (1974) preserved Nosema pyrausta infected Ostrinia nubilalis by lyophilization and vacuum drying. There was some loss of infectivity in both lyophilized and vacuum dried materials. Pilley (1976) observed some loss in viability of Nosema necatrix spores that were lyophilized and stored in the dark at room temperature for one day, but some infectivity was retained for up to two years.

The use of various compounds and natural substances as protectants has had considerable value in the preservation of

spermatozoa by freezing. For instance, Ott and Horton (1971) found that sperm of steelhead trout (Salmo gairdneri) remained viable for one week after deep freezing with an extender containing 12 percent (v/v) DMSO. Bull sperm were well preserved by the same method and protectant (Lovelock and Bishop, 1959). Moreover, Entwistle and Martin (1972) reported that ram sperm survived for 2 weeks after deep freezing, when a solution of 6 percent (v/v) egg yolk, 7.5 percent (v/v) glycerol and synthetic diluents was used as a cryoprotectant. Both boar and human sperm were successfully stored at -196°C in the presence of the same protective agent (Salamon, 1973; Salamon et al., 1973; Wilmut et al., 1973).

Statement of Objective

Storage properties are an important consideration in the evaluation of an insect pathogen as a potential biological control agent. Useful quantities of a pathogen must be accumulated before it can be distributed. Storage is essential to that accumulation. Storage is also important simply to experimentation with a pathogen. Maintenance of a constant supply of fresh material is expensive in time, space and effort. Also, the more frequently an agent is passed, the greater the possibility for contamination of stock and for modification of host preference and reduction of virulence. The objective of the experiments reported here-in was to compare several methods for the preservation and storage of spores of H. parasiticum produced in A. aegypti in order to determine the best one.